UNIVERSIDADE DE LISBOA
Faculdade de Medicina

MOLECULAR PROFILING IN NEUROTYPICAL YOUNG ADULTS
(i) mental health stratification and (ii) characterization of the neuronal-salivary proteome and neuropeptide/neuroimmune response.

Jorge Emanuel Martins

Orientadores: Prof. Doutor Mário Pinto Simões
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Neurociências

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To children with neurodevelopmental disorders and disabilities,
their families,
and to neuroscientists…

Abstract

Martins, J. E. (2021). Molecular profiling in neurotypical young adults: (i) mental health stratification and (ii) characterization of the neuronal-salivary proteome and neuropeptide/neuroimmune response.

(Under the direction of Simões, M. & Barros, M.)

SCOPE: Molecular biology represents a promising method for *in vivo* diagnosis and screening of neuropsychiatric conditions, particularly due to the recent methodological advances in the analytical omics sciences. However, in this thesis, the neuropsychophysiology of healthy young adults without neuropsychiatric or neurodevelopmental conditions is rather explored. For that purpose, a strategy for a combined mental health and molecular stratification was developed, in order to define subtypes of functioning, strata or phenotypic traits, in a neurotypical sample. The definition of these functional subphenomes is important for the adequate research of biomarkers of susceptibility. This classical thesis is published as a dissertation and is structured in two fundamental sections: Mental Health Stratification and Molecular Stratification, comprising three chapters each. In this work, a stratification model to describe mixed correlates of mental health and molecular functioning is presented, and, for that matter, different sets of results were presented to identify the successive stages of characterization.

Section I - Mental Health Stratification

INTRODUCTION: As already published by the author in *Progress in Biophysics and Molecular Biology* (Hipólito and Martins, 2017), there are reasons to doubt that cognitive life is exclusively neurocentred. A living system remains alive by perceiving and relating with its environment through interactions that become meaningful in ways that are hardly and merely exclusive to the brain. There are two fundamental models to understanding the phenomenon of cognitive life. One is the computational model, which is based on the symbolic thinking paradigm. The other is the biological organism model. The common difficulty attributed to these paradigms is that their reductive tools allow the phenomenological aspects of experience to remain hidden behind yes/no responses (behavioral tests), or brain 'pictures' (neuroimaging). Hence, one of the problems regards how to overcome methodological difficulties towards a non-reductive investigation of cognitive life. Furthermore, a healthy cognitive life is commonly associated to well-being, in neurotypical subjects. However, how such relation is established requires further evidence. Do cognitive life influence or modulate well-being? How does subjective experience modulate mental health in neurotypical subjects? (Re, Palma, Martins and Simões, 2016)

OBJECTIVES: The idea that cognitive life is completely reducible to brain function is usually suggested when one thinks that "there is a gap between the mind and the world", or more specifically between conscious experience and molecular expression (Re, Ventura, Vitiello, Simões and Martins, 2015). The first section of this PhD thesis aims thus to make a non-reductive study of mind, and to do so a phenomenological examination will be used for a mental health profiling. The examination will comprise

a psychological intervention and a psychological assessment and both will be used to acquire systematic profiles of mental health functioning.

METHODS: Neurophilosophical arguments and empirical evidence against a hard reduction in the study of mind were reviewed. In addition to this, as attempted to show, systemic protein networks, brain and non-brain located, might play a role in cognitive life. To support this argument, it is studied the first-person experience of 128 (19-to-25-year-old) healthy university students, non-medicated and with no serious or uncontrolled conditions of the kidney, liver, lung and heart, chronic musculoskeletal, rheumatologic or metabolic diseases which might affect the nervous system. The university students were recruited from the University of Lisbon (i. Faculty of Medicine, ii. Faculty of Economics, iii. Faculty of Sciences and iv. The School of Social and Political Sciences), the allocation was randomized, the assignment was parallel, and the blinding was single in Section I and triple in Section II of the thesis. Phenomenal and clinical data were stratified and characterized according to students' mental health functioning and provided the ground for the subsequent third-person study, in the second section of this PhD thesis. The psychological intervention employed was a single cognitive behavioral intervention, structured to increase awareness and wakefulness by a cognitive exercise of psychophysical training and autobiographical-self memory-recalling (Martins et al., 2016b). For the psychological assessment, a set of four psychological scales (*Abnorme Psychischer Zustände* States of Consciousness, Subjective Happiness Scale, Mindful Attention Awareness Scale, and Self-consciousness Scale – Revised version) were applied afterwards for a mental status examination. This assessment, conducted as a standardized and structured clinical interview, provided temptative experiential profiles based upon subjects' evaluations of their own conscious experiences and self-constructs.

RESULTS: Firstly, the conceptual analysis (Pacheco, Martins and Simões, 2019), as an empirical method, shows that combining phenomenal experience and molecular networks is consistent with a parallel and complement non-neurocentred view of cognition. Although brain representations and studies are the dominant metaphor, there is no exclusive empirical support for that postulate. Secondly, the overall protocol (psychological intervention and psychological assessment), used on this thesis, points towards the capacity to stratify phenotypic states and traits in young neurotypical adults. This protocol estimated the process and content of thought, and perception; the mood and affect; the cognitive awareness and attention; and the insight and judgment of the 128 healthy subjects (μ(age) = 21,495; 50% males) with no neurological and psychiatric pathologies. The experimental procedure, i.e., the single cognitive behavioral intervention, permitted to isolate mental subphenomes associated with autobiographical-self memory-recalling, positive affectivity and increased self-awareness. The sham intervention was used to isolate mental subphenomes associated to a simple cognitive task of maintaining attention, alertness and concentration during a class. Four different neurotypical subgroups with distinct mental status, and psychological profiles, were found. A first subgroup obtaining the higher scores in the psychological assessment (*Abnorme Psychischer Zustände* States of Consciousness, Subjective Happiness Scale, Mindful Attention Awareness Scale, and Self-consciousness Scale – Revised version) had a phenotype characterized by having a highly developed self-awareness, exhibiting a high-level cognition and a highly narrative autobiographical interpretation and self-understanding. A second subgroup with a distinct phenotype was characterized by having an elevated self-consciousness,

exhibiting positive pre-reflective bodily sensations and emotional states, as well as a significant focused cognition. A third subgroup with a characteristic phenotype was well defined by having an increased reflective self-experience, presenting with an overdeveloped conscious thought. A fourth subgroup obtaining the lower scores in the psychological assessment had a phenotype characterized by having a pre-reflective self-experience, displaying simple, immediate and non-observational access to conscious experience. Thirdly, it is shown a coherence between the psychological assessment and the psychological intervention, and consequently, the mental health characterization shows adequacy for the profiling of the mental status. Hence, four different subgroups, i.e., four different mental health strata, are screened by the developed clinical and statistical strategy, which comprehended a proportionate stratified random sampling. Correspondingly, the 128 stratified subjects are expected to have a homogeneous molecular response, i.e., a possible specific biomarker signature.

CONCLUSION: The first section of this work fulfills an *a priori* pre-molecular assessment and stratification for the subsequent molecular typing. It is expected that the characterization of the neuronal-salivary proteome may be used as an *in silico* proteomic biobank for the clinical study of the nervous system, and *in vivo* molecular referential for the neuromolecular examination of young neurotypical adults.

Section II - Molecular Stratification

INTRODUCTION: The neuronal proteome can be probed in salivary samples (Martins et al., 2016c). While neuropsychiatric states result from an altered neuroproteome, neurotypical conditions also have a characteristic molecular signature. Most of the molecular correlates to those conditions are identified in brain cerebrospinal fluid or blood samples. However, neuronal protein networks identifiable in the salivary protein profile may be useful to characterize the physiology of neurotypical mind and cognition. Cognitive-immune coupling is a possible low-level biomolecular structure of high-level neuronal cognition. The neuropeptide and neuroimmune responses have been linked to mental health states and stimuli, as part of the neuro-immune interface, and are constituted by peptides with a small molecular weight range of approximately [9.1;30]kDa. Moreover, neuro-immune homeostasis has been proposed as a physiological evidence of neurological/mental health or well-being. For some authors, this physiology is maintained, on the one hand, by regulation of small peptides like Orexin, β-Endorphin, Oxytocin and Substance P, and on the other hand, by Th17 cell type response and biochemical messengers.

OBJECTIVES: The second section of this work will focus first on a database literature review (Sousa, Martins, Rosa, Barros and Correia, 2016) of the neuronal proteome found both in neurotypical subjects and in the conditions reviewed in the ICD-10 chapters ‘Diseases of the Nervous System’ and "Mental and Behavior Disorders", the Neuro.OralCard. The neuronal-salivary protein profile will be characterized in order to produce knowledge in biomarker research of neuropsychiatric conditions probed in saliva. An *in silico* characterization and a bioinformatics clustering analysis (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017) by PANTHER and Cytoscape functional clustering will be executed to evaluate the neuronal-salivary proteome. Secondly, a pipeline salivary protein profile study, which consists of a total protein profiling (Saavedra Silva, Sousa, Silva, Martins, Esteves, Fernandes, Rosa, Correia, Barros, 2017), will test a small and representative sample of the 128 neurotypical young

adults, aiming to show valid consistency as a salivary probing technique. Subsequently, a full molecular approach on selected and stratified 92 neurotypical young adults will be executed, with the objective to show specific protein profiles present in saliva. Lastly, a final stratified sample of 20 neurotypical young adults will be probed, aiming to study neuro-immune correlates.

METHODS: As a method, initially a neuromolecular database was acquired. This database offered an update of the OralCard (Arrais et al., 2013), revising 2416 neuronal proteins identified in neuroproteomics, from which 765 are present, detected and identifiable in the saliva proteome. Afterwards, saliva proteomics, through capillary electrophoresis and multiplexed simultaneous immunodetection mixed analysis, was used as a technique to evaluate, in a first phase, the total protein profile and, in a second phase, the neuropeptide and neuroimmune response (Martins et al., 2016a). In the first phase and taking in consideration an error variable correspondent to the lack of accuracy offered by capillary electrophoresis, molecular profiles were identified and significantly correlated with the previously identified mental health strata, in the first section of the PhD thesis. Subsequently, a Graphical User Interface toolbox, the MODeLING.Vis, was developed for the data mining of the acquired neuroproteomic data and to address the problematic of the low discriminative power of capillary electrophoresis. The developed toolbox permitted data exploration and hypothesis testing of the biomolecular data. In the second phase, the neuromolecular profiling of the stratified and selected sample of 20 neurotypical young adults was concluded through a combined multiplex panel: the *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the *Bio-Plex Pro™ Human Th17 Cytokine Assay*.

RESULTS: In a first analysis, this descriptive study shows that, in literature, 85% of the neuronal proteins are primarily correlated with a neuropsychophysiological condition. From those, only 13% are in fact identified, quantified and proposed as biomarkers of susceptibility. Afterwards, a functional enrichment that was executed in comparison to the *Homo sapiens* (Human - taxon 9606) proteome, demonstrates that proteins in brain circuits are predominantly involved in metabolic functions, immune system processes and neuropeptide transmission. The outcome of this first analysis is the acquisition of the total protein profiles (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017) and the preliminary quantification of the [9.1;30]kDa molecular weight range of proteins, which are present in the salivary samples of the 92 neurotypical young adults. In this first analysis, the acquired total protein profiles and more specifically the characteristics of the protein distribution in the [9.1;30]kDa show correlation with the corresponding four mental health profiles already identified. Moreover, this light molecular weight range, in literature, is characteristic of small neuroimmune molecules and neuropeptides and, thus, offers a solution for probing into the neurocognitive response. From this first analysis and obtained results, 20 neurotypical young adults are then selected and stratified as expressing the most significant molecular profile in the [9.1;30]kDa molecular weight range. The outcome of this second analysis is that those 20 neurotypical young adults have a homogeneous salivary response of Orexin, β-Endorphin, Oxytocin, Substance P and Th17 cell biochemical messengers. These 20 final subjects have a reasonable distinct neuropeptide response and anti/pro T-helper cell type 17 equilibrium. Likewise, they represent a significant and stratified sample of the four distinct mental health profiles identified in the first section of the PhD thesis. As a supplementary milestone, it is developed (i) a molecular biology atlas for a better understanding of the neuronal

proteome functions, the MolecularCog(G) Atlas and (ii) a molecular function GO-term analysis of the immune and neuronal preferential interaction networks: the Neuro-Immune Network and the Human Th17 Cytokine Database.

CONCLUSION: The second section of this work shows some evidence of distinct mental functioning in young neurotypical adults, which is associated with distinct protein expression and a characteristic neuropeptide and neuroimmune response. These mental health-molecular strata can be evaluated for the investigation of biomarkers of susceptibility present in saliva. The identification of salivary biomarkers should take carefully into account the problematic of protein stability and degradation profile, as well as post-translational modifications and the degradation products. On the one hand, biomarkers in saliva may be able to provide insight into the health of the brain and the central nervous system. On the other hand, the identification of salivary proteins also present in brain samples needs to be taken into account with extreme caution and accurate correlations should be wisely considered. In the case of this descriptive study model, preliminary explanatory correlations are made between: (i) the subjective experience, (ii) the total protein profile, (iii) Orexin, β-Endorphin, Oxytocin, and substance P, and (iv) the Th17 cell type response and its biochemical messengers. These preliminary correlations precedes a full explanatory quantification of those mental health/molecular constructs. Therefore, causal relationships should be further addressed with improved and gold standard quantification protocols, and by so, developing better understanding in biomarkers of susceptibility of brain and mental health. Finally, this dissertation illustrates, to a certain degree and as a *proof of concept*, a cognitive-immune coupling in a neurotypical sample and opens venue for a further causalistic analysis (Hipólito and Martins, 2018).

Section I And II - Overall

OUTCOMES: The Intervention and Assessment Protocols effectively isolate significant ($p \leq 0.05$) mental subphenomes in the sample of 128 neurotypical young adults, and consequently permits the characterization of four neurotypical profiles with distinct mental and resulting molecular phenotypes: (i) the Self Awareness subgroup, (ii) the Self Consciousness subgroup, (iii) the Reflective Self subgroup, and (iv) the Pre-Reflective Self subgroup.

LIMITATIONS: This study is a descriptive research that might possibly be used in the research of salivary biomarkers of mental health states, and should be validated with causalistic explanations with a higher sample size and higher internal validity. The cost of such a study, in this 128 neurotypical sample, draws important scientific limitations for its development in a PhD framework, but motivates and gives significance to the effort used for the mental and molecular stratification strategy.

SELF AWARENESS SUBGROUP: Characterized by a self awareness phenotype, with a significant ($p \leq 0.05$) mental and molecular correlation. This subgroup shows a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a low increase in the [9.8;10.3]kDa and high decrease in the [13.7;17.5]kDa. This reflects the higher molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. More specifically, this phenotype is characterized by a significative increase in Orexin, β-Endorphin and Oxytocin levels, which reflects the elevated neuropeptide response related to the augmenting of specific autonomic and central

nervous system functions. In this subgroup, this is responsible for the increased reward function and arousal, but also for the elevated associative learning and incentive salience. Furthermore, this neuropeptide response is associated with the increased positive emotions and happiness, the reduced fear and anxiety, and the augmented predisposition to social interaction. Moreover, there is a corresponding effect of this cognitive load on the total inflammatory load. In the interleukin trait and state profiles this subgroup had the lowest pro-inflammatory expression of the 15 analytes studied for the Th17 cell type response, tending rather to an anti-inflammatory response. This is represented by the overexpression of IL-10, IL-21, IL-25 and IL-33. Henceforth, the most significative expressed biological processes of the immune system are the regulation of isotype switching and the positive regulation of lymphocyte-mediated immunity.

SELF CONSCIOUSNESS SUBGROUP: Characterized by a self consciousness phenotype, with a significant ($p \leq 0.05$) mental and molecular correlation. This subgroup shows a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a moderate increase in the [9.8;10.3]kDa and a high increase in the [13.7;17.5]kDa. This reflects the moderate molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. Neurophysiologically, and more specifically the significative increase in the Oxytocin levels, reflects the increased attention and elevated capacity for learning. Furthermore, this neuropeptide response, in this phenotype, is associated with pleasure and with the increased predisposition to social learning. Moreover, there is a complementary effect of this cognitive load on the total inflammatory load. In the interleukin trait and state profiles this subgroup has a low expression of the 15 analytes studied for the Th17 cell type response, tending to no marked immune response. This is represented by the regulation of IL-4, IL- 10, IL-21, IL-25 and IL-33. Henceforth, the most significative biological processes of the immune system are the adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, the regulation of immunoglobulin production, and the co-regulation of lymphocyte-mediated immunity.

REFLECTIVE SELF SUBGROUP: Characterized by a reflective self phenotype, with a significant ($p \leq 0.05$) mental and molecular correlation. This subgroup shows a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a low decrease in the [9.8;10.3]kDa and in the [13.7;17.5]kDa. This reflects the decrease in the molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. More importantly, the significative decrease in Orexin and Oxytocin levels reflects the decreased neuropeptide response related to the diminishing of certain autonomic and central nervous system functions. In this subgroup and phenotype, this is responsible for the decreased activation of the reward system, but also for the lower associative learning and incentive salience. Additionally, this neuropeptide response is associated with the diminished predisposition to positive emotions and tendance to anhedonia, to increased anxiety, and to the decreased tendency to social interaction. Moreover, there was a complementary effect of this cognitive profile on the total inflammatory load. In the interleukin trait and state profile this subgroup has the highest expression of the 15 analytes studied for the Th17 cell type response, showing a relative pro-immune phenotype. This is represented by the overexpression of IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L and TNF-α. Henceforth, the most significative biological processes of the immune system were the positive regulation of (i)

immunoglobulin production and secretion, (ii) osteoclast differentiation, and (iii) cytokine-cytokine receptor interaction.

PRE-REFLECTIVE SELF SUBGROUP: Characterized by a pre-reflective self phenotype, with a significant ($p \leq 0.05$) mental and molecular correlation. This subgroup shows a distinct profile in [9.1;30]kDa molecular weight range and more specifically no increase or decrease in the [9.8;10.3]kDa and very high increase in the [13.7;17.5]kDa. This reflects the marginal molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. Neurophysiologically, in this subgroup and phenotype, this is responsible for a slight decreased attention, arousal and diminished learning. Additionally, this neuropeptide response is associated with a certain apathy and the decreased predisposition to social learning. Rather on this subgroup, there is an effect of a demanding cognitive load on the total inflammatory load. Consequently, in the interleukin trait and state profile this phenotype has a moderate expression of the 15 analytes studied for the Th17 cell type response, tending to a light pro-immune response. This is represented by the expression of IL-1β, IL-6, IL-17F, IL-22, IL-31, sCD40L and TNF-α. Henceforth, the most significative biological processes of the immune system are the positive regulation of (i) T cell proliferation, (ii) immunoglobulin production, and (iii) osteoclast differentiation by pro-inflammatory interleukins.

Resumo

Martins, J. E. (2021). Perfis moleculares em jovens adultos neurotípicos: (i) estratificação cognitiva e (ii) caracterização do proteoma neuronal-salivar e resposta neuropeptídica/neuroimmune.

(Sob a orientação de Simões, M. & Barros, M.)

ENQUADRAMENTO: A biologia molecular representa um método promissor para diagnóstico e *screening in vivo* de condições neuropsiquiátricas, particularmente devido aos recentes avanços metodológicos de análise das ciências *ómicas*. Neste trabalho de tese, é explorado a neuropsicofisiologia de jovens indivíduos saudáveis sem doença neuropsiquiátrica ou do neurodesenvolvimento. Para tal, desenvolveu-se uma estratégia que combina saúde mental e estratificação molecular, de forma a definir subtipos de funcionamento, *strata* ou traços fenotípicos, numa amostra neurotípica. A definição de traços fenotípicos de funcionamento é importante para a adequada investigação de biomarcadores de suscetibilidade. Esta tese clássica é publicada como uma dissertação e é estruturada em duas seções fundamentais: Estratificação de Saúde Mental e Estratificação Molecular, compreendendo três subcapítulos cada. Neste trabalho é apresentado um modelo de estratificação para descrever correlatos mistos de saúde mental e funcionamento molecular, e, para esse efeito, são apresentados diferentes conjuntos de resultados para identificar as sucessivas etapas de caracterização.

Secção I – Estratificação de Saúde Mental

INTRODUÇÃO: Como já publicado pelo autor em Progress *in Biophysics and Molecular Biology* (Hipólito e Martins, 2017)[1], há razões para duvidar que a cognição seja exclusivamente neurocentrada. Um sistema permanece vivo, relacionando-se com seu ambiente através de interações que se tornam significativas de maneiras que são dificilmente exclusivas apenas ao cérebro. Existem dois modelos fundamentais para entender o fenómeno da vida cognitiva. Um deles é o modelo computacional, que se baseia no paradigma do pensamento simbólico. O outro é o modelo do organismo biológico. A dificuldade comum atribuída a esses paradigmas é que suas ferramentas de análise exigem que os aspetos fenomenológicos da experiência consciente permaneçam impercetíveis atrás de respostas sim/não (testes comportamentais) ou 'imagens' cerebrais (neuroimagem). Assim, um dos problemas centrais diz respeito a como superar as dificuldades metodológicas para a execução de investigação não redutora da vida cognitiva. Além disso, uma cognição saudável é habitualmente associada ao bem-estar, em jovens adultos neurotípicos. No entanto, a forma como essa relação é estabelecida requer justificação científica. Modula a vida cognitiva o bem-estar? Como é que a experiência consciente modula a saúde mental em jovens adultos neurotípicos? (Re, Palma, Martins e Simões, 2016)

[1] Hipólito, I., & Martins, J. (2017). Mind-life continuity: A qualitative study of conscious experience. *Progress in Biophysics and Molecular Biology*. Volume 131, 432-444, doi: 10.1016/ j.pbiomolbio.2017.09.004.

OBJETIVOS: A ideia de que a vida cognitiva é inteiramente redutível à função cerebral é geralmente sugerida quando se pensa que "há uma interrupção entre a mente e o mundo", ou mais especificamente entre a experiência consciente e a expressão molecular (Re, Ventura, Vitiello, Simões e Martins, 2015). A primeira seção da tese de doutoramento tem como objetivo desenvolver um estudo não redutivo da mente, e para o executar, uma avaliação fenomenológica será usada como estratégia para a obtenção de perfis de saúde mental. A avaliação incluirá uma intervenção psicológica e um exame psicológico, e ambos serão usados para adquirir perfis sistemáticos de funcionamento de saúde mental.

MÉTODOS: Argumentos neurofilosóficos e evidências empíricas contra um estudo redutivo da mente foram revistos. Além disso, tal como se tentou mostrar, redes de proteínas sistémicas localizadas no cérebro, ou fora, podem desempenhar uma ação fundamental na vida cognitiva. Para apoiar esse argumento, é estudada a experiência consciente de 128 estudantes universitários saudáveis, com idades compreendidas entre 19 a 25 anos de idade, não medicados e sem doenças crónicas, graves ou descontroladas dos rins, fígado, pulmão e coração, bem como doenças músculo-esqueléticas crónicas, reumatológicas ou metabólicas que possam afetar o sistema nervoso. Os estudantes universitários foram recrutados na Universidade de Lisboa (i. Faculdade de Medicina, ii. Faculdade de Economia, iii. Faculdade de Ciências e iv. Escola de Ciências Sociais e Políticas). Ao nível do desenho do estudo, este foi randomizado, paralelo, *single-blinded* na Secção I e *triple-blinded* na Secção II da tese. Os dados fenomenológicos foram caracterizados e estratificados de acordo com o funcionamento da saúde mental dos alunos e forneceram o suporte para o estudo molecular, na segunda secção desta tese de doutoramento. A intervenção psicológica utilizada foi uma intervenção única do tipo cognitivo-comportamental, estruturada de modo a aumentar a consciência e vigília através de um exercício cognitivo de treino psicofísico e da evocação da memória autobiográfica (Martins et al., 2016b). Para a avaliação psicológica, um conjunto de quatro escalas psicológicas *(Abnorme Psychischer Zustände States of Consciousness, Subjective Happiness Scale, Mindful Attention Awareness Scale,* e *Self-consciousness Scale – Revised version)* foram aplicadas posteriormente para o exame do estado mental. Essa avaliação, conduzida como uma entrevista clínica padronizada e estruturada, forneceu perfis experienciais empíricos com base nas avaliações dos sujeitos sobre as suas próprias experiências conscientes e vivência do eu.

RESULTADOS: Em primeiro lugar, a análise conceptual (Pacheco, Martins e Simões, 2019), como método empírico demonstra que a combinação de dados fenomenológicos e de redes moleculares sistémicas é consistente com uma visão paralela e complementar da cognição não neurocentrada. Embora os estudos e representações cerebrais sejam a metáfora dominante, não há suporte empírico exclusivo para esse postulado. Em segundo lugar, o protocolo geral (intervenção psicológica e avaliação psicológica), usado nesta tese, aponta globalmente para a capacidade de estratificar estados e traços fenotípicos em jovens adultos neurotípicos. Este protocolo estimou a forma e o conteúdo do pensamento e da perceção; o humor e o afeto; a atenção e a vigilância cognitiva; o *insight* e autoconsciência dos 128 indivíduos saudáveis (μ (idade) = 21.495; 50% do sexo masculino) sem patologias neurológicas e psiquiátricas. O procedimento experimental, ou seja, a intervenção cognitivo-comportamental, permitiu isolar subfenomas mentais associados com a memória autobiográfica, a afetividade positiva e consciência do eu aumentada. A intervenção *sham* foi utilizada para isolar

subfenomas mentais associados a uma tarefa cognitiva simples, associada à capacidade de atenção, vigília e concentração durante uma aula. Quatro subgrupos neurotípicos diferentes com estados mentais e perfis psicológicos distintos foram encontrados. Um primeiro subgrupo que obteve as pontuações mais altas na avaliação psicológica (*Abnorme Psychischer Zustände* States of Consciousness, Subjective Happiness Scale, Mindful Attention Awareness Scale, and Self-consciousness Scale – Revised version) teve um fenótipo caracterizado por uma autoconsciência altamente desenvolvida, exibindo uma cognição de alto nível e uma interpretação e auto compreensão autobiográfica altamente narrativa. Um segundo subgrupo com um fenótipo distinto foi caracterizado por ter uma autoconsciência elevada, exibindo sensações corporais e estados emocionais pré-reflexivos positivos, bem como uma cognição focalizada significativa. Um terceiro subgrupo com um fenótipo característico foi bem definido por ter uma autoexperiência reflexiva aumentada, apresentando um pensamento consciente excessivamente desenvolvido. Um quarto subgrupo que obteve as pontuações mais baixas na avaliação psicológica teve um fenótipo caracterizado por ter uma autoexperiência pré-reflexiva, apresentando um acesso simples, imediato e não observacional à experiência consciente. Em terceiro lugar, foi mostrada uma coerência entre a avaliação psicológica e a intervenção psicológica e, consequentemente, a caracterização da saúde mental, executada nesta secação, mostrou-se adequada para a estratificação do estado mental. Assim, quatro subgrupos diferentes, ou seja, quatro diferentes estados de saúde mental, são selecionados pela estratégia clínica e estatística desenvolvida, que compreendeu uma amostragem aleatória estratificada e proporcional. Correspondentemente, espera-se que os 128 indivíduos estratificados tenham uma resposta molecular homogénea, ou seja, uma possível assinatura de biomarcadores que é característica.

CONCLUSÃO: A primeira seção deste trabalho cumpre os critérios de uma avaliação pré-molecular e de uma estratificação *a priori* para a análise molecular subsequente. Espera-se que a caracterização do proteoma neuronal-salivar possa ser utilizada como biobanco proteómico *in silico* para o estudo clínico do sistema nervoso (Martins, Hipólito, Barros, Simões, *submetido),* e referencial molecular *in vivo* para o estudo neuromolecular de jovens adultos neurotípicos.

Secção II – Estratificação Molecular

INTRODUÇÃO: O proteoma neuronal pode ser sondado em amostras salivares (Martins et al., 2016c). Enquanto os estados neuropsiquiátricos resultam de um neuroproteoma alterado, as condições neurotípicas também têm uma assinatura molecular característica. A maioria dos correlatos moleculares dessas condições são identificados no líquido cefalorraquidiano cerebral ou em amostras de sangue. No entanto, redes de proteínas neuronais identificáveis no perfil proteico salivar podem ser úteis para caracterizar a fisiologia da mente e cognição neurotípica. O acoplamento cognitivo-imune é uma possível estrutura biomolecular *low-level* da cognição neuronal de *high-level*. As respostas neuropeptídicas e neuroimunes têm sido correlacionadas com estados e estímulos de saúde mental, como parte da interface neuroimune, e são constituídas por péptidos com um peso molecular baixo de aproximadamente [9,1;30] kDa. Além disso, a homeostasia neuroimune tem sido proposta como uma evidência fisiológica de saúde neurológica/mental ou bem-estar. Para alguns autores, esta fisiologia é mantida, por um lado, pela regulação de pequenos péptidos como Orexina,

β-Endorfina, Oxitocina e Substância P, e por outro lado, pela resposta celular do tipo Th17 e seus mensageiros bioquímicos.
OBJETIVOS: A segunda secção deste trabalho incidirá primeiramente numa revisão de literatura da base de dados (Sousa, Martins, Rosa, Barros e Correia, 2016) do perfil proteico neuronal encontrado tanto em sujeitos neurotípicos como nas condições revistas nos capítulos da CID-10 "Doenças do Sistema Nervoso" e "Perturbações Mentais e do Comportamento", apelidada Neuro.OralCard. O proteoma neuronal-salivar será caracterizado com o objetivo produzir conhecimento no domínio de investigação de biomarcadores salivares de condições neuropsiquiátricas sondadas na saliva. Uma caracterização *in silico* composta por uma análise bioinformática por *clusters* (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017) através do PANTHER, bem como por uma análise bioinformática funcional através do Cytoscape, será executada para avaliar o proteoma neuronal-salivar. Em segundo lugar, um estudo do perfil proteico salivar, que consiste na obtenção do perfil total de proteínas (Saavedra Silva, Sousa, Silva, Martins, Esteves, Fernandes, Rosa, Correia, Barros, 2017), analisará uma pequena e representativa amostra dos 128 jovens adultos neurotípicos, com o objetivo de demonstrar a consistência válida da técnica de deteção salivar. Posteriormente, uma abordagem molecular completa em 92 jovens adultos neurotípicos selecionados e estratificados será efetuada, com o objetivo de evidenciar os perfis totais de proteínas característicos presentes em saliva. Por último, será examinada a amostra estratificada final de 20 jovens adultos neurotípicos, com o objetivo de estudar os correlatos neuroimunes.

MÉTODOS: Como método, inicialmente procedeu-se a aquisição de uma base de dados neuromolecular. Esta base de dados ofereceu uma atualização do OralCard (Arrais et al., 2013), revendo 2416 proteínas neuronais identificadas em neuroproteómica, das quais 765 estão presentes, detetadas e identificadas no proteoma salivar. Seguidamente, através de uma técnica de proteómica salivar, por meio de análise mista de eletroforese capilar e imunodetecção simultânea multiplexada, foi avaliado, numa primeira fase, o perfil total da proteína e, numa segunda fase, a resposta neuropeptídica e neuroimmune (Martins et al., 2016a). Na primeira fase, não descartando a existência de uma variável de erro que corresponde à imprecisão da técnica de eletroforese capilar, os perfis moleculares foram identificados e significativamente correlacionados com os estratos de saúde mental previamente identificados, na primeira secção da tese de doutoramento. Seguidamente, uma caixa de ferramentas do tipo de interface gráfica de utilizador, chamada MODeLING.Vis, foi desenvolvida para a mineração dos dados de neuroproteómica adquiridos e para responder à problemática do baixo poder discriminativo da eletroforese capilar. A caixa de ferramentas desenvolvida permitiu a exploração de dados e o teste de hipóteses dos dados biomoleculares. Na segunda fase, o perfil neuromolecular da amostra estratificada e selecionada de 20 jovens adultos neurotípicos foi concluído através da utilização de painel multiplex combinado: o *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* e o *Bio-Plex Pro™ Human Th17 Cytokine Assay.*

RESULTADOS: Numa primeira análise, este estudo descritivo demonstra que, na literatura, 85% das proteínas neuronais estão correlacionadas primariamente com uma condição neuropsicofisiológica. Dessas proteínas neuronais, apenas 13% são de facto identificadas, quantificadas e propostas como biomarcadores de suscetibilidade. Seguidamente, um enriquecimento funcional dessas proteínas que foi executado em comparação com o proteoma do *Homo sapiens* (Human - taxon 9606), evidencia que

as proteínas nos circuitos cerebrais estão predominantemente envolvidas em funções metabólicas, em processos do sistema imunitário e na transmissão de neuropéptidos. O resultado desta primeira análise é a aquisição dos perfis totais de proteínas (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais e Barros, 2017) e a quantificação preliminar das proteínas da gama de peso molecular [9,1;30]kDa, que estão presentes nas amostras salivares dos 92 jovens adultos neurotípicos. Nesta primeira análise, os perfis totais de proteína adquiridos e mais especificamente as características da distribuição proteica no intervalo de [9,1;30]kDa, mostram correlação com os quatro perfis de saúde mental correspondentes e identificados anteriormente. Além disso, essa gama de baixo peso molecular é descrita na literatura como característica de pequenas moléculas neuroimunitárias e de neuropéptidos, e, assim sendo, essa característica oferece uma solução para estudar a resposta neurocognitiva. A partir desta primeira análise e dos resultados obtidos, 20 jovens adultos neurotípicos são então selecionados e estratificados por expressarem o perfil molecular mais significativo na gama de pesos moleculares entre [9,1;30]kDa. O resultado desta segunda análise é que esses 20 jovens adultos neurotípicos têm uma resposta salivar homogénea de Orexina, β-Endorfina, Oxitocina, Substância P e dos mensageiros bioquímicos das células Th17. Estes 20 sujeitos finais têm uma resposta neuropeptídica razoavelmente distinta e um característico equilíbrio anti/pro inflamatório mediado pelas células T-helper 17. Da mesma forma, estes 20 sujeitos representam uma amostra significativa e estratificada dos quatro perfis distintos de saúde mental identificados na primeira secção da tese de doutoramento. Como *milestone* suplementar, é desenvolvido (i) um atlas de biologia molecular para uma melhor compreensão das funções de proteoma neuronal, o MolecularCog (G) Atlas, e (ii) uma extração das funções moleculares das redes preferenciais de interação imunológica e neurológica: a *Neuro-Immune Network* e a *Human Th17 Cytokine Database*.

CONCLUSÃO: A segunda secção deste trabalho mostra alguma evidência que o funcionamento mental distinto em jovens adultos neurotípicos está associado a uma expressão proteica distinta, bem como uma característica resposta neuropeptídica e neuroimune. Estes estratos moleculares de saúde mental podem ser avaliados em investigação de biomarcadores de suscetibilidade presentes na saliva. A identificação de biomarcadores salivares deve levar cuidadosamente em consideração a problemática da estabilidade proteica e do perfil de degradação, bem como as modificações pós-traducionais assim como os relevantes produtos de degradação. Por um lado, a identificação de biomarcadores na saliva pode fornecer informações sobre a saúde mental e sobre sistema nervoso central. Por outro lado, a identificação das proteínas salivares também presentes em amostras cerebrais deve ser levada em consideração com extrema sabedoria e cautela de forma a conservar a veracidade das possíveis correlações existentes entre elas. No caso do nosso modelo de estudo descritivo, correlações explicativas preliminares são feitas entre: (i) a experiência subjetiva, (ii) o perfil total de proteínas, (iii) a Orexina, β-Endorfina, Oxitocina e Substância P, e (iv) a resposta celular do tipo Th17 e os seus mensageiros bioquímicos. Essas correlações preliminares precedem uma quantificação explicativa completa desses constructos de saúde mental/moleculares. Portanto, as relações causais inferidas devem ser abordadas mais detalhadamente utilizando os protocolos de quantificação *gold standard* mais sensíveis e, assim, desenvolver uma melhor compreensão dos biomarcadores de suscetibilidade de saúde mental no cérebro. Finalmente, esta dissertação ilustra, até certo ponto e como prova de conceito, um acoplamento cognitivo-imune em uma

amostra neurotípica e define as bases para uma análise causalista adicional (Hipólito e Martins, 2018).

Secção I And II – Resumo Geral

OUTCOMES: Os Protocolos de Intervenção e de Avaliação isolam de forma efetiva os subfenomas mentais significativos ($p \leq 0,05$) na amostra dos 128 adultos jovens neurotípicos e, consequentemente, permitem a caracterização dos quatro perfis neurotípicos com fenótipos mentais e moleculares distintos: (i) o Subgrupo *Self-Awareness*, (ii) o Subgrupo *Self-Consciousness*, (iii) o Subgrupo *Reflective Self* e (iv) o Subgrupo *Pre-Reflective Self*.

LIMITAÇÕES: Este estudo é uma investigação descritiva que pode ser utilizado na pesquisa de biomarcadores salivares em estados de saúde mental, mas para isso, deve ser validado através de explicações causalísticas com maior validade interna e maior *sample size effect*. O custo total de tal estudo numa amostra de 128 jovens neurotípicos conduz a importantes limitações económicas e científicas na orientação do presente trabalho de doutoramento. Contudo, a possibilidade de desenvolver este tipo de investigação, mesmo com todas as limitações que este estudo acarreta, mostra o interesse científico, a motivação e o esforço do autor para o desenvolvimento desta estratégia de estratificação mental e molecular.

SUBGRUPO *SELF-AWARENESS*: É caracterizado por um fenótipo de elevada consciência do eu, com uma correlação mental e molecular significativa ($p \leq 0,05$). Este subgrupo mostra um perfil distinto na gama de pesos moleculares [9,1; 30] kDa e, mais especificamente, um ligeiro aumento em [9,8; 10,3] kDa e um decréscimo acentuado em [13,7; 17,5] kDa. Isso reflete a elevada expressão molecular dos perfis do traço e estado fisiológico da Orexina, da β-Endorfina, da Oxitocina e da Substância P. Mais especificamente, este fenótipo é caracterizado por um aumento mais significativo nos níveis da Orexina, β-Endorfina e Oxitocina, o que reflete a elevada resposta neuropeptídica que está relacionada ao aumento de funções que são específicas do sistema nervoso autónomo e central. Neste subgrupo, esta característica é responsável pelo aumento da função de recompensa e vigília, e também pela elevada aprendizagem associativa e saliência incentiva. Além disso, essa resposta neuropeptídica está associada ao aumento de emoções positivas, da felicidade, da diminuição do medo e da ansiedade, bem como ao aumento da predisposição à interação social. Mais ainda, existe um efeito correspondente dessa carga cognitiva na carga inflamatória total. No traço e estado fisiológico das interleucinas, este subgrupo mostrou uma expressão pró-inflamatória reduzida nos 15 analitos estudados da resposta celular do tipo Th17, com uma tendência para desencadear uma maior resposta anti-inflamatória. Isso é representado pela expressão aumentada das IL-10, IL-21, IL-25 e IL-33. Assim sendo, os processos biológicos expressos mais significativos do sistema imunitário são a regulação de *switching* de isótopo e a regulação positiva da imunidade mediada por linfócitos.

SUBGRUPO *SELF-CONSCIOUSNESS:* É caracterizado por um fenótipo de autoconsciência ponderada, com uma correlação mental e molecular significativa ($p \leq 0,05$). Este subgrupo mostra um perfil distinto na gama de pesos moleculares de [9,1; 30] kDa e, mais especificamente, um aumento moderado em [9,8; 10,3] kDa e um aumento acentuado em [13,7; 17,5] kDa. Isso reflete uma expressão molecular

moderada dos perfis de traço e estado fisiológico da Orexina, da β-Endorfina, da Oxitocina e da Substância P. Neurofisiologicamente e mais representativamente, o aumento significativo dos níveis da oxitocina, refletem-se no aumento da atenção e na elevada capacidade de aprender característica deste subgrupo. Além disso, essa resposta neuropeptídica nesse fenótipo está associada ao prazer e ao aumento da predisposição para a aprendizagem social. Mais ainda, existe um efeito complementar dessa carga cognitiva sobre a carga inflamatória total. No traço e estado fisiológico das interleucinas, este subgrupo mostrou uma expressão pró-inflamatória menos evidente nos 15 analitos estudados da resposta celular do tipo Th17. Esta resposta é representada pela regulação das IL-4, IL-10, IL-21, IL-25 e IL-33. Assim sendo, os processos biológicos mais significativos do sistema imunitário são a resposta imunitária adaptativa, baseada na recombinação somática de recetores imunes construídos a partir dos domínios da superfamília de imunoglobulinas e a regulação da produção de imunoglobulinas, assim como a co-regulação da imunidade mediada por linfócitos.

SUBGRUPO *REFLECTIVE SELF*: É caracterizado por um fenótipo autorreflexivo, com uma correlação mental e molecular significativa ($p \leq 0,05$). Este subgrupo mostra um perfil distinto na gama de pesos moleculares de [9,1; 30] kDa e, mais especificamente, uma ligeira diminuição em [9,8; 10,3] kDa e em [13,7; 17,5] kDa. Isto reflete a diminuição na expressão molecular dos perfis de traço e estado fisiológico da Orexina, da β-Endorfina, da Oxitocina e da Substância P. Mais importante, a diminuição mais significativa dos níveis da Orexina e da Oxitocina refletem a diminuição da resposta neuropeptídica que está relacionada à diminuição de certas funções do sistema nervoso autónomo e central. Nesse subgrupo e neste fenótipo, essa diminuição é responsável pela diminuição da ativação do sistema de recompensa, e pela menor capacidade de aprendizagem associativa e saliência incentiva. Além disso, esta resposta neuropeptídica está associada a uma diminuição da predisposição para as emoções positivas e a uma tendência à anedonia, ao aumento da ansiedade e à diminuição da disposição à interação social. Além disso, houve um efeito complementar deste perfil cognitivo na carga inflamatória total. No traço e estado fisiológico das interleucinas, este subgrupo mostrou uma expressão pró-inflamatória aumentada dos 15 analitos estudados da resposta celular do tipo Th17, mostrando um fenótipo pró-imune relativo. Isso é representado pelo aumento da expressão de IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L e TNF-α. Assim sendo, os processos biológicos mais significativos do sistema imunitário foram baseados na regulação positiva da (i) produção e secreção de imunoglobulinas, (ii) diferenciação de osteoclastos e (iii) na interação citocina-receptor de citocina.

SUBGRUPO *PRE-REFLECTIVE SELF:* É caracterizado por um fenótipo pré-reflexivo, com uma correlação mental e molecular significativa ($p \leq 0,05$). Este subgrupo mostra um perfil distinto na gama de pesos moleculares de [9,1; 30] kDa e, mais especificamente, uma ausência de aumento ou diminuição em [9,8; 10,3] kDa e um aumento significativo em [13,7; 17,5] kDa. Essa alteração reflete uma expressão molecular marginal dos perfis de traço e estado fisiológico da Orexina, da β-Endorfina, da Oxitocina e da Substância P. Neurofisiologicamente, neste subgrupo e fenótipo, esse perfil de neuropéptidos é responsável por uma ligeira diminuição da atenção, da vigília e da aprendizagem. Além disso, esta resposta neuropeptídica está associada a uma certa apatia e à diminuição da predisposição à aprendizagem social. Neste subgrupo, há então um efeito de uma carga cognitiva exigente sobre a carga inflamatória total. Consequentemente, no traço e estado fisiológico das interleucinas, este subgrupo

mostrou uma expressão pró-inflamatória moderada dos 15 analitos estudados da resposta celular do tipo Th17, mostrando uma tendência para uma resposta pró-imune leve. Esta tendência é representada pela expressão das IL-1β, IL-6, IL-17F, IL-22, IL-31, sCD40L e TNF-α. Assim sendo, os processos biológicos mais significativos do sistema imunitário são a regulação positiva da (i) proliferação de células T, (ii) da produção de imunoglobulinas e (iii) da diferenciação de osteoclastos por interleucinas pró-inflamatórias.

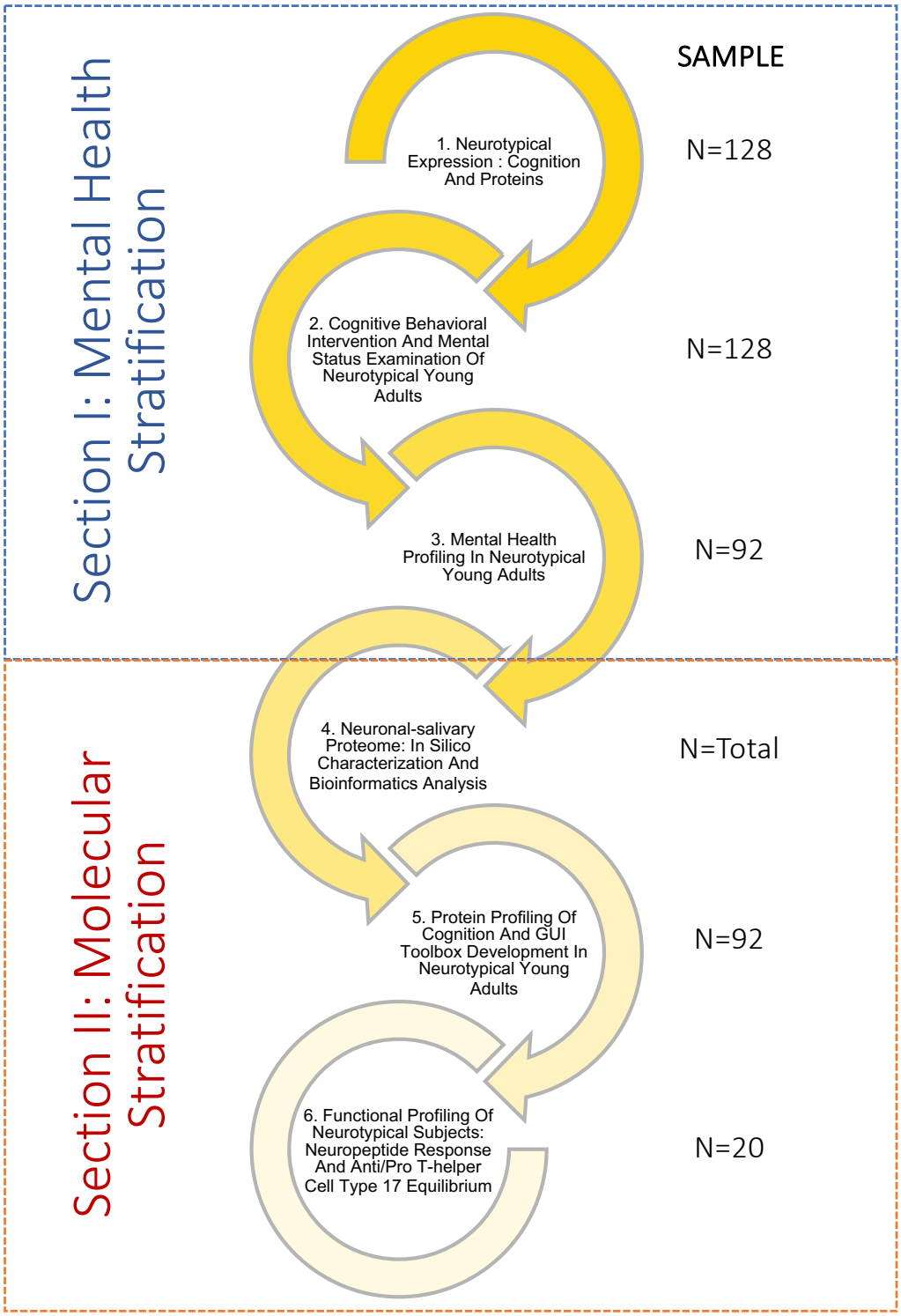


Figure α. Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults.
The 6 different chapters of this PhD thesis are presented, each chapter corresponds to a stage of stratification. The N of subjects are equally presented for each stage.

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Foreword

Expectations

This PhD thesis aims to study a sample of neurotypical young adults, with no neurological and psychiatric pathologies or uncontrolled disorders of the kidney, liver, lung, heart, musculoskeletal, rheumatologic or metabolic, or severe chronic or terminal diseases, which might affect the nervous system.

The study is a descriptive research, used to describe combined cognitive and molecular characteristics of a convenience sample of 128 healthy 19-to-25-year-old university students, recruited from the University of Lisbon (i. Faculty of Medicine, ii. Faculty of Economics, iii. Faculty of Sciences and iv. The School of Social and Political Sciences).

This research does not focus on the causality of the occurred characteristics. Rather than that, it addresses what the cognitive and molecular characteristics of a sample of neurotypical[2] young adults are (Shields and Rangarajan, 2013)[3]. Likewise, this research does not pretend to merely correlate observable data, but to explore its methodological strategy, instead. As such, a descriptive research precedes a full explanatory research.

Henceforth, the limitation of this work is that it cannot be used as the basis of a causal and direct relationship between the cognitive and the molecular characteristics of our neurotypical sample.

Nevertheless, and in spite of the low internal validity of this kind of research, it made it possible to advance with a top-down[4] molecular biology characterization. In fact, the sample size and its characteristics, and the proteomic techniques and methodology offer considerable external validity.

Conceptual Framework

The question raised by this work is if it is possible to probe into neurotypical cognitive states using top-down biomolecular techniques offered by SalivaTec lab, Institute of Health Sciences. It is a *proof of concept*, executed on a limited doctoral budget, aiming for further financial support as a *postdoc* project and to be validated as a method in neuropsychiatric studies, e.g., neurodevelopmental disorders[5].

The work of this PhD thesis comprises two fundamental sections, Section I: Mental Health Stratification and Section II: Molecular Stratification.

Section I: Mental Health Stratification (**Figure 1**) is a neurophenomenological evaluation obtained by a standardized and structured clinical interview. This evaluation

---

[2] not affected with a neurodevelopmental disorder or characteristic of typical neurological development (Merriam-Webster, Inc., retrieved August 12, 2020).

[3] Shields, P. M., & Rangarajan, N. (2013). *A playbook for research methods: Integrating conceptual frameworks and project management*. New Forums Press.

[4] proceeding by breaking large general aspects (as of a problem) into smaller more detailed constituents (Merriam-Webster, Inc., retrieved August 12, 2020).

[5] a condition that is typically marked by delayed development or impaired function especially in learning, language, communication, cognition, behavior, socialization, or mobility (Merriam-Webster, Inc., retrieved August 12, 2020).

examined subjects reports by the application of psychological scales designed to access their subjective experience.

Section I englobes the first 3 chapters:

Chapter 1. Neurotypical Expression: Cognition and Proteins;

Chapter 2. Cognitive Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults;

Chapter 3. Mental Health Profiling In Neurotypical Young Adults.

Each of the 3 chapters are works published, or *in submission*.

These three chapters offer the setting of the theoretical background and the reasons to combine phenomenological[6] with molecular assessments. The outcome was the clinical stratification of the neurotypical subjects, i.e., the identification of mental health strata.

Chapter 1 briefly reviews cognition and proteins expression in neurotypical subjects and the *rationale* of using molecular biology as methodology.

Chapter 2 focuses on the Intervention Protocol and the Psychological Assessment. It was published as original research by the author in first co-authorship and is presented *Facsimile* in Annex A: Original Research as chapter 2 of the PhD Thesis. The referred article was published in *Progress in Biophysics and Molecular Biology*[7] with an impact factor of 3,427.

Chapter 3 explores the clinical and statistical strategy to find mental health strata on a neurotypical sample.

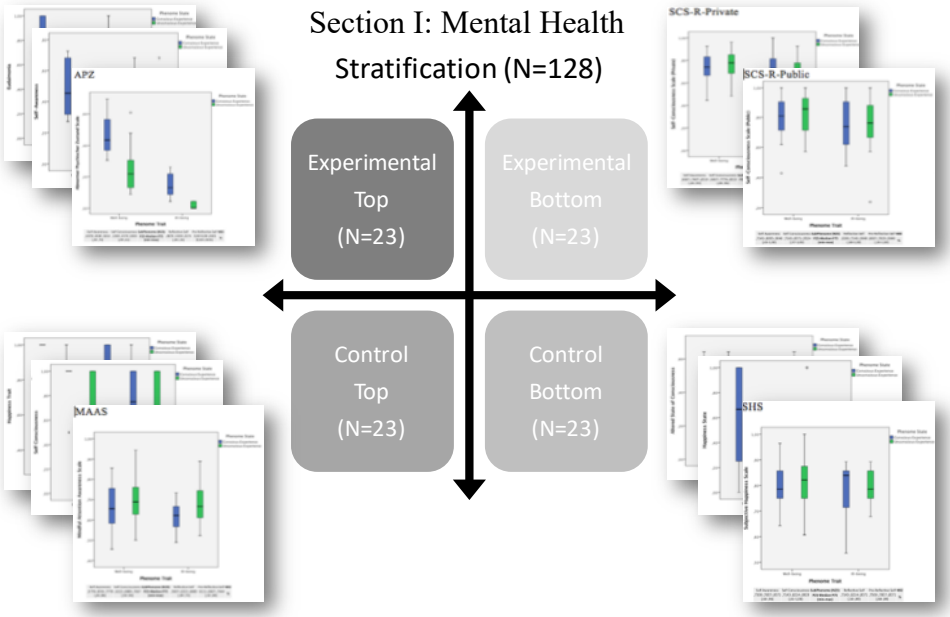


Figure 1. Section I: Mental Health Stratification
Review of the strategy for the stratification used in section I.
From a total of 128 young neurotypical adults,
92 subjects were selected to advance to the molecular stratification (section II).

[6] descritions of the formal structure of the objects of awareness and of awareness itself (Merriam-Webster, Inc., retrieved August 12, 2020).

[7] Hipólito, I., & Martins, J. (2017). Mind-life continuity: A qualitative study of conscious experience. *Progress in Biophysics and Molecular Biology*. Volume 131, 432-444, doi: 10.1016/ j.pbiomolbio.2017.09.004.

Section II: Molecular Stratification (**Figure 2**) is the molecular characterization of the neuronal-salivary protein profile and neuropeptide[8]/neuroimmune[9] response, by *in silico* analysis, total protein profile and simultaneous multiplexed immune detection.

Section II englobes the last 3 chapters.

Chapter 4. *In silico* characterization of the Neuronal Oral Proteome and bioinformatics analysis;

Chapter 5. Protein Profiling of Cognition and GUI Toolbox Development in Neurotypical Young Adults;

Chapter 6. Anti/pro T-helper cell type 17 equilibrium and neuropeptide response in cognitive functional profiling.

Each of the 3 chapters is also a complete work, and they are all *in submission*.

These three chapters offer the parallel molecular profiling of our neurotypical sample with SalivaTec lab tools. The outcome was the molecular stratification of the neurotypical subjects, i.e., the identification of molecular states and traits.

Chapter 4 offers (i) a theoretical revision of susceptibility neuronal-salivary biomarkers, (ii) *in silico* proteomic biobank[10] for the clinical study of the nervous system, and (iii) a molecular reference for correlation with our sample. To attain that objective, in subchapter 4 it is submitted a database with *in vivo* and *in vitro* neuroproteomic studies. Afterwards, a supervision of the discovered proteins is attained by bioinformatics tools. If sets of proteins were identified and sufficiently reported in previously neuropsychiatric studies, those sets of proteins were annotated as possible molecular responses or expressions of our neurotypical sample.

Chapter 5 focuses in the neuronal protein networks identifiable in the salivary protein profile, which may be useful to characterize neurotypical cognition. To attain that objective, in subchapter 5, molecular pathways to physiological states and traits are purposed. The neurotypical sample is probed with a widespread and extensive molecular technique, i.e., capillary electrophoresis[11], so as to find protein profiles. The aim of that analysis is to identify categorical differences in the protein profiles that are correspondent to our neurotypical strata. In order to do so, a visualization strategy with a MATLAB™ toolbox was created. If categorical differences were to be found in an unsupervised learning analysis, the question would be if they corresponded to the previously identified mental-health strata and matched electrophoretic signatures. This unsupervised analysis permitted the clustering of proteins in profiles with a more precise and exact molecular weight range. Those profiles were corresponding with the direct electrophoretic profiles of our mental health strata, while offering higher discriminative power.

Chapter 6 evaluates the distinct neuropeptide and neuroimmune responses and extracts their molecular function and corresponding interaction networks. To attain that objective, in chapter 6 it is used a top-down biomolecular technique named multiplex[12]-

---

[8] an endogenous peptide that influences neural activity or functioning (Merriam-Webster, Inc., retrieved August 12, 2020).

[9] a branch of immunology that deals especially with the interrelationships of the nervous system and immune responses (Merriam-Webster, Inc., retrieved August 12, 2020).

[10] a storage place for biological samples (such as human tissue, blood, or DNA) that may be used especially for future medical research (Merriam-Webster, Inc., retrieved August 12, 2020).

[11] electrophoresis performed in a thin tube subject to capillarity (Merriam-Webster, Inc., retrieved August 12, 2020).

[12] being or relating to a system of transmitting several messages or signals simultaneously on the same circuit or channel (Merriam-Webster, Inc., retrieved August 12, 2020).

biometric[13] immunoassay[14]. This highly sensible and sensitive technique permits the adequate quantification of the categorical differences found in chapter 5. More specifically, it permitted to significatively probe into the sets of proteins identified in chapter 4 and put into evidence in chapter 5. Two main classes of proteins were quantified: small immune molecules and neuronal signaling molecules.

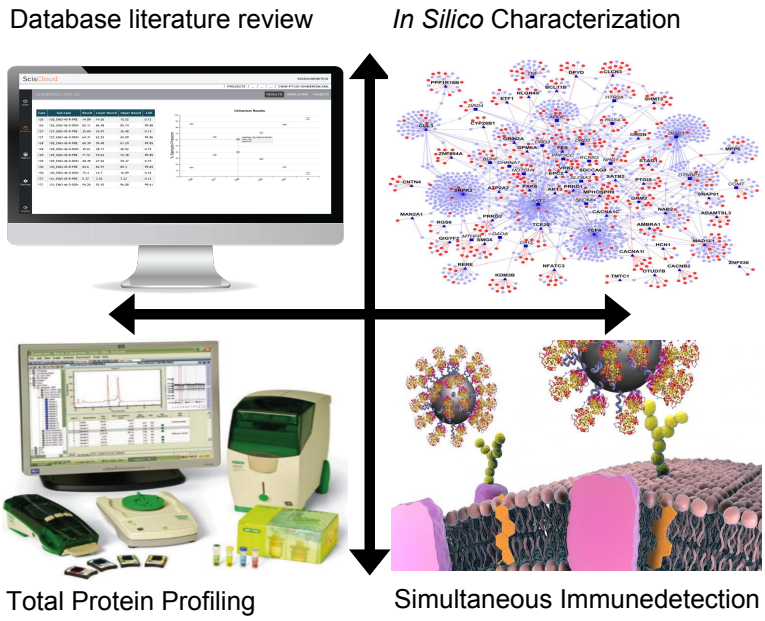


Figure 2. Section II: Molecular Stratification
Review of the different stages of molecular stratification and characterization.
From a total of 92 young neurotypical adults,
20 subjects were selected for the simultaneous immunedetection.

The analysis developed in the doctorate permitted the quantification of specific and exact protein networks and to deepen one's knowledge on the neurophysiology of young neurotypical adults.

This combined mental health and molecular profiling has been published in the II Mind-Brain College Meeting, at the University of Lisbon (Martins et al., 2016c), entitled "Molecular cognition strategy". The publication is accessible online, in a non-restricted access server, in the LIMMIT lab, Faculty of Medicine, University of Lisbon: https://www.limmit.org/uploads/2/6/8/4/26841837/mindbraincollege26out2016molecularcogstrategy.pdf (retrieved August 12, 2020; *in* Annex K: External Support).

In this publication, the author and supervisors explored the strategy used in this PhD for neuronal-salivary protein profile[15] characterization, identification of salivary cognitive-molecular networks and multi-omics datamining[16] solutions.

Finally, in the conclusion of this work, the limitations and outcomes of this descriptive research are addressed.

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[13] measurement of living tissue or bodily structures (Merriam-Webster, Inc., retrieved August 12, 2020).
[14] a technique or test used to detect the presence or quantity of a substance (such as a protein) based on its capacity to act as an antigen (Merriam-Webster, Inc., retrieved August 12, 2020).
[15] the complement of proteins expressed in a cell, tissue, or organism by a genome (Merriam-Webster, Inc., retrieved August 12, 2020).
[16] the practice of searching through large amounts of computerized data to find useful patterns or trends (Merriam-Webster, Inc., retrieved August 12, 2020).

On the one hand, its technical limitations, e.g. the lack of discriminative and quantification power of the capillary electrophoresis used in Chapter 5, led the author to develop adequate data visualization and exploration tools to adequately probe into the *mind-life* strata of the neurotypical young adults.

On the other hand, its outcomes led to the interest of targeting this strategy to study a sample of neuroatypical toddlers, in early-diagnosis and screening.

For that purpose, the author has published a book chapter (Hipólito and Martins, 2018) and intends to develop his *postdoc* by taking advantage of the Horizon 2020 grant: "Molecular insight into Autism Spectrum Disorder (ASD)", coordinated by Universidade Católica Portuguesa and approved by the Coordinated Research Infrastructures Building Enduring Life (CORBEL) consortium. This grant, obtained by the research group the author integrates, aims to study patients with an atypical child development.

As published by Anttila and colleagues (2018)[17], "The high degree of genetic correlation among many of the psychiatric disorders adds further evidence that their current clinical boundaries do not reflect distinct underlying pathogenic processes… this suggests a deeply interconnected nature for psychiatric disorders, in contrast to neurological disorders, and underscores the need to refine psychiatric diagnostics… with cognitive measures."

[17] Anttila, V., Bulik-Sullivan, B., Finucane, H. K., Walters, R. K., Bras, J., Duncan, L., ... & Patsopoulos, N. A. (2018). Analysis of shared heritability in common disorders of the brain. *Science*, 360 (6395), eaap 8757.

Introduction

1. Neurotypical Expression: Cognition And Proteins

Keywords: enactive theory; molecular networks; organism-environment coupling; proteomics; molecular biology; subjective experience.

Cognitive life does not happen solely inside the skull. Indeed, cognition is seen as an expression of a mind-brain continuum (Hipólito and Martins, 2017). Research in molecular biology and cognitive science[18] offers sufficient reasons today to look for a continuity between life and mind, against the traditional ways of conceiving cognitive life as detached from the organism and its environment. Yet, science has been dominated by the commitment to objectively reduce sentient life to physics. Cognition cannot be reduced to brain processes. There are at least some cognitive elements that are not explained by the brain alone. Questions such as the relationship between mind and brain, the nature of consciousness (Re, Ventura, Vitiello, Simões and Martins, 2015, *in* Annex J: Conceptual Analysis), and how we perceive the world, have come to be seen as not answerable by the brain alone. Reasons for such a conclusion come from theoretical arguments (Hipólito, 2018), and empirical investigation, for example in minimal cognition.

This chapter aims to show that even though brain models are the dominant metaphor, until today we do not have empirical evidence for the idea that the brain operates with representations. In fact, research from biology of cognition point towards a different direction. In line with this, we advocate that protein networks might play a role in cognitive life.

The chapter starts by reviewing the second-generation cognitive science proposals coming from enactive and embodied theory that rejects a reduction of cognition to brain computations. The second section attempts to show that such a framework is consistent with recent findings in molecular biology, suggesting a relation between subjective experience and protein networks distributed in the environment. As suggested, this might result from the exchange between the organism-environment coupling. The following section puts forward an empirical method for the investigation of cognitive life understood as organism-environment coupling that combines both enactive theory and proteomics[19]. Ultimately, the empirical method develops the experimental design that we applied in the empirical research, on the nature of the relation between subjective experience and molecular networks.

1.α. Enacting The World: From The Body All The Way Up And Out

Interacting with the world presupposes a body that moves, perceives and acts in the world. Not just through the senses, but through the whole organism, with each of

[18] an interdisciplinary science that draws on many fields (such as psychology, artificial intelligence, linguistics, and philosophy) in developing theories about human perception, thinking, and learning (Merriam-Webster, Inc., retrieved August 12, 2020).

[19] The proteome is the entire set of proteins expressed by a genome, cell, tissue or organism at a certain time. Proteomics is the study of the proteome. The term has been applied to several different types of biological systems. Therefore, a cellular proteome is the collection of proteins found in a particular cell type under a particular set of environmental conditions. A complete proteome can be conceptualized as the complete set of proteins from all of the various cellular proteomes. A human proteome is the complete proteome of the human species (Altelaar, Munoz and Heck 2013; Kim et al., 2014).

its parts integrated with each other, developing an integral and dynamic experience. The interaction is the subjective experience defined by these relational aspects.

Second-generation cognitive science questions traditional theories that conceive cognition as secluded inside the skull, with an indirect access to the world. The idea is that humans perceive in an active exploration of the environment. Accordingly, the ecological niche[20] offers specific possibilities for a specific embodied agent to act or intervene – this is grasped by the notion of *affordance*. The challenge for science, if this is valid is to adopt a conception of nature that does not reduce the embodied agent to a set of computational-neuronal processes.

In moving, perceiving and acting, humans explore a world of affordances. That is, a vivid experience that is instantly constituted between the subject and the environment in a particular situation (Gibson, 1979). This is the idea that a glass is graspable because there are organisms capable of grasping it. Some claim that computational neuroscience has the ability to plausibly explain the phenomenology of affordances. Jakob Hohwy (2018), for example, suggests that "computational cognitive science has the resources to accommodate insights from phenomenology", that is, "to capture the relational states of the world" (paragraph 12). Could phenomenological aspects of affordances be reduced to computational neuroscience? The next section overviews and critically assesses the computational proposal for reducing the exploration of affordances in the body, within the world of brain functions.

1.β. Underlying Molecular Level: Organism-Environment

In the enactive approach, the paradigm is a cell with biomolecular components that constitute the biological individual. Cognitive capacities are extraordinary without having to be complex (Rebertus, 1988; Fish et al., 2006).

Single celled organisms also present cognitive behavior (Marder, 2012; Uria-Diez and Pommerening, 2017). Bacteria are able to perceive, navigate and act in the environment. Likewise, they sense and respond to an astonishing variety of environmental signals, such as amino acids, sugars, oxygen, pH, osmolarity, temperature, light, secondary metabolites, molecular waste products (e.g., ammonia), environmental DNA, and other microbes (both conspecifics and other species), and even physical objects such as tiny, chemically inert beads (Dworkin, 1983).

Mammalian E. coli anticipates what Mitchell and colleagues (2009) call "adaptive prediction", when exposed to nutrient sugars in the gastrointestinal tract. This suggests "evidence in bacteria for capacities encompassed by the concept of cognition" (Lyon 2015, 264). Bacteria are the most basic forms of animal life. They are able to perceive, navigate, and act in the world without having a brain and, consequently, without representing the environment. These ecological niches raise questions of whether cognition should be thought by the architecture of isomorphic representations.

From a functional or anatomical point of view, there is no evidence for the idea of representations, despite the philosophical advantage that cognition would be intelligible by the rules of symbolic language offering tractable strategies to empirically investigate it. Even if the progress of contemporary cognitive science towards understanding the essential principles of operation of cognition is embryonic, one thing

[20] In ecology, a niche is a term describing the relational position of a species or population in an ecosystem. More formally, the niche includes how a population responds to the abundance of its resources and enemies (e. g., by growing when resources are abundant, and predators, parasites and pathogens are scarce) and how it affects those same factors (e. g., by reducing the abundance of resources through consumption and contributing for the population growth of enemies by falling prey to them).

is impossible to deny - in some forms of life, cognition is not representational. These findings are consistent with the enactive theory in which the agent has its own individuality in virtue of its autopoietic organization, that is, of its self-producing and self-maintaining networks. This organization is determined by its own membrane boundary, which actively regulates itself according to the environment (Bitbol and Luisi, 2004; Bourgine and Stewart, 2004; Di Paolo et al., 2017; Maturana and Varela, 1980). Crucially, it is in virtue of this self-organized system that the organism is qualified as a self-defining and self-determining autonomous agent (Thompson and Stapleton, 2009).

The core form of biological autonomy relies on the linkage between sensory affecters and effectors within the body, and thereby integrates the organism as a mobile and unified autonomous sensory-motor agent. For this reason, the nervous system establishes and sustains a sensory-motor cycle, whereby what the organism senses varies directly on how it moves, and how it moves depends directly on what it senses.

Moreover, biological selfhood, in its cellular form, emerges from the autopoietic system, whilst sensory-motor selfhood arises as a consequence of how the nervous system assimilates its somatic structures. Importantly, the organization of the system is shaped by the system itself. Such self-produced organization defines the system's identity and determines a perspective or point of view in relation to the environment.

Systems organized in this way enact or bring forth what counts as information for them (Thompson, 2005); they are not transducers or functions for converting input instructions into output products, and, hence, it is reasonable to invoke the concepts of selfhood[21], consciousness[22], and the sense of agency[23] to describe them.

1.δ. The Molecular-Inactivist Method: Protein Networks Expressing Subjective Experience

1.δ.1. Brief Definitions: Phenome And Proteome

The aim of this section is to put forward an empirical method for the investigation of cognitive life understood as organism-environment coupling that combines proteomics and enactive theory. But before that, we offer a brief definition of the basic concepts in molecular biology[24]. As a method, molecular biology comprises a series of operational protocols used for performing research, high-throughput[25] analytic techniques (Romualdi, 2009), e.g., transcriptomics, metabolomics, proteomics, to propose specific testable hypotheses about an experimental validation, and then using the newly acquired quantitative description of biological processes to refine the studied theory (Alberghina and Westerhoff, 2005). Moreover, it may endorse a dynamical

[21] total character distinguishing an individual from others (Merriam-Webster, Inc., retrieved August 12, 2020).

[22] the state of being characterized by sensation, emotion, volition, and thought (Merriam-Webster, Inc., retrieved August 12, 2020).

[23] initiating, executing, and controlling one's own volitional actions in the world.

[24] a branch of biology dealing with the ultimate physicochemical organization of living matter and especially with the molecular basis of inheritance and protein synthesis (Merriam-Webster, Inc., retrieved August 12, 2020).

[25] method for scientific experimentation using data processing/control software and sensitive detectors, that allows a researcher to rapidly identify active compounds, antibodies, or genes that modulate a particular biomolecular pathway.

systems theory[26] of the molecular model (Voit, 2012). Finally, it offers a "multi-data for scientific phenomenon defined by the strategy of pursuing the integration of complex data about the interactions in the studied biological model from diverse experimental sources, using interdisciplinary tools (Baitaluk, 2009). By discovering how a function arises in dynamic interactions, molecular biology addresses the missing links between molecules and physiology. Two main approaches of molecular biology can be distinguished:

i. Top-down molecular biology identifies molecular interaction networks on the basis of correlated molecular behavior observed in "omics" studies and it is a method to characterize cells using systems-wide data. These models are often phenomenological but serve to discover new insights into the molecular network under study (Bruggeman et al., 2007).

ii. Bottom-up molecular biology does not start with data, instead it examines the mechanisms through which functional properties arise in the interactions of known components with a detailed model of a molecular network and its properties. In this approach, molecular networks can be quantitatively studied leading to predictive models (Bruggeman and Westerhoff, 2007).

1.δ.2. Macro-Functional Networks Vs. Micro-Functional Networks

A phenomics study involves the human proteome[27] analysis, i.e., the entire set of proteins expressed by a genome, cell, tissue or organism at a certain time, under a particular set of environmental conditions (Kim, 2014). Consequently, a proteomics (Altelaar, Munoz and Heck, 2013) study design may characterize a neuronal-salivary proteome, reflected in a specific fluid, i.e., saliva, which a) is representative of all cellular expressions of the organism system, and b) whose probing or sampling implies a minute system invasion. Mental functioning and more specifically the different states and traits of consciousness (Martins et al., 2016b, *in* Annex G: Hypotheses and Theory) can be seen as a phenome state and trait: the sum of all the phenotypic traits associated to that physiological state, studied as molecular biology methodology.

Consciousness has been studied as macroscopic cartography of synaptic connections in a macro-network, the neural networks perspective. However, and at the intracellular scale, micro-networks should be addressed as they regulate the physiological mechanisms for the preferential attachment of synapses and assumedly for the somatic correlate of the enactive approach.

Brain relies on flexible architecture networks molded by local and non-local remodeling of proteins and synapses of excitatory and inhibitory neurons. This dynamic protein network structures a network topology. "The neural networks are up or down, regulated via intracellular metabolic, immunity and regulatory networks of the genome-transcriptome-proteome interface" (Foster, 2015).

1.δ.3. Neural Correlates Vs. Somatic Biomarkers Problematic

Few findings describe the specific biomarkers that can be elicited through inducting a psychological state, or state of consciousness, for instance by a

[26] an area of mathematics used to describe the behavior of the complex systems and can be used to study the nature of systems that arise in biology, employing differential equations.

[27] a phenome is the set of all phenotypes expressed by a cell, tissue, organ, organism, or species, representing the total sum of its phenotypic traits. Phenomics is the study of the phenome and how it is determined, particularly when studied in relation to the set of all genes (genomics) or all proteins (proteomics) (Siebner, Callicott, Sommer and Mattay, 2009).

cognitivebehavioral intervention. Few relationships with psychoneuroimmunology[28] models (Sobrinho et al., 2003) were presented in the past.Therefore, different ways of reaching, perpetuating and intensifying a specific state of consciousness, i.e., mental state, should be scientifically approached, because they have rarely been associated with biochemical markers.

In fact, specific mental states associated with an increased self-consciousness have only been extensively and specifically associated to the endogenous N,N-dimethyltryptamine pathways (Barker and Strassman 2012; Barker et al., 2013), obtained by 2D-LC-MS/MS. Biochemical approaches like this could, in principle, produce biomarkers of mental states that make it possible to report the phenomenological experience (Owen et al., 2006). Therefore, this scientific gap leads to thoroughly explore other neural and somatic correlates of consciousness, possibly through salivary molecular biomarkers (Martins et al., 2016a).

1.δ.4. "Omics" Methodologies And Cognition

One of the subjects of study in molecular biology is psychoneuroimmunology (**Figure 3**).

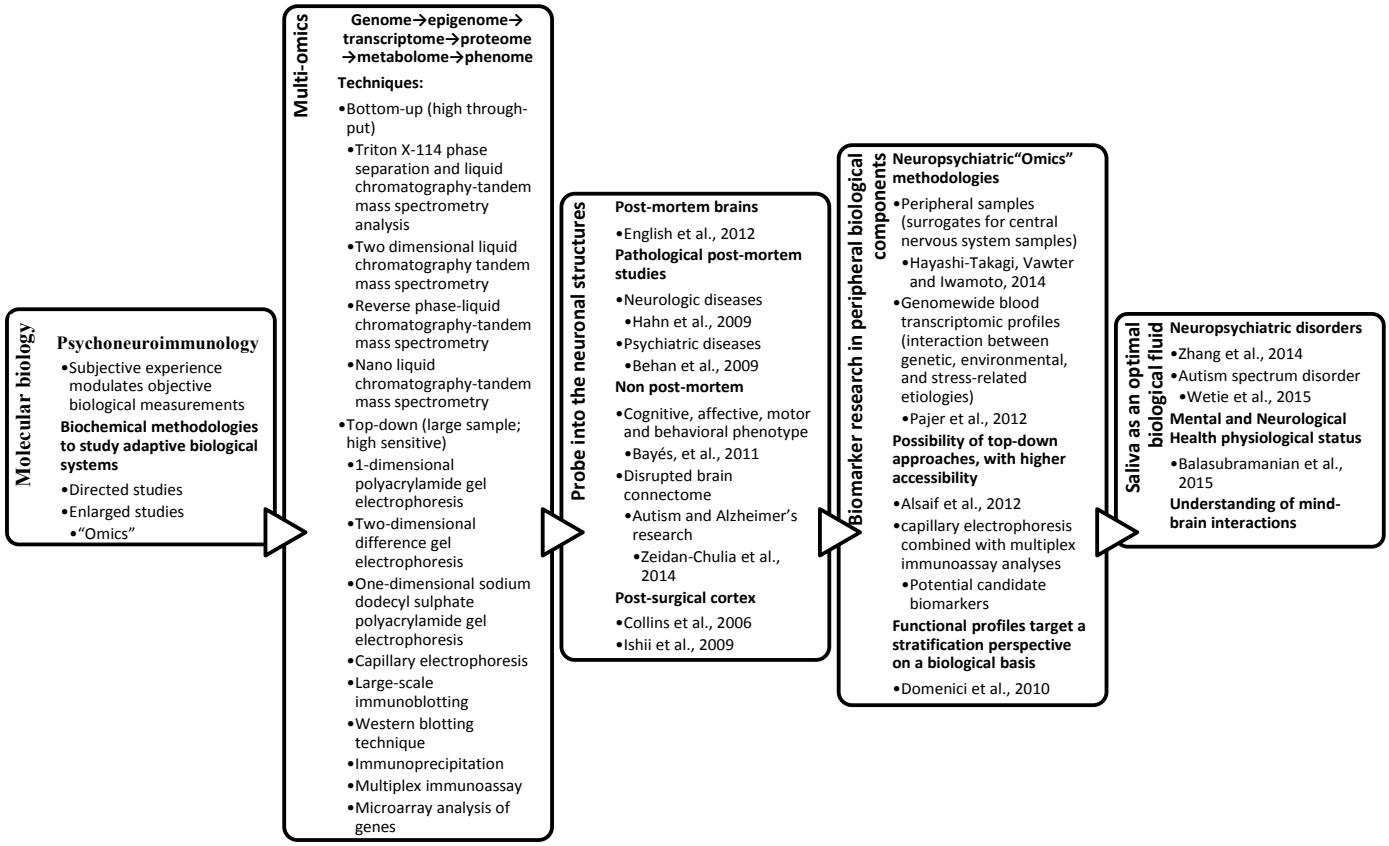


Figure 3. Revision Of "Omics" Methodologies And Its Applications To The Study Of Cognition
Review of the molecular biology approach to study the neuropsychiatric conditions and psychoneuroimmunology, since 2006. The spectrum of multi-omics techniques are presented, and more specifically the advances in the study of the neuronal structures. Likewise, the state-of-the-art on biomarker research in peripheral components is presented, and more importantly the use of saliva as a diagnostic tool.

[28] a branch of medicine that deals with the influence of emotional states and nervous system activities on immune function especially in relation to the onset of disease (Merriam-Webster, Inc., retrieved August 12, 2020).

Figure 3 presents the organization of the proteomic data used for the study of the nervous system in the last decade.

Omics is a subfield of biomedical sciences that intends to study the interplay between mind, body, environment and behavioral characteristics (Ulvestad, 2012); specifically, how the subjective experience [29] modulates objective biological measurements.

The subjective experience is biologically relevant since it has an impact on all adaptive systems, including the endocrine, immune, and neurological ones, not only for the unfolding of an organism's healthy status, but also for the development of malfunctional traits.

There are two biochemical approaches or methodologies to study adaptive biological systems: a) directed studies, also known as classical biochemical studies and b) enlarged studies, also known as "omics" biochemical studies.

"Omics" methodologies have been recently gathering a complex and extensive quantity of data that allowed biomedical sciences to assimilate the concept of psychoneuroimmunology, and to address it in a molecular biology framework. Consequently, methodologies, such as the one proposed by Ritchie and colleagues (2015), assess biological systems via multiomics [30], this is, from the genome, epigenome, transcriptome, proteome and metabolome to the phenome.

"Omics" studies started to characterize different biological substrates. Nevertheless, only when it started to probe into the neuronal structures (**Figure 3**), was it possible to relate it to the whole human biological system.

A first study in humans was conducted in *post-mortem* brains (English et al., 2012), and defined neuroproteomics as a "powerful platform for targeted and hypothesis-driven research, providing comprehensive insights into cellular and subcellular disease states, genes and environmental effects".

The analysis of subproteomes, such as neuronal structures, has become increasingly important in clinical proteomics, discovering otherwise undetectable proteins which might be possible markers for disease. For instance, the quantification of the membrane proteins is one of those subproteome classes that are specifically important for neuropsychiatric disorders.

Through Triton X-114 phase separation and liquid chromatography-tandem mass spectrometry analysis, membrane proteins were enriched and identified, and it eased the quantification of glutamate receptors, G proteins, voltage gated and calcium channels, myelin and synaptic proteins. Furthermore, post-surgical cortex proteomics studies offered a better and more physiological characterization of the neural proteome, specifically myelin and the postsynaptic density, which is a protein dense specialization attached to the postsynaptic membrane.

Consequently, studies performed by Ishii and colleagues (2009) described the proteome of myelin, formed by oligodendrocytes in the central nervous system, which is critical for axonal functions.

The understanding of the molecular mechanisms of myelination and the pathogenesis of human myelin disease has been partly limited by the relative lack of identification of its functional characterization. 1-dimensional polyacrylamide gel

[29] refers to the emotional and cognitive impact of a human experience as opposed to an objective experience which are the actual events of the experience. While something objective is tangible and can be experienced by others subjective experiences are produced by the individual mind.

[30] a biological analysis approach in which the data sets are multiple "omes", such as the genome, proteome, transcriptome, epigenome, metabolome, and microbiome. In other words, the use of multiple omics technologies to study life in a concerted way.

electrophoresis and liquid chromatography/tandem mass spectrometry identified proteins into biological subgroups and discussed their relevance in myelin biogenesis and maintenance.

Additionally, Collins and colleagues (2006) characterized the composition of the postsynaptic proteome that provided a framework for understanding the overall organization and function of the synapse.

Analysis by liquid chromatography tandem mass spectrometry and large-scale immunoblotting identified proteins in purified postsynaptic densities. For instance, 9 in AMPA-receptor immuno-purifications, 100 in isolates using an antibody against the NMDA receptor subunit NR1, and 170 by peptide-affinity purification of complexes with the C-terminus of NR2B. In total, 1124 synaptic proteins were found as a basis for future research in synaptic biology, providing, not only useful information in the characterization of physiological healthy synaptic functioning, but also in brain disease and mental disorder studies.

Pathological *post-mortem* studies followed, and Hahn and colleagues (2009) finally encompassed this set of studies into the characterization of specific neuropsychiatric diseases. They suggested a "molecular genomic technique of various trans-synaptic processes for pathophysiological mechanisms of neuropsychiatric illnesses".

Therefore, two-dimensional liquid chromatography tandem mass spectrometry observed known postsynaptic proteins by mass spectrometry, and afterwards, immunoprecipitation and immunoblot methods revealed physiologically expected protein-protein interactions and specific proteome microdomains. These microdomains, found by Behan and colleagues (2009), in the dorsolateral prefrontal cortex, were strongly implicated in the pathogenesis of schizophrenia and bipolar disorder. Within these microdomains, abnormalities in glutamatergic neurotransmission and synaptic function were extensively described.

Two complementary proteomic methods were used: two-dimensional difference gel electrophoresis and one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by reverse phase-liquid chromatography-tandem mass spectrometry.

As a final result, from an extensive proteome, these combined high-throughput bottom-up techniques, found three proteins with roles in synaptic function: syntaxin-binding protein 1, brain abundant membrane-attached signal protein and limbic system-associated membrane protein, for post western blotting validation of potential biomarkers of schizophrenia and bipolar disorder.

Complementarily, non-*post-mortem* proteomics (Bayés, et al., 2011) also characterized the postsynaptic density from human neocortex and identified 1,461 proteins and genomic mutations causing 133 neuropsychiatric diseases, possibly reflecting a whole cognitive, affective, motor and behavioral phenotype underpinned by this combined genomic and proteomic research. This wide-ranging study opened doors to the cognitive functional profiling.

Likewise, a disrupted brain connectome (Zeidan-Chulia et al., 2014) supported autism and Alzheimer's research, respectively seen as neurodevelopmental and degenerative diseases. The amyloid-β precursor protein-a has been shown to be elevated in both. "A focused microarray analysis of genes belonging to NOTCH and WNT signaling cascades, as well as genes related to Alzheimer's disease and apoptosis pathways in cerebellar samples from autistic individuals, provided further evidence for pathological relevance for these cascades in both diseases".

Based on these results, a model of NMDA glutamate receptor-mediated ERK activation of α-secretase activity and mitochondrial adaptation to apoptosis was explained as the early brain overgrowth and disruption of synaptic plasticity and connectome[31] in autism. This study shows the relevance of an analytical biomolecular consideration in the assessment of autism's cognitive impairment and in the impact that proteomics studies have in the understanding of social cognition (Hipólito and Martins, 2018; Hipólito, 2016).

"Omics" methodologies have been used to understand neuropsychiatric diseases in other biological components (Hayashi-Takagi, Vawter and Iwamoto, 2014). Peripheral samples (**Figure 3**), such as blood and skin, have been used in neuropsychiatric research as surrogates for central nervous system samples. Although the validity of the data obtained from peripheral samples has been questioned when compared to other state-of-the-art techniques, such as neuroimaging, omics methodologies seem to increase its value.

In "omics" studies, however, the utility of peripheral samples is mandatory, as a non-invasive and accessible biological substrate, for both:

a) the development of functional and physiological profiles of disease state and trait pathways;

b) tailor-made therapies, such as multimodal assays.

Moreover, these biomolecular advances may be inter-complementary to neuroimaging, in terms of the high sensibility and specificity of their diagnostic capabilities.

The understanding of the neurobiological underpinnings of neuropsychiatric disorders and its cognitive structures may improve its evaluation and treatment, which is still mostly based on a subjective assessment of symptoms. Therefore, "functional profiles from peripheral samples target a stratification perspective on a biological basis" (Domenici et al., 2010).

One of the possible peripheral sample fluids that may be used is plasma. In plasma, Domenici and colleagues (2010) identified possible unspecific biological markers, which included a series of cytokines, chemokines and neurotrophins, for major depression – insulin and MMP-9, and for schizophrenia – BDNF and EGF.

Furthermore, research for potential functional networks profiles of cognitive and affective impairment combined multi-omic techniques (Pajer et al., 2012). Early-onset major depressive disorder multi-omic analysis combined results from genome wide[32] blood transcriptomic profiles, representing the interaction between genetic, environmental, and stress-related etiologies of major depressive disorder.

Similarly, the use of different biological fluids (Alsaif et al., 2012), serum and plasma, allowed top-down proteomic approaches, with higher accessibility. This top-down proteomics, such as capillary electrophoresis combined with multiplex immunoassay analysis, may offer a more efficient methodology when compared to bottom-up techniques, in terms of large sample high-throughput high-sensitive study designs.

[31] a comprehensive map of neural connections in the brain, and may be thought of as its "wiring diagram". A connectome includes the mapping of the major neural connections within an organism's nervous system.

[32] study of genetic variants in different individuals to see if any variant is associated with a trait. Typically focus on associations between single-nucleotide polymorphisms and traits like major human diseases, but can equally be applied to any other genetic variants.

Alsaif and colleagues (2012) showed, by multiplex immunoassay analysis, 190 different proteins in these biofluids, which were identified as potential candidate biomarkers in bipolar disorder patients.

Opportunely, the use of saliva (**Figure 3**) has shown to be an optimal biological fluid for multi-omics science, in terms of accessibility (Zhang et al., 2014). Saliva is a biofluid comprising secretion of the salivary glands, oral mucosa cells, blood and gingival crevicular fluid.

Analogously and equivalently to serum, saliva contains standardized biomolecules, present in other biofluids, such as DNA, mRNA, microRNA, protein, metabolites and microbiota. This homogeneity prompts saliva as a suitable peripheral sample.

A salivary omics study conducted in a person with autism spectrum disorder (Wetie et al., 2015; Hipólito and Martins, 2018) showed that proteomics with salivary biofluids, using mass spectrometry, identified initial putative biomarkers.

Saliva, thenceforward, started to provide an easily accessible biomaterial for analysis. Wetie and colleagues (2015), using nano liquid chromatography-tandem mass spectrometry, found a full proteome, including, for instance, elevated prolactin-inducible protein, lactotransferrin, neutrophil elastase and polymeric immunoglobulin receptor.

These results indicate that this is an efficient method for the identification of psycho-neuro-immuno-endocrinological functional profiles characterizing behavior and cognitive states and traits.

Having the psycho-neuro-immuno-endocrinological functional profile in consideration, the healthy physiological status should also be evaluated. This physiological status, previously described, is "related to the self and the conscious experience concept" and, as published by Balasubramanian and colleagues (2015), it has "salivary protein functional networks".

In that study, obtained with salivary proteomics, molecular alterations were discovered by mass spectrometry, e.g., deleted in malignant brain tumor-1, and subsequently confirmed by western blotting technique. These data were the first to demonstrate the feasibility of stimulating and detecting salivary protein biomarkers in response to a cognitive stimulus, through the techniques mentioned previously.

Concluding, a new field of "omics" studies may step forward into the understanding of mind-brain interactions. By the same token, there have already been neuro-psycho-pharmacological breakthroughs in the characterization of receptoromics and psychoactive compounds (Re, Palma, Martins and Simões, 2016, *in* Annex H: Review).

"Neuro-psycho-pharmacological research is not only the study of perception, emotion, and cognition, but also the discovery of molecular mechanisms and targets likely to yield insights into the biomolecular underpinnings of human consciousness or mind[33]" (Roth et al., 2004).

Additionally, it is likely that the elucidation of these molecular targets is of the utmost importance for central nervous system drug discovery and portrayal of its proteome encoding receptors – the neural receptorome: 5-HT1A, 5-HT1D, 5-HT2, 5-HT2B, 5-HT2C, 5-HT5A, 5-HT6, 5-HT7, α2A, α2C, β2, H1, σ2, D1, D3, I1, κ, μ, CB1.

The scientific knowledge gathered by these techniques and methods has endorsed insights into specific phenomenological experiences, such as neuropsychiatric

[33] the complex of elements in an individual that feel, perceive, think, will, and especially reason. The organized conscious mental events and capabilities in an organism. (Merriam-Webster, Inc., retrieved August 12, 2020).

disorders or physiological healthy states, prompting opportunities for new diagnostic strategies.

1.δ.5. Analytical Proteomic Techniques

Neuropathophysiological mechanisms studied functional protein networks, e.g., astrocyte Serum S-100 family (Martens, Raabe and Johnsson, 1998), correlated with the prediction of regaining consciousness after global cerebral ischemia (Miao et al., 2007).

"Physiological mechanisms have described that phenomes composed by strong emotional states also modify the salivary protein biomarker profile" (Chiappelli, Iribarren and Prolo, 2006). These are examples of potential proteins obtained by 2D-LC-MS/MS, or other proteomic methods.

In addition, peptides such as encephalins might also serve as additional parameters for influencing and reflecting baseline states and traits of cognition, as measured by, for instance, a phenomic/proteomic approach (Susan, Greenfield and Collins, 2005). To our knowledge, no research seems to have combined analytical proteomic techniques with changes in physiological states resulting from the self-conscious experience[34] (Martins et al., 2016a). Recently, stress biomarkers that reflect mental states have been identified in saliva (Okochi, Fujiki and Yamanaka 2015).

So far, we have attempted to show that the physiological approach through the molecular biomarker analysis of saliva might be a genuine and interesting framework for the objective research of consciousness, as the "sensitivity and utility of assessing central nervous system conditions using peripheral or distal human biomaterials is becoming increasingly possible" (Wormwood et al., 2015).

A molecular biology approach may, ideally, generate associated functional protein networks related to specific phenotypic states and traits, i.e., the different functional and physiological states of mental health. Moreover, the subjective experience might be objectively assessed through proteome analysis in a phenomics study design. Cognitive life is about the interactions between a living organism and its environment through a dynamic interaction and it can conceivably be measured by changes in the total protein profile and networks.

2. Cognitive Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults

Keywords: autopoiesis; cognitive behavioral intervention; conscious experience; psychological assessment.

A challenging question in science remains to be answered, viz. why does physical sensory stimulation have to be accompanied by subjective experience[35]?

[34] in addition to its manifestation in first-personal thinking, self-consciousness is also present in various forms of sensory and non-sensory experience. Self-conscious experience has representational content and is first-personal.

[35] Conscious experiences such as: when an idea comes to mind; when a childhood memory suddenly returns; when we enjoy a piece of music, or taste a good wine; when we read a novel, or write a letter; or when we watch the sunset, or visit an art gallery, and so on.

Subjective phenome[36] which constitute an important mental texture is difficult to describe and have thus far been excluded from scientific investigation.

This is a very difficult topic for experimental research, particularly because science starts by quantification, and assumes that psychological experience can be expressed in purely quantitative terms (Weber's law). Of course, "scientists recognize that only because something escape quantification, it does not mean they are to be dismissed from science" (Varela, 1997).

How, then, do we explain subjective phenomena in a non-reductive examination of the human mind[37]? Psychology, neuroscience, philosophy, and physics, among other areas, study the nature of the subjective phenomenon and their progressive specialization has marked scientific progress. This specialization and separation were necessary, but it has inevitably worked against the aim of a plurally constituted science (Simeonov, 2010). Owing to this, in this thesis, it is proposed to look at a different perspective within the study of the mind and subjective experience in a sample of healthy participants. This perspective is based on the principle of interdependence between constituting disciplines studying mind, behavior and proteins. In this study, it is our aim to show empirically how theoretical cooperation between these different schools may shed light not only on the development of mental and behavioral disorders, but also its relation to the diseases of the nervous system. A plurally constituted approach can be rigorously formulated to accommodate qualitative analysis and quantitative measurements. In this study, it is combined a psychological assessment with a molecular measurement of protein profiles, with the aim of contributing to the phenomenological investigation of subjective experience. This is a study in which we focus on the intervention methodology adopted, viz. cognitive-behavioral interventions (Kendall and Hollon, 2013). The chapter begins by introducing the hard problem of consciousness, discussing the various answers it offers with regard to the phenomenological investigation of subjective experience. A focus is given in particular to the *neurobiology of consciousness*[38] and *enactive*[39] hypotheses.

2.α. The Hard Problem Of Consciousness

It is uncontroversial that consciousness is present when, for example, a student attends a class, or in a spectator's excitement at a football game. These activities can be described molecularly as processes whereby excitation on the sensory surface is conducted by nerve fibers to nerve centers, switched over to efferent nerves, and resulting in muscle contraction or gland secretion. Ordinary man, however, knows nothing of the molecular processes of conscious experience, but he certainly knows *what it is like* to experience a football game or university class. The problem of consciousness has occupied the best human minds for thousands of years, and has generated two most common ways of facing the *hard problem of consciousness* in science, the (1) *physicalist* and the (2) *dualistic* view.

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[36] conscious experiences such as: when an idea comes to mind; when a childhood memory suddenly returns; when we enjoy a piece of music, or taste a good wine; when we read a novel, or write a letter; or when we watch the sunset, or visit an art gallery, and so on.

[37] thesis that claims that human mind is an ontological and causal network that is fundamentally physical and, however, cannot be reduced to nor fully explained by basic physical science.

[38] hypothesis that aims to find cellular and molecular mechanisms and improve the general concepts of consciousness.

[39] hypothesis defined as the manner in which a subject of perception creatively matches its actions to the requirements of its situation.

1. The whole problem is illusory. This response holds that there are not two kinds of substance or modes of existence, matter, and mind, but only one, viz. matter. Matter is composed of whirling atoms that, owing to their great numbers and time available, from all sorts of combinations, among them those we call animals and human beings. Thus, it is scientific conviction that thinking, and feeling are merely movements of atoms, even including the feeling of awe accompanying the moment of remarkable scientific discoveries. This materialistic conception of mind has served science well, building up physical and physiological knowledge. Nevertheless, difficulty resides in the fact that between life and mind this conception of consciousness makes an arbitrary discrimination with regard to scientific dignity. They have accepted matter and rejected mind, while each of them may in fact disclose as much of the truth as the other.

2. The second solution draws a Cartesian line between life and inanimate nature belonging together. However, this response does not solve but rather *re-emphasizes* the problem. Numerous speculative attempts have been made to overcome dualism. But pretending that a new name provides a solution to it may do a great deal of harm to science were it to be widely accepted.

Cartesian dualism is theoretically rejected in contemporary science, although, as previously mentioned, in practice we see too often a gap between the body and the mind in science. The procedure is either to look for the subjective phenomena in the brain (bottom-up paradigm), or to theoretically reject the subjective phenomena while investigating brain function (top-down paradigm). Science cannot empirically deny subjectivity as an ontological phenomenon, nor can it verify or falsify subjective phenomena.

Subjective phenomena have been identified as the *hard problem of consciousness* (Chalmers, 1995). The *hard problem* concerns why physical stimulation must be presented to consciousness with a *subjective feel*. As David Chalmers frames problem (1995, p. 3).

It is widely agreed that experience arises from a physical basis, but we have no good explanation of why and how it so arises. Why should physical processing give rise to a rich inner life at all? It seems objectively unreasonable that it should, and yet it does.

The subjective aspect of experience, elicited from sensory stimulation, was illustrated by Frank Jackson (1982) in a thought experiment known as “the knowledge argument” against physicalism. It can be summarized as follows. Mary is a neurophysiologist who happens to be color blind from birth, but nevertheless specializes in perception and color. Imagine that she knows all there is to know in physical terms about perception before she in fact perceives red for the first time. Would she know *what it is like* to perceive red? Notwithstanding all the knowledge Mary has about the neurophysiology of sight however detailed, it may be this knowledge cannot give her the experience of what it feels like to see a red rose.

Jackson’s thought experiment attempts to establish, first, that there are non-physical properties, and second that knowledge of these properties can only be attained through conscious experience. Thus, Jackson’s thought experiment attempts to refute the claim that all knowledge is physical knowledge. To the contrary, conscious experience must involve non-physical properties because someone with complete physical knowledge about some phenomenon might yet lack knowledge about *how it feels* to experience the phenomenon itself. From this it follows that Mary does not know *what if feels like* (Nagel, 1974) to see red until she in fact sees red, sc. until she has had the conscious, phenomenal experience of red.

Easy problems, on the other hand, are those that fall within the scope of neuronal and cognitive function. These are usually studied through statistical and computation measurements. Such measuring techniques include behavioral models of yes/no responses (button-pushes, time-responses, etc.), and neuroimaging. Cognitive functions, such as memory, attention, sensory stimulation, are empirically investigated and explained by the standard methods and tools of cognitive science, viz. by computational or neural mechanisms (Chalmers, 1995, p. 2).

On these models, to explain access (to consciousness) and reportability (language), for instance, we need only specify the mechanisms by which information about internal states is retrieved and made available for verbal report. To explain the integration of information, we need only exhibit mechanisms by which information is brought together and exploited by later processes. For an account of sleep and wakefulness, for example, an appropriate neurophysiological account of the process responsible for an organism's contrasting behavior in those states will suffice. As Chalmers (1995, pp. 2-3) explains: "In each case, an approximate cognitive or neurophysiological model can clearly do the explanatory work […] If these phenomena were all there was to consciousness, then consciousness would not be much of a problem."

In cognitive science, the *hard problem of consciousness*, or of subjective phenomena, comes up again and again. Indeed, it is well- known to those who study brain function, such as LeDoux, Antonio Damásio, Gerhard Roth, and Francisco Varela.

There are thus two fundamental models to understanding the phenomenon of mind. The first is the computational model, based on symbolic thinking paradigm; the second is the biological organism model, based on the natural life paradigm. The latter involves the thought that we should understand mind from the perspective of life and evolution. In what follows, a brief introduction and discussion will be made on the two versions proposed within the biological model (*neurobiology of consciousness* and *enactive approach*)

2.β. Neurobiology Of Consciousness

In recent years, neuroscientists have intensively studied the cerebral activity correlated with conscious experience. Patterns of cell activity have been detected from PET and fMRI scans, which are commonly seen as showing the mind in action. It is said that it is possible to visualize where mental arithmetic occurs or where words are formed, for instance (albeit what we are in fact witnessing are changes in oxygen levels in the blood during these mental activities).

The embryonic neurobiology of consciousness was introduced by Crick and Clark in their *The Astonishing Hypothesis* (1994), followed by *The Quest for Consciousness* by Koch (2004), which further lays down paths to be explored in the next several decades.

Koch is optimistic that careful scientific research will provide the answers, but is less impressed with the theoretical limits imposed by philosophers. Although Koch admits that philosophers have frequently formulated questions that challenge scientists, philosophical constraints are often overlooked (Martinez-Conde, 2004). Their model proposes a neurobiological account of experience.

Koch narrows down the neural correlates of consciousness for visual consciousness to coalitions of neurons in and around the inferior temporal cortex, projecting to the frontal lobes and supported by feedback activity from the cingulate

and frontal cortices. The function of consciousness, he states, is to provide an "executive summary" of the world's status to the planning stages of the brain (i.e., the frontal lobes).

Thus, consciousness may be an intermediate-level process (see Jackendoff's, 1987) limited to intermediate brain areas. However, a main difficulty regards on how to ontologically conceptualize, "visual consciousness". In empirical investigation, one should be cautious when establishing ontological correlations of levels of reality. It seems the object of Koch's study is 'vision awareness', that is, the relation between attentional function and vision stimulation, which would fall naturally within the purview of the *easy problems*. It has, however, nothing to do with how subjects experience a certain phenomenon.

In other words, it fails to explain the phenomenological experience accompanying sensory stimulation.

Antonio Damásio proposes a theory about the neural basis of consciousness that contests Koch's idea (Damásio, 1994, 2010). On Damásio's view, consciousness is a private, first-personal phenomenon that can be studied by combining cognitive and neurobiological methods in order to describe its cognitive nature, behavioral correlates, possible evolutionary origin, functional role, and finally its neuroanatomical and neurophysiological underpinnings (Damásio, 1998).

Phenomenal conscious content is, in Damásio's view, a matter of associations that are processed in different brain areas at the same time. More specifically, what makes a conscious state feel like something rather than nothing is explained as a fusion of mind and body, in which neurons become "extensions of the flesh." Phenomenal consciousness is enabled as the result of a procession of neural maps of inner and outer worlds. Indeed, this is what Damásio names *self-consciousness*. Without a self, the mind would lose its orientation: thoughts would be freewheeling, unclaimed by an owner, and we would almost certainly look unconscious (Damásio, 2010; chap. III).

Damásio makes a distinction between three levels of "self", which build upon each other to construct the kind of full-blown consciousness that humans are privileged to experience.

On his view, the most basic form of self - the "protoself" - is a neural map of an organism's internal status, i.e., related to primordial feelings of the body present in the normal, waking brain ((Damásio, 2010; chap. III, p. 191).

The "core self" is what further extends the "protoself", namely by incorporating interactions between an organism and its environment. It provides personhood, and firmer senses of being.

At the highest level, there is the "autobiographical self", which adds information about an organism's past and its expectations about the future. With language as a useful medium, memory and reasoning are enhanced. With this enhancement, narratives become possible, giving the "core self" the sense of being a protagonist, that is, an "autobiographical self". The autobiographical self is the construct that will be stimulated with the intervention protocol of this thesis.

Damásio's theory is largely centered on the notion and levels of self as exemplified by the brain. His theory relies on neuroimaging techniques and determines that experience results from internal or external physical stimuli. This view therefore requires understanding experience on its reductive construal. Damásio (1994, 2010) rejects dualism by bringing consciousness back to the body (sc. brain function). The reduction of experience to brain function returns us squarely to a reductionist view of consciousness.

2.δ. The Enactive Approach And Autopoiesis

The Enactive[40] approach in cognitive science is a biological model that emerges from the theory of *autopoiesis*. *Autopoiesis*[41] aims to correlate the data from brain, mind, and consciousness studies (particularly neuroscience laboratory work), with phenomenological insights in the understanding and explanation of subjective experience, and ongoing reflection on one's own personal consciousness (Varela et al., 1993; Varela, 1996, 1999; Hayward and Varela, 1992).

In other words, the internal structure of the organism changes and adapts according to its environment. The structure of the living organism, together with its previous history of perturbations, determines the reactions that new perturbations will induce. Changes, mutations, and evolution are here seen as the result of maintaining the internal structure of an autopoietic organism.

Cognition, as seen here, contrasts with the representational/cognitivist model. This is because it consists in a mutual interaction between the inner structure of the system and the environment, in the sense that the environment is 'created' during interaction itself.

According to this theory, the very notion of consciousness derives from the autopoietic organization of life: there is no human life without consciousness, and there is no consciousness that is not embodied in organic life.

This perspective culminated in the neurophenomenological method (Varela, 1996), which is, according to Varela (1997, in Rudrauf et al., 2003): "Grounded on a pragmatic will to progressively and systematically reduce the distance between subjective and objective […] a way of narrowing the gap between the mental and the physical."

2.ε. Self And Cognitive Behavioral Intervention

The idea of self is seen as the enclosed capacity "to order my own thoughts and my own life, to use reason as an instrument to control and order my own life … what's really important is not the particular content of our feelings or thinking but the power to control it reflexively (Varela, 1997, p. 18). The self is thus internalization of our experience: feelings and so forth arise in response to certain experiences (Brockman, 1996).

Studies conducted in university students demonstrated that the self-training, as the paradigm employed in cognitive behavioral interventions, derives from cognitive modeling and imagery rehearsal. As behavior therapy techniques were altered to incorporate cognitive factors, the likelihood of achieving metacognitive access[42] also increased (Kendall and Hollon, 2013).

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[40] theory that argues that cognition arises through a dynamic interaction between an acting organism and its environment.

[41] *Autopoiesis* is a theory concerning the inextricable link between life and cognition, insofar as one cannot exist without the other. As Luisi and Houshmand (2010, pp. 85-86) elaborate: "[The] starting point is the interaction between the autopoietic unit and the environment. The living unit is characterized by biological autonomy and at the same time is strictly dependent on the external medium for its survival."

[42] awareness or analysis of one's own learning or thinking processes (Merriam-Webster, Inc., retrieved August 12, 2020).

According to Evan Thompson (2014), one sort of cognitive incorporation was possible by focused attention interventions, as the one used in this thesis. These interventions are well developed and achieved in cognitive behavioral intervention, where the subject attends to features of experience that are ordinarily ignored or neglected. In other words, the participant can notice things that he infrequently does (Thompson, 2014). Thus, focused attention interventions can be understood as a kind of phenomenological training that can serve the scientific study of mind (Crane, 2017). The objective of this type of intervention is to enable participants to relate to their thoughts as objects of awareness, i.e., as arising "mental events" (Segal and Teasdale, 2018).

Henceforward, the psychological intervention, i.e., a cognitive behavioral intervention, used in this study is often described in literature as a systematic process of familiarizing the subject with a present character of a mental event, cultivating his capacity for sustained and attentive awareness (Thompson, 2006).

3. Mental Health Profiling In Neurotypical Young Adults

Keywords: biomarker research; clinical biostatistics; mental health profiling; neurophenomenology; neurotypical; self-report measures.

Whereas mental health is seen as an expression of a mind-brain *continuum* (Hipólito and Martins, 2017), healthy individual cognition remains an intriguing question for neuroscience, cognitive science and health sciences (Re, Ventura, Vitiello, Simões and Martins, 2015).

In this work, we formulate the hypothesis that cognition is embodied[43] in rich mental constructs (Hipólito, 2015a). Understanding mental health involves the understanding of experiential, embodied, socially and culturally situated factors, which contribute to make each person's experience what it is (Gomez-Marin and Mainen, 2016).

To understand what mental health is, in the healthy individual, it is necessary to address not only its molecular expression, but its clinical/psychological features first (Hipólito and Martins, 2018). These features should reflect "a state of physical, mental, and social well-being and not merely the absence of disease or infirmity" (WHO, 1948).

Cognitive profiling has been important in the stratification of neurologic disorders such as, for instance, Parkinson Disease (Biundo et al., 2014). Likewise, the cognition of neurotypical subjects, i.e., their cognitive phenotypes and endophenotypes[44] (Bearden et al., 2016), reflects specific clinical and mental health constructs (Pacheco, Martins and Simões, 2019, *in* Annex F: Book Chapter Review).

A healthy neuropsychophysiological functioning (Shafer, 2015) may be described, by molecular biology, as a healthy phenome, i.e., the healthy set of all phenotypes expressed by an organism, representing the total sum of its phenotypic traits (Siebner, Callicott, Sommer and Mattay, 2009).

A phenome is usually composed of functional states and traits (Lawrence, 2005) and can, typically, be studied by its subphenomes or functional subgroups.

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[43] theory that many features of cognition are shaped by aspects of the entire body of the organism.

[44] is a term used to separate behavioral symptoms into more stable phenotypes with a clear genetic connection.

Measurements of brain response are based on the premise that different cognitive states are accompanied by different neurophysiological states. A state alludes to altered sensory, perceptive, and self-referential awareness[45], whereas, a trait refers to the lasting neurological and mental changes (Austin, 1998; Shapiro, 1984; West, 2015).

The cognition of the healthy individual may reflect a more focused and aware mental state and trait. These states and traits are thought to be characterized by good physiological functioning and neuroplasticity, possibly leading to new brain correlates (Lutz et al., 2008).

In fact, those states and traits induce long-term changes in self-consciousness, observed both in high- and low-level cortical representations (Cahn and Polich, 2009). Moreover, it has been shown that those cognitive states have an important role in self-knowledge (Simões, 2002).

These functional changes can be induced by psychological interventions, which modulate conscious experience. Both natural and induced changes have been described in literature (Cahn and Polich, 2006).

The natural ones correspond to what is named trait mindfulness; whereas the induction ones involve a second individual that leads the induction and facilitates the achievement of a desired mental state or trait (Cahn and Polich, 2009). These are well studied and have been recognized as an effective clinical tool (Sobrinho et al., 2011).

These referred techniques are also a vehicle for inducting subjective states of happiness and well-being (Hagerty et al., 2013), which have been described as constructs associated to health in neurotypical subjects.

One way of attaining this cognitive state and trait is by a cognitive behavioral intervention (Segal and Teasdale, 2018). Trait mindfulness is described as sustainable awareness aiming at a non-reactive consciousness (Kabat-Zinn, 2003; Farb et al., 2007).

In fact, trait mindfulness[46] (Bishop et al., 2004; Hanley and Garland, 2014; DaSilveira, DeSouza and Gomes, 2015; Asai et al., 2016) is a physiological state and trait distinct from exclusive pre-reflective or reflective self-experience. This phenome state might be induced with a cognitive behavioral intervention (Segal and Teasdale, 2018), which has two interlinked components:

(1) a sustained reflective self, to the present moment (awareness), related to the quality of the conscious experience in the very moment of its occurrence (Cardaciotto et al., 2008);

(2) an open and acceptant attitude towards the experience (arousal), the pre-reflective self-experience.

This specific mental state (Halsband et al., 2009; Hinterberger, Kamei and Walach, 2011) concedes a continuous process of internal and external self-monitoring[47], which has been shown to lead to neuropsychophysiological changes (Hagerty et al., 2013; Simões et al., 1998; Hinterberger, Schöner and Halsband, 2011).

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[45] suggesting that the human being interprets incoming information in relation to himself, using his self-concept as a background for new information.

[46] A mindful trait, i.e., trait mindfulness, promotes physical and mental well-being with positive emotional traits (Brown and Ryan, 2003), and modulates the limbic-neocortical emotional systems connectivity (Aftanas and Golosheykin, 2005; Bishop et al., 2004).

[47] monitor of self-presentations, expressive behavior, and nonverbal affective displays. It is defined as a personality trait that refers to an ability to regulate behavior to accommodate social situations.

Until now there is no valid and reliable instrument that probes into the healthy individual cognition, but only into singular functional subconstructs (e.g., well-being or *eudaemonia* (Ryan & Huta, 2009)).

Yet, different publications have altogether probed into subconstructs that are exclusive of mental health functioning in neurotypical subjects (Pacheco, Martins and Simões, 2019). For a clinical evaluation of mental health and experiential variables, self-report measures are usually used, assessing those state and trait variables.

In this chapter, we propose a method used as a clinical and statistical strategy for profiling those neurotypical subjects.

Additionally, this method, i.e., mental health profiling, aims to address the problematic of adequate accuracy[48] and replicability[49]. Accuracy and replicability are usually the challenge of standard molecular predictive research when probing into clinical profiles (Malottki et al., 2016).

This strategy may be an advantage in finding strata (Pylkkönen, Drugman and Bisani, 2016), called subphenomes in this study and used for mental and cognitive subgrouping of the neurotypical subjects. For instance, we aim to define specific mental strata like the ones that led to the mental health constructs already purposed by Da Silveira, De Souza and Gomes (2015).

These mental health strata or profiles are central in stratified medicine research (Trusheim, Berndt and Douglas, 2007), and more specifically in the subfields of psychiatry (Schumann et al., 2014) and neurology (Matthews et al., 2014).

3.α. Problematic Of The Reliability Of Psychological Measuring

Psychological measures (Parsons, Kruijt and Fox, 2018) have not yet developed a tradition of routinely examining reliability. Test-retest reliability assessment is crucial in the development of psychometric tools, helping to ensure that measurement variation is due to replicable differences between people regardless of time, target behavior, or user profile.

Reliable measures are essential to draw robust inferences from statistical analyses, while subpar reliability has severe implications for the measures' validity and interpretation. Without examining and reporting the reliability of psychological measurements, it is near impossible to ascertain whether results are robust or have arisen largely from measurement error.

Thus, report measurement reliability should be a routine practice so more reliable assessment tools can be developed. Parsons, Kruijt and Fox (2018) purposed different statistical strategies, i.e., coefficient procedures as the ones used in chapter 3 of this thesis, to account for test-retest reliability and estimate internal consistency. Consequently, with adequate test-retest reliability analyses, common errors may be avoided.

As a gold standard and as performed in this thesis, it is thus imperative that the materials (psychological measures) that are used in the assessment protocol provide sufficient psychometric information to estimate the accuracy of inferences and better inform the results of the cognitive behavioral intervention. As known, psychological research and clinical practice rely heavily on psychometric testing for measuring

[48] degree of conformity of a measure to a standard or a true value (Merriam-Webster, Inc., retrieved August 12, 2020).

[49] refers to whether the results from the experiment can be replicated if repeated exactly the same way, providing statistical evidence that shows that the results can be used to predict outcomes in other experiments. An example of evidence that can suggest replicability is the probability value, or p-value.

psychological constructs that represent not only symptoms of psychopathology, but also individual difference characteristics or cognitive profiles (Aldridge, Dovey and Wade, 2017).

Nevertheless, and in order to better defend the reliability of the psychological states and traits and the measures used in this thesis, this problematic will be further discussed and tested in the materials and method of chapter 3.

Effectively, the mental status of the subjects studied in this work is maintained over time, and remain similar, if the same conditions of the Intervention Protocol are applied. Note that the psychological variable studied is the memory traces of autobiographical memory, which is a measure that "remains constant in neurotypical individuals without psychiatric disease, such as schizophrenia, or neurodegenerative conditions, such as dementia" (Sutin, 2008).

In this thesis, the stability of the memory traces of autobiographical memory is therefore maintained because there were no external stimuli, not properly controlled or monitored.

Furthermore, the mental state's stability is ensured in the absence of the cognitive behavioral intervention, as well as in the sham intervention used in this thesis, since it is a fundamental nature or characteristic of a mental state if no external stimulation is given. In the experimental procedure, because there was only a single cognitive behavioral intervention (Segal and Teasdale, 2018), the modification (or "instability") of the psychological measures is to be attributed to this intervention.

4. Neuronal-Salivary Proteome: *In silico* Characterization And Bioinformatics Analysis

Keywords: biomarkers of susceptibility; clustering analysis; Cytoscape; database literature review; *in silico*; gene ontology (GO); neuronal proteome; salivary proteome; PANTHER.

4.α. Saliva, A Biological Fluid

Saliva is a human fluid that continuously bathes the mucosa of the oral cavity (Rosa et al., 2012). This fluid is essential for the maintenance of oral health (Rathnayake et al., 2013) and is characterized by "having numerous biological functions, such as: antimicrobial and antiviral activity; acting as a barrier against pathogens; tissue lubrication; buffer capacity; and prominent in food intake and tasting" (Shultz, Cooper-White, Punyadeera, 2013).

In addition to the important role in maintaining homeostasis of the oral cavity system, saliva is considered a means for monitoring health and disease states, both in the oral cavity and, more interestingly, in systemic tissues and organs (Giannobile and Wong, 2011). In general, a healthy adult produces on average 500-1500 mL of saliva per day at a rate of approximately 0.5 mL/min.

Several physiological and pathological conditions (such as: oral mucosal bleeding, multiple sclerosis, epilepsy, cystic fibrosis) can modify their production quantitatively and qualitatively (Pfaffe et al., 2010). Not only smelling and tasting stimulate the production and secretion of saliva, but also psychological and hormonal states. Moreover, drug intake, age difference, hereditary influences, oral hygiene and physical exercise may influence saliva chemical and molecular composition (Nunes et al., 2015).

Saliva secretion comes from three large pairs of salivary glands: parotid (20%), submandibular (65-75%) and sublingual (7-8%) (Yoshizawa et al., 2013). These are responsible for the production of approximately 90% of the total saliva, whereas the minor salivary glands secrete the remainder (Greabu et al., 2009). The major glands are located in and around the mouth and throat, whereas the smaller glands (approximately 600) are located throughout the oral cavity (lip, tongue, palatoglossus and palatal mucosa) (Rosa et al., 2012).

Like blood, and its subproduct fluid, saliva is composed of a huge variety of electrolytes, vitamins, hormones, enzymes, antibodies, growth factors, antimicrobial constituents, cytokines and other proteins (Javaid et al., 2016). Total saliva, or whole saliva, may, therefore, be analyzed to assess the existence of systemic diseases.

Total saliva is a mixed fluid, which is derived not only from the major and minor salivary glands, but also from non-salivary sources, containing gingival crevicular fluid, desquamated epithelial cells, bronchial secretions, but, more importantly, serum and blood derivatives, bacteria, viruses, fungi and their subproducts (Thirrunavukkarasu, 2010).

As regards to salivary flow, salivary composition and concentration of each protein, there is an interindividual variation present, depending on different internal factors, like the circadian rhythm, or environmental factors, like the proximity of meals. Salivary flow properties may also be affected by other factors such as age, oral microbial enzymes, protease activity, systemic diseases, nutrition, gender and alcohol consumption (Walsh, 2007).

Several constituents can be found in saliva, in smaller amounts (sodium, magnesium, chloride), higher amounts (potassium, calcium, bicarbonate, phosphate) and in similar concentrations (uric acid), when compared to blood (Castagnola et al., 2011).

The mechanisms by which these molecules and substances arise in the salivary fluid from the blood are multiple and may occur through passive diffusion and active transport (intracellular pathways), and by ultrafiltration (extracellular pathway) within the salivary glands or from the gingival crevicular fluid (Malathi et al., 2014).

4.β. Saliva, A Diagnostic Tool

As previously mentioned, saliva has been recognized as a physiological "mirror" of body health, as it integrates constituents that can be measured and used to assess health and systemic disease states.

As a diagnostic fluid, saliva presents some distinct advantages compared to serum, particularly because it can be collected non-invasively and by non-specialized individuals. Moreover, this can be achieved with relatively simple equipment, providing a “promising cost-benefit approach for the screening of a large number of people, and top-down proteomics studies” (Al Kawas, Rahim and Ferguson, 2012).

For the study of neuropsychiatric conditions, the non-invasive collection is of utter importance because it dramatically reduces anxiety and discomfort. As proposed by Miller and colleagues (2010), the collection does not alter the physiological and mental state of the subjects, when sampled. Moreover, it increases individuals’ willingness to perform single analysis or multiple analysis, with shorter intervals.

These collection conveniences can be used in challenging clinical situations, such as obtaining samples from children, neuroatypical subjects (Hipólito and Martins, 2018) or patients with disabilities, in which collection through the blood could be a difficult act to perform (Al Kawas, Rahim and Ferguson, 2012).

In addition, technically, it is also easier than blood to handle during diagnostic procedures as it does not coagulate, thus reducing the number of manipulations required. Besides, and from a clinical security perspective, for health professionals, it is considered as a minimal risk of infection during the collection (Castagnola et al., 2011).

Considering that proteins are the most important constituents of saliva, a comprehensive analysis, through proteomics studies in recent years, has led to the identification of the whole proteic content of saliva. This comprehensive identification is considered "essential to characterize the functions of salivary proteins and to reveal potential biomarkers involved in different pathophysiological conditions" (Zhang et al., 2013). However, the description of the state of the art of the proteomes is not only to be able to use this information to better understand biological systems, but prominently to use it in clinical settings.

An overall analysis of human salivary proteomes can provide a comprehensive spectrum of general health, through changes in the expression of proteins (Lee and Wong, 2009), but it can also reflect a spectrum of neuronal proteins produced in the central and peripheral nervous system.

To achieve these neuropsychiatric nosology objectives, the identification of the presence of proteins is not yet considered sufficient, that is to say, these objectives require a better characterization of the neuronal-salivary proteome (Miller et al., 2010).

With the important advances in the technology used in the field of proteomics, namely mass spectrometry[50], it was possible to identify and characterize proteins and peptides in a more accurate approach (Al-Tarawneh, 2011). This precise approach is leading to a wide range of applications for neuroproteomics and it is becoming ideal for the detection of molecular biomarkers, i.e., proteins, mRNA and DNA (Helmerhorst and Oppenheim, 2007). In this way, human neuronal-salivary proteomes represent a field of biomedical research, both for the diagnosis and for the monitoring of various neuropsychophysiological conditions (Miller et al., 2010; Al Kawas, Rahim and Ferguson, 2012).

4.δ. Saliva, A Probe Into Neuropsychiatric Health And Conditions

A biological process detected in the neuronal-salivary proteome may be seen as a (dys)regulation of a specific neuronal pathway. This (dys)regulation may generate a specific etiology of the neuronal system (Ansermet and Magistretti, 2004; Zhang et al., 2013; Schafer et al., 2014).

In this work, we propose a revision of all the molecular biology studies probing into the nervous system, with the objective of generating an embedded type database. Database literature reviews have been proposed, systematically, as a useful tool in molecular biology (Bolender and Bluhm, 1992). Our database presents an overall representation of the neuropsychiatric diseases. The pathologies are identified by their MESH ID (Lowe and Barnett, 1994) and comprehend the etiologies of ICD-10's[51] (WHO, 2016) chapter VI "Diseases of the nervous system (G00-G99)" and chapter V

[50] an instrumental method for identifying the chemical constitution of a substance by means of the separation of gaseous ions according to their differing mass and charge (Merriam-Webster, Inc., retrieved August 12, 2020).

[51] International Statistical Classification of Diseases and Related Health Problems 10th Revision.

"Mental and behavioral disorders (F00-F99)". So, both ICD-10 G and F dimension are generally characterized (**Figure 4**).

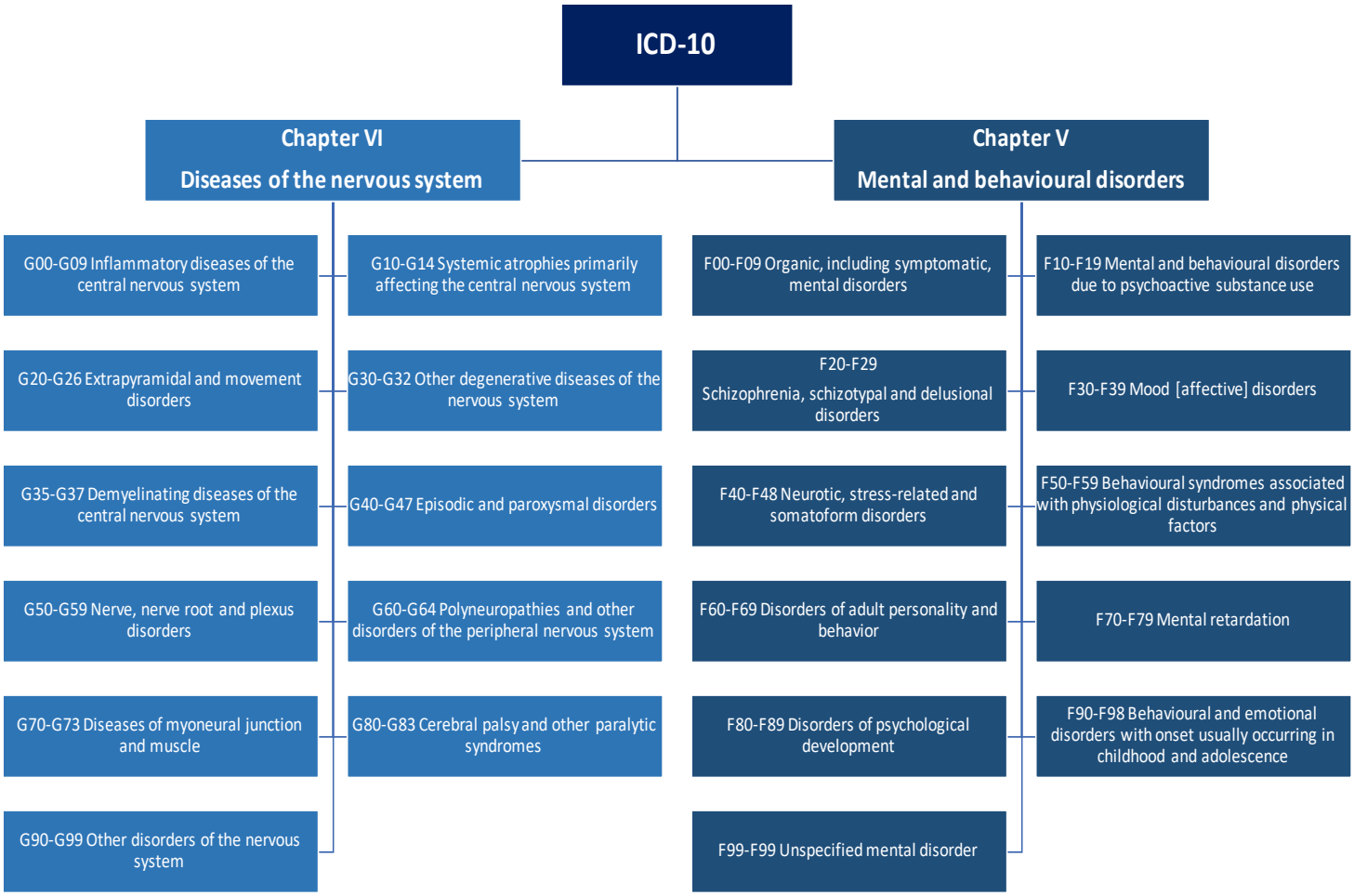


Figure 4. Organigram ICD-10
A review of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) conditions included in the database of this thesis.
Chapter VI and Chapter V were included for the overall representation of the neuropsychiatric diseases.

Hence, an overview of the pathologies affecting the central and the peripheral nervous systems are correlated to their molecular identification. That molecular identification is mapped to a correspondent UniprotKB code (UniProt Consortium, 2016).

On the one hand, the database elusively addresses the molecular expression, via UniprotKB codes of neurological pathways of: G00-G09 Inflammatory diseases of the central nervous system; G10-G14 Systemic atrophies primarily affecting the central nervous system; G20-G26 Extrapyramidal and movement disorders; G30-G32 Other degenerative diseases of the nervous system; G35-G37 Demyelinating diseases of the central nervous system; G40-G47 Episodic and paroxysmal disorders; G50-G59 Nerve, nerve root and plexus disorders; G60-G64 Polyneuropathies and other disorders of the peripheral nervous system; G70-G73 Diseases of the myoneural junction and muscle; G80-G83 Cerebral palsy and other paralytic syndromes; and G90-G99 Other disorders of the nervous system.

On the other hand, biological pathways, via UniprotKB coding, are also elusively proposed for psychiatric pathologies: F00-F09 Organic, including symptomatic, mental disorders; F10-F19 Mental and behavioral disorders due to psychoactive substance use; F20-F29 Schizophrenia, schizotypal and delusional disorders; F30-F39 Mood [affective] disorders; F40-F48 Neurotic, stress-related and somatoform disorders; F50-F59 Behavioral syndromes associated with physiological disturbances and physical factors; F60-F69 Disorders of adult personality and behavior; F70-F79 Mental retardation; F80-F89 Disorders of psychological development; F90-F98 Behavioral and emotional disorders with onset usually occurring in childhood and adolescence; and F99-F99 Unspecified mental disorders.

The same analysis is rather carried out to address the molecular correlates of a non-pathological state, i.e., of neurotypical subjects, or of a healthy state without a neuropsychiatric condition. The fundamental objective, which is further explored in the next chapters, is to propose an easy access database for hypothesis making of not only neuropsychiatric pathologies, but also in healthy mental functioning.

5. Protein Profiling Of Cognition And GUI Toolbox Development In Neurotypical Young Adults

Keywords: cognition; data-mining; data exploration; data visualization; neurobiology; total protein-profiling.

In this work, we place the hypothesis that not all causal, or constitutive factors of experience, are to be found simply in the brain. Cognition is embodied (Hipólito, 2015b), and comprehends understanding how the brain is communicating with the body. These relationships could be reflected by the salivary total protein profile. Hence, the salivary protein profile may then be useful when stratifying a mental health state.

The proteome, which is a part of the phenome[52], is the entire set of proteins expressed by a cell, tissue, organ or organism at a certain time. Proteomics is the study of the proteome[53]. Changes in the protein profile, such as the increase or decrease of a protein, or its absence, indicate that the underlying molecular mechanisms are also compromised or altered. The evaluation of the total protein profile present in a sample of tissue, or biological fluid, of an individual exposed to different challenges allows the verification of the proteome. This verification of whether there is a typical or non-typical protein profile may characterize the phenome response to a specific challenge. Additionally, the identification and quantification of which proteins are altered allows their use as response biomarkers. In summary:

(i) the determination of the total protein profile;

(ii) the subsequent quantification of the altered proteins enables the creation of a strategy to evaluate, at a molecular level, biopsychological phenomena.

[52] a phenome is the set of all phenotypes expressed by a cell, tissue, organ, organism, human being (Siebner et al., 2009), and phenomics is the study of the phenome.

[53] proteins are the product of gene expression and have a fundamental role in executing instructions and enabling molecular mechanisms. These molecular mechanisms are activated as a response to a specific internal or external challenge or signal in each individual. Therefore, proteins are what executes the phenome. If the proteome expressed under different challenges is characterized, a precious tool for phenome identification is found.

Saliva is an important biological fluid (Martins et al., 2016a) that provides various functions and is produced by multiple salivary glands. Saliva is composed of proteins such as enzymes, enzyme inhibitors, growth factors, cytokines, immunoglobulins, and other glycoproteins (de Almeida et al., 2008). Saliva is a filtration of blood, reflecting the physiological conditions of the body (Sugimoto et al., 2010); thus, it could be used to monitor clinical *status* and predict systemic diseases. Compared with blood, saliva offers distinct advantages for diagnostic or research purposes; its collection is cost-effective, safe, easy and non-invasive (Rosa et al., 2016). Moreover, several molecular biomarker candidates have been identified by analyzing the proteome or transcriptome of saliva (Hu et al. 2007, 2008; Zimmermann and Wong, 2008).

The proteomics and transcriptomics are part of omics sciences, just like genomics, epigenomics, metabolomics, lipidomics and interactomics. Omics allow "large-scale studies of genes and interactions and it is the foundation and the driving force of molecular biology" (Wang and Bodovitz, 2010). Omics[54] is a scientific subject that endeavors to quantify all the molecular elements of a biological system and (i) to assess their interactions and (ii) to integrate them into graphical network models. Those models serve as predictive hypotheses to explain emergent behaviors (Hood et al., 2004). Henceforth, omics strives to identify, quantify and characterize all the components in cellular systems with spatiotemporal resolution, and thereby dissect the intracellular pathways and networks. Currently, major technologies for proteomics mainly include mass spectrometry (MS) and protein arrays (Wang and Bodovitz, 2010).

Salivary protein profiling is defined in general as identifying the group of proteins expressed in saliva, under a specified set of conditions at a particular time. The profile is then compared to the expression of reference samples. This information can be useful in the discovery of biomarkers, in diagnosis, as well as, in understanding response mechanisms at the protein level (Kingsmore, Nallur and Schweitzer, 2003). The protein profile is obtained using proteomic techniques like capillary electrophoresis. This technique separates proteins based on principles similar to those of standard slab-gel electrophoresis, but with the advantages of faster run times, higher resolution and greater sensitivity.

Classically, capillary electrophoresis profiles represent "a direct measurement of protein via the peptide bonds, an accurate method for quantifying their relative concentrations" (Wijnen and van Dieijen-Visser, 1996). However, sufficiently sensitive and reproducible electrophoretic saliva-based diagnostics are not yet available, specifically in probing into mental health.

Classical total protein profiling has already been validated (Esteves et al., 2017) and proposed for different biomedical applications. It can be used for phenotypic stratification, as proposed by recently by SalivaTec Lab (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017; Cruz et al., 2018). The total protein profile acquired by capillary electrophoresis offers a practical and cost-wise solution for obtaining a simple protein profile, with a significant sensibility and specificity (Esteves et al., 2017). However, this classical technique lacks the discriminative and quantification power of modern proteomic techniques, i.e., mass spectrometry (not used in this thesis due to financial matters) or simultaneous immune detection (further explored in Chapter 6).

[54] in cellular and molecular biology, forming nouns with the sense "all constituents considered collectively"

Hereafter and to propose salivary protein profiles (Saavedra Silva, Sousa, Silva, Martins, Esteves, Fernandes, Rosa, Correia, Barros, 2017) with a higher sensibility, bioinformatics applications, i.e., toolboxes, offer an integrated software environment for better protein profile analysis. They provide access to proteomic data formats, analysis techniques, and specialized visualizations for proteograms (Henson and Cetto, 2005).

Experion™ Automated Electrophoresis System, i.e., the system offered straightforwardly by the manufacturer, has been used for multiple clinical applications because of its usefulness to easily offer a graphical visualization of proteomic bands (Kim et al., 2013). It can be used as an *out-of-the-box* feature for biomarker research. However, it lacks better tools for data visualization and exploration.

Contrastingly, MATLAB Toolbox is a computer interface, specifically designed for analysis of matrix-based data sets, which is typically applied in the automation and standardization of image analysis routines. This interface is a useful tool that can aid in the exploration, interpretation and visualization of data in molecular biology, i.e., proteome, transcriptome or genome (Kumar, Singh and Dubey, 2015).

Recently, Ottman and colleagues (2017) used Experion™ Automated Electrophoresis System[55] to access RNA quality in combination with MATLAB Toolbox. Those tools, working together, permitted the simulation and construction of proteomic models. Likewise, Hou and colleagues (2018) have used Cytoscape™, a data visualization bioinformatics tool, combined with MATLAB Toolbox, for data mining and to analyze interactome networks, i.e., interaction between proteins. Similarly, to optimize the analysis of the numerous data generated by Experion™ system, we wrote an algorithm, using the programming platform of MATLAB, not only for data visualization and exploration, but also for hypothesis-driven biomarker research.

SalivaTec lab, Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), has recently proposed bioinformatics solutions (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017; Cruz et al., 2018) to address the problematic in this study. Hence, this approach aims (i) to complement the already published results and (ii) to address other specific difficulties, i.e., profiling mental health. The use of total protein profile electrophoretograms has been scarcely used in the study of mental health due to their limitative discriminative and quantification power. Indeed, only few studies purpose it, e.g., Sultana and colleagues (2010).

In this study, we propose an initial relationship between an acquired neurophenomenological bank (Hipólito and Martins, 2017; Section I of this PhD) and a protein profile counter-bank (Section II of this PhD). As published by Varela, Thompson and Rosch (1991) in their embodied cognition theory, a phenotypic state or trait can be studied as to how the body is shaped by human cognition.

6. Functional Profiling Of Neurotypical Subjects: Neuropeptide Response And Anti/Pro T-Helper Cell Type 17 Equilibrium

Keywords: cognitive-immune coupling, neuro-immune interface; neuroinflammatory response; neuropeptide response; TH17 cell type response; Treg.

[55] automated platform for protein analysis that incorporates LabChip technology into an integrated system that performs multiple electrophoresis steps in one.

The neuro-immune interface is mainly composed of the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis and the vagal withdrawal. The failure of this adaptive system to resolve inflammation affects well-being and neuropsychiatric health (Elenkov, 2008).

Specifically, according to Reed and Raison (2016), intrapersonal and interpersonal cognitive factors may exacerbate or buffer the health effects of this interface.

While intrapersonal factors include emotion and psychological regulation, interpersonal factors include behavior and relationship aspects. These factors are, thus, central for the maintenance of allostasis, which is the process of achieving stability, or homeostasis, through physiological and cognitive adaptation.

Hence, the neuro-immune interface may comprise the dynamic relation between physiology and cognition. Moreover, physiological processes, like growth and reproduction, energy intake, metabolism and neuronal development are regulated via environmental stressors, which communicate with the immune and the inflammatory systems, and vice versa.

Hereafter, this co-adapted interface communicates with the entire body, including the brain, to evoke the adaptive responses to fit external and internal conditions.

This phenomenon is evolutionarily conserved, enacted in the environment (Thompson, 2005) and emerges from principal mechanisms of bidirectional [neuronal-immune] communication (Verburg-van Kemenade, 2016).

Inflammation, as the result of this bidirectional communication, through interleukin (IL) paracrine signaling, comprises a continual ubiquitous cascade of complex signaling pathways and processes.

Those pathways and processes are initiated and maintained by the immune system, *per se*, affecting almost all aspects of physiology (Vassileva, 2014), including neuropsychophysiology.

Cytokines, as effectors of that complex cascade mediate and control the immune and inflammatory responses. Neuropeptides, on the other hand, modulate those effectors and that response (Lotz, Vaughan and Carson, 1988). Furthermore, that neuroinflammatory cascade regulates signal interactions of adaptive responses, maintaining homeostasis (Becher, Spath and Goverman, 2017) and regulating synaptic plasticity (Di Filippo, 2008). This regulation is essential for neuropsychiatric health (Holmgren, 2014), e.g., in neuroatypical subjects (Vargas et al., 2005, Hipólito and Martins, 2018), and also for well-being (Salim, 2016).

Those signals are mediated by the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system (Chrousos, 1995). This axis is mainly regulated by hormones and neuropeptides in an equilibrium which actually facilitates or inhibits inflammation.

By doing so, this neuro-immune interface regulates not only (i) reproduction, growth and immunity, but also (ii) behavior and emotional and cognitive functions (Chrousos and Gold, 1992). Chrousos and colleagues affirm that a dysfunction of this interface may occur via dysregulation of (i) the anti- versus pro-inflammatory systems, (ii) the Th (Lymphocyte T helper) 1 versus Th 2 cytokines, or (iii) the Th17 pathways.

More recently, this dysfunctionality was also associated with the neuropeptide response (Veiga-Fernandes and Mucida, 2016). Specifically, multidirectional neuro-immune interactions occur at barrier surfaces between the nervous and the immune systems, affecting homeostasis and the course of neurodegenerative and neurodevelopmental conditions.

Hence, the neuro-immune interface may play a role not only in the pathogenesis of somatic pathologies, but also in mental and cognitive states, e.g., depression, as a physiological and mental expression of enactive cognition (Hipólito and Martins, 2017; chapter 1).

6.α. The Neuro-Immune Interface

Contemporary immunology is established by the knowledge generated by molecular, developmental and evolutionary molecular biology, which surpasses clonal selection principles (Vaz, 2011).

The central problem in immunology is that systems cannot be controlled in a linear-causal manner, because, by definition, systems follow their own internal rules of operation. In fact, the immune system "exhibits activities that are spontaneous and non-linear, and consequently, is defined as an open-system in relation with a dynamic environment" (Varela, 1989). The embodiment of the immune system into the organism results of all the interaction of its components with the environment, which consequently leads to the self-maintenance and the development of specific lower order cognitive skills[56].

In spite of being usually defined by its inflammatory role, the immune system may instead be seen as a part of the molecular reciprocity between the organism's structure, its actions and the environment (Thompson and Varela, 2001). The nervous and the immune systems might, therefore, be seen as the chief regulators of homeostasis, through the neuro-immune interface and its tissue-resident and patrolling immune cells.

The two systems are not only physically connected through resident immune cells in the central nervous system and peripheral nerve terminals, but together they also share each other's 'languages'. The effectors of those systems are molecules, such as: a) cytokines (Steinman, 2004; McAllister and van de Water, 2009; Ben Menachem-Zidon et al., 2011; Heijtz et al., 2011; Gabay et al., 2011; Yirmiya and Goshen, 2011); b) neurotransmitters: acetylcholine, glutamate, dopamine, and serotonin (Koval et al., 1997; Steinman, 2004; Ganea et al., 2006; Pocock and Kettenmann, 2007; Levite, 2008; Kong et al., 2010); and c) neuropeptides (Gonzalez-Rey, Chorny and Delgado, 2007).

In fact, the human neuronal proteome has 2416 proteins maintaining brain circuitry. When performing a functional analysis of that proteome, a significative and relevant percentage of those proteins exert a neuropsychophysiological function associated with metabolic functions, immune system processes and neuropeptide transmission (chapter 4).

Recently, increasing evidence has considered the neuro-immune interface as primarily beneficial for homeostasis (Kipnis et al., 2012; Cohen et al., 2006; Ziv et al., 2006), through behavioral and cognitive learning (Brynskikh et al., 2008; Derecki et al., 2010). Therefore, this neuro-immune interface has also been described as cognitive-immune coupling (Varela et al., 1988)

As an example of cytokine interference in the cognitive-immune coupling, transcriptomes from different organisms show elevated interferon-γ (IFN-γ) / Janus kinase/signal transducers and activators of transcription (JAK-STAT) - dependent gene signatures in social cognition. This signaling pathway might mediate a co-evolutionary

[56] lower order cognitive skills include recalling (memorize) facts, figures, and basic processes; knowing vocabulary and definitions; understand and illustrating information.

link between behavior and an efficient anti-pathogen and adaptive immune response (Filiano et al., 2016).

As an example of neuropeptide interference in the cognitive-immune coupling, neuropeptides, such as somatostatin, calcitonin gene-related peptide, neuropeptide Y, and substance P, directly induce a distinct secretion of cytokines, e.g., IL-2, interferon-γ, IL-4, and IL-10.

In the absence of any additional factors, Th1 and Th2 cell populations are directly stimulated and driven to a characteristic secretion of Th2 cytokines from a Th1 T cell line and vice versa. Such an atypical phenomenon cannot be induced by classical antigenic stimulation. This suggests that neuropeptides interact with their specific T cell-expressed receptors in a particular signature, i.e., in a distinct Th phenotype (Levite, 1998).

As an example of this cognitive-immune coupling, IL-4 has been extensively studied in its critical role in higher cognitive functions of neurotypical subjects, such as memory and learning (Gadani et al., 2012).

6.β. Cognitive-Immune Coupling In Neurotypical Subjects

Neurotypical subjects have a characteristic cognitive-immune coupling (Croft, 2015). This typical coupling, according to Croft (2015), is essential for neurodevelopment and well-being. As mentioned, a specific neuroimmune physiology is maintained by a specific cognitive state and trait and vice versa (Nusslock and Miller, 2016).

Likewise, for Nusslock and Miller (2016), this physiological signature is a lifespan characteristic of an adequate development, well-being and *eudaemonia*[57].

Such *eudaemonia* can be correlated with biological homeostasis via the concept of corporeality. In Merleau-Ponty's work (1976) and Thompson's developments (2005), corporality is a primary modality of existence, the ground for and between physiology and cognition.

Emotions like anxiety, depression or fear have not only an internal representation and a perceptive dimension (Ansermet and Magistretti, 2004), but also a clear physiological influence (chapter 1). Heretofore, Solomon and colleagues (1974) had described the mechanism through which emotions cause perturbations in physiology, precisely in the immune system. Later, in 1998, Koh showed that a clinical level of anxiety seems to reduce immune function, whereas a subclinical level of anxiety seems to enhance immunity.

Eudaemonia or well-being, as a mental health trait, is characterized as having a lower pathophysiological response. For instance, it is a "mental health state associated with (i) lower daily salivary cortisol, pro-inflammatory cytokines, cardiovascular risk analytes, and (ii) longer duration of physiologic NREM sleep" (Ryff, Burton and Love, 2004).

Additionally, this mental health state has been associated with specific immune biomarkers, mirroring a more stable neuro-immune system configuration (Barak, 2006).

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[57] eudaemonia does not mean an emotional state, neither an utilitaristic perspective of happiness. Instead, it should be regarded as "good living" (chapter 3) and well-being. Therefore, eudaemonia does not mean a particular state or moment, but the whole life that tends towards the pursuit of a healthy living, which promotes well-being. It is a character trait based on self-consciousness (Kraut, 2016) and, possibly, also a physiological trait.

Salsman and colleagues (2013) have recently developed a cognitive assessment in order to better evaluate and understand the potential role of well-being and *eudaemonia* in chronic neurological conditions. Likewise, we have already published a mental health assessment for the stratification of neurotypical subjects (Hipólito and Martins, 2017; chapter 3). This mental health stratification is important for the better understanding of the neuro-immune system configuration in a sample of healthy subjects.

Those healthy subjects had distinct cognitive profiles and, possibly, a different cognitive-immune coupling. In this profiling, we evaluated representations and perceptions associated with self-awareness, self-consciousness and *eudaemonia* (chapter 2).

In this chapter, we bring forth the perspective that *eudaemonia* is the "organism's potential to achieve its best condition: a well-being that emerges from a cognitive and neuronal homeostasis" (Ramocki and Zoghbi, 2008). For Ramocki and Zoghbi (2008), synaptic modification and the remodeling of neuronal networks continually mediate cognitive development and health.

This cognitive homeostasis (**Figure 5**) is attained by constructs like self-awareness, self-consciousness, reflective self and pre-reflective self (Hipólito and Martins, 2017; chapter 3). These constructs will be evaluated, in chapters 2 and 3, by the use of the following psychological tests[58]: APZ (Simões et al., 1986), SHS (Pais-Ribeiro, 2012), MAAS (Gregório and Gouveia, 2011) and SCS-R (Neto, 1986).

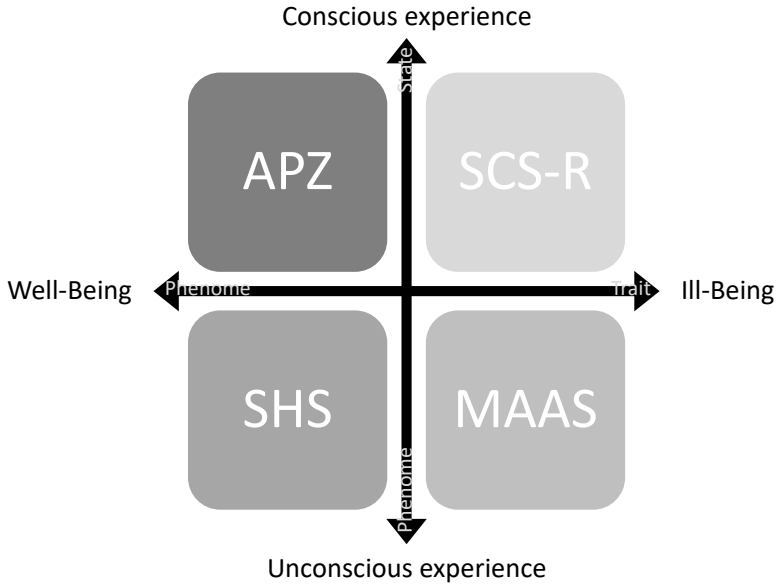


Figure 5. Cognitive Homeostasis
Illustration of the psychological scales used in the Assessment Protocol in Section I and the evaluated constructs. A two-axis representation is presented to demonstrate the phenome state and trait evaluation. The vertical axis represents the construct of a more self-conscious experience vs. a less self-conscious experience. The horizontal axis represents the complementary construct (postulated in this thesis) of a higher tendency to well-being vs. a lower tendency to well-being.

Concluding, we quest for inflammatory and neuropeptide profiles that can be pinpointed as physiological mirrors (chapter 1; chapter 5) of that constant and

[58] four psychological instruments, validated for the Portuguese population, were used in this work: the Abnorme Psychischer Zustaende States of Consciousness (APZ), the Subjective Happiness Scale (SHS), the Mindful Attention Awareness Scale (MAAS), and theSelf-consciousness Scale - Revised version.

continuous synaptic remodeling and pruning. “Positive emotions have been significantly associated with health” and with the neuro-immune interface (Barak, 2009). In order to do so, we have firstly performed a molecular stratification via total protein-profiling of the neurotypical subjects. The outcome of that analysis was the importance of quantifying the [9.1;30]kDa molecular weight range, as, in this PhD study, this light weight range had a specific molecular signature.

The hypothesis that is addressed is that this range may be characteristic of small neuroinflammatory molecules and neuropeptides (chapter 5). Moreover, the [9.1;30]kDa molecular weight range is conceivably identifiable in saliva and may operably be useful to characterize neurotypical molecular networks (chapter 4).

6.δ. Neuro-Immune Homeostasis And Cognitive Health

In neurotypical subjects, homeostasis within the immune system (Elenkov, 2008) is largely dependent on cytokines, particularly IL-10 (Sanjabi, Zenewicz, Kamanaka, and Flavell, 2009). Cytokines are mediators of chemical messages between immune and non-immune cells. These messages, in the brain-immune system communication, may be supervised by cognition (Elenkov et al., 2005).

The central nervous system and the immune system are the two major adaptive systems of the body (Elenkov et al., 2000), affecting the well-being of the individual. These adaptive systems may be subjectively assessed by behavioral and cognitive tests (chapter 3), as well as, objectively assessed by indices of physiologic health. “All organisms must maintain a complex dynamic equilibrium, by synergistic and antagonistic actions, which are constantly challenged by internal or external adverse forces termed as stressors” (Cohen, Tyrrell, and Smith, 1991; Chrousos, 2009).

Homeostasis is threatened by these stressors, and, thus, re-established by various physiological and behavioral adaptive responses (chapter 1). For Menary (2010), these adaptive responses have been defined as cognitive responses and characterized as embodied, embedded, extended and enacted.

Neuropeptides (Chrousos and Gold, 1992), e.g., neuropeptide Y (Reichmann and Holzer, 2016), have major roles in the regulation of both basal homeostasis and adaptive response to stress (Elenkov, 2008). This stress response is mediated by the stress system (Chrousos, 2009), formed by interconnected effectors, which include:

(i) hypothalamic hormones (Chrousos, 1995);

(ii) the *locus coeruleus* and autonomic norepinephrine centers in the brainstem (Elenkov et al., 1996);

(iii) the pro-opiomelanocortin-derived peptides (Elenkov and Chrousos, 1999).

Likewise, stress response is also mediated by the targets of those effectors, which include 2 main cognitive systems: the fear/reward and the neuro-immune systems (Elenkov, 2008).

An optimal basal activity, i.e., homeostasis within the immune system, is also generated by the combination of immune and metabolic fluctuations and changes in the Th1/Th2 balance. Those fluctuations are synchronized by the responsiveness of the stress system, and are consequently essential for health and well-being. Contrastingly, desynchronization may generate behavioral, mental and neurological conditions (Elenkov, 2008). The downregulation of the stress system (Elenkov, 2008), through induction of a Th2 shift, in combination with the increase of the ‘anti-inflammatory’ efferent vagus activity (Elenkov et al., 2000) in visceral organs, may actually protect the organism from systemic “overrunning” with type 1/pro-inflammatory response (Wilder, 1995; Tracey, 2002).

On the other hand, depending on the inflammatory context, an upregulation of stress systems may actually facilitate inflammation, through induction of IL-1, IL-6, and TNF-α and through activation of the Corticotropin-releasing hormone/substance P/histamine axis (Elenkov, 2005). Moreover, like stress response, the neuro-immune interface is time-limited and stimuli tailored. Abnormalities of the 'systemic anti-inflammatory feedback' and/or 'hyperactivity' of local pro-inflammatory factors (i.e., cytokines) may cause a dysfunction of this interface.

This dysfunction (Elenkov and Chrousos, 1999) may play a role in:

(i) the health/pathology dichotomy of neuropsychiatric conditions;

(ii) the well-being of neurotypical subjects.

6.ε. Neuropeptide Response And Neuro-Immune Interface

Dawidson and colleagues (1996) have identified salivary neuropeptides in neurotypical subjects with classical radioimmunoassay[59]. However, with the recent developments in neuropeptidomics, there is a wider and global characterization of neuropeptides. Mass spectrometry, or simultaneous immunodetection[60] of salivary samples, offers optimum qualitative and quantitative capabilities (Yin et al., 2011).

Indeed, neuronal proteins present in the salivary protein profile (Chapter 4 of this PhD) are associated with 79 specific biological processes, 60 precise pathways, 32 particular cellular components, and 41 distinctive molecular functions, which are maintained by a full neuronal proteome composed of 55 main protein classes. Besides, a significant percentage of those functions are associated with neuropeptide and immune response.

Neuropeptides comprise a complex set of small signaling molecules found in the nervous system, though able to cross the blood-brain barrier (Banks, 2015). They are involved in regulatory functions, behavioral control and cognitive functions in the nervous system (Südhof, 2017).

However, they are also part of the neuro-immune interface (Lotz et al., 1988), releasing inflammatory cytokines and interleukins (Dallos et al., 2006). In 1991, Heijnen, Kavelaars and Ballieux, considered β-Endorphin as the first molecule with cytokine and neuropeptide properties. In 2012, Scharrer, Smith and Stefano stated that neuropeptides were widely produced by cells of the immune system. Since then, emerging neuropeptide targets have been proposed in immune research (Chandrasekharan, Nezami and Srinivasan, 2013); for instance, with mechanism mediating inflammation through mast cell activation (Kritas et al., 2014a).

This neuropeptide-cytokine coupling has then been proposed as a model for the study of neuroatypical subjects (Hipólito and Martins, 2018) and neurodevelopmental conditions (Tostes et al., 2012). Likewise, this neuroimmune crosstalk was associated with several neurological diseases (Tian et al., 2012), e.g., complex regional pain syndrome (Kingery, 2010). Simultaneously, this coupling was associated with mental disorders (Kritas et al., 2014b). As an example, this specific type of signaling was identified as a biomarker for posttraumatic stress disorder (Baker, Nievergelt and O'Connor, 2012).

In this chapter, the premise is to understand the relation of the neuropeptide response in cognitive-immune coupling. Therefore, it is proposed a panel of 4 specific

[59] immunoassay of a substance that has been radioactively labeled (Merriam-Webster, Inc., retrieved August 12, 2020).

[60] simultaneous immunological detection used to identify specific proteins blotted to membranes.

neuropeptides in the molecular weight range [9.1;17]kDa, which is hypothesized as more relevant for the type of cognitive (Hipólito and Martins, 2017; chapter 3) and molecular response (chapter 5) probed in this PhD. We wanted to evaluate neuropeptides present in neurotypical subjects with a clear association with (i) neuropsychiatric health and well-being, and (ii) Th17 or Th1/Th2 type response.

More recently, a study evaluated a sample of cancer patients using multiplexed simultaneous immunodetection with a combined neuropeptide and TH17 cytokine assay (Millipore, UK) (Fancourt et al., 2016), as it was done in this thesis.

Specifically, and for the purpose of our investigation, we used a *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Magnetic Bead Panel - Neuroscience Multiplex Assay*, as Manzardo and colleagues (2016), but to study the neuropeptide dimension of neuro-immune interface.

6.ζ. HNPMAG-35K Multiplexed Assay

The Human Neuropeptide Magnetic Bead Panel is constituted by 4 neuropeptides: Orexin, β-Endorphin, Oxytocin and Substance P.

Orexin (13,363 kDa, Uniprot: O43612) has a renowned association with arousal and sleep (Nishino et al., 2000), feeding behavior (Sakurai et al., 1998) and the reward function (Harris, Wimmer and Aston-Jones, 2005). Immunohistochemical[61] studies mapped their wide brain expression, with a higher distribution in the perifornical nucleus and the dorsal and lateral hypothalamic areas (Peyron et al., 1998).

Harris, Wimmer and Aston-Jones (2005) found synaptic projections from the *locus coeruleus* to the reward-associated brain regions, including the *nucleus accumbens* and ventral tegmental area. This suggests that Orexin has physiological functions associated with the neuroendocrine system, regulating circadian-dependent and circadian-independent behavior (España, Plahn and Berridge, 2002) and, thus, sleep and energy homeostasis (Sakurai, 2005).

Orexin deficiency is associated with human leukocyte antigen and T cell receptor polymorphisms (la Herrán-Arita, Alberto, and García-García, 2014), and hypocretin neuron loss is associated with pro-inflammatory Th17 cytokines: IL-6, IL-17 and IL-23 (Debebe et al., 2014).

β-Endorphin (4.5 kDa, Uniprot: P01189#PRO_0000024975) has an intrinsic relation with the *nucleus accumbens* - ventral tegmental area - dorsolateral prefrontal cortex circuitry. Hence, it acts as an effector of the reward system and is released in situations of incentive salience, associative learning and positive emotions, such as pleasure (Adinoff, 2004).

β-Endorphin may be synthesized in immunocytes (Sibinga and Goldstein, 1988) and exert a tonic inhibitory effect on the immune system (Panerai and Sacerdote, 1997), regulating Th1/Th2-Type response switch.

Oxytocin (12,722 kDa, Uniprot: P01178) secreted in the pituitary gland has a well-known relationship with stress response and is a molecular constituent of the hypothalamo-pituitary-adrenal axis (Viero et al., 2010).

Neurobiological models have been proposed to show how mental health faces environmental stress (Ozbay et al., 2008) and maintains health and well-being (Farhud, Malmir and Khanahmadi, 2014).

[61] of or relating to the application of histochemical and immunologic methods to chemical analysis of living cells and tissues (Merriam-Webster, Inc., retrieved August 12, 2020).

Oxytocin is released to reduce fear/anxiety and to increase social interaction (Walker, F. et al., 2016). This anti-inflammatory neuropeptide has an effect on innate immune responses. In neurotypical subjects, Oxytocin decreases the neuroendocrine and cytokine chronic activation, e.g., TNF-α, IL-1 receptor antagonist, IL-4, IL-6, due to the pharmacological modulation of the cholinergic anti-inflammatory pathway (Clodi et al., 2008).

Substance P (15,003 kDa, Uniprot P20366) has a recognized relationship with the immune system, specifically in the crosstalk between endocrine cells and peptidergic nerves.

Substance P is secreted by sensory nerves, dendritic cells and inflammatory cells such as macrophages, eosinophils, lymphocytes, and acts by binding to the neurokinin-1 receptor. Substance P has proinflammatory effects on immune and epithelial cells and participates in neurogenic inflammation (O'Connor et al., 2004).

Substance P is involved in the control and augmenting of multiple autonomic functions; being the focus of intense fundamental and neuroscientific research as a central neurotransmitter, neuromodulator[62], and immunomodulator[63]. It is distributed throughout the central nervous system and co-located in serotonin, norepinephrine, and dopamine transmissions (Schwarz and Ackenheil, 2002).

6.η. Treg/Th17 Equilibrium In The Neuro-Immune Interface

Th17 pathways are mediated by Tregulatory (Treg) and Toll-like receptors (TLRs) (Sutmuller, Garritsen and Adema, 2007). There has been a growing body of evidence of the involvement of TLRs and consequently Treg/Th17 equilibrium, in neurogenesis, neurite outgrowth and neuron survival. This involvement suggests that TLRs might also have an impact on the cognitive processes in health or disease (Okun, Griffioen and Mattson, 2011).

It was recently shown that TLRs, for instance, have broad effects on cognitive performance (Okun et al., 2010), spatial reference, working memory, and in contextual fear and anxiety memory (Sloane et al., 2010).

The expression of TLRs shifts Treg/Th17 equilibrium (Tang and Bluestone, 2008) to Th17 dominance. This dominance is achieved via TLR4-IL-4 vs. TLR4-IL-6 pathway, which exacerbates inflammation (Li et al., 2014), regulating the differentiation and function of Th17 effector cells (Littman and Rudensky, 2010).

On the one hand, this equilibrium is a highly inflammatory immune response, activating cytokines IL-6, IL-21, and IL-23 along with transforming growth factor beta (TGF-β) and IL-1β (Korn et al., 2009). This immune response is generally mediated via the signal transducer and activator of transcription 3 (STAT3).

On the other hand, this equilibrium is mediated by Treg cells, via expressing the transcription factor forkhead box P3 (Foxp3). Foxp3 emerged as a dedicated suppressor of diverse immune responses and inflammation and as a vital and critical gatekeeper of immune homeostasis.

Th17 cytokines exhibit a variety of pro-inflammatory effects (Ghilardi and Ouyang, 2007; Dong, 2008), but are co-regulated by Treg cells that suppress the immune effector cells. This suppression occurs mainly by Foxp3 (Wing et al., 2008),

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[62] something (as a polypeptide) that potentiates or inhibits the transmission of a nerve impulse but is not the actual means of transmission itself (Merriam-Webster, Inc., retrieved August 12, 2020).

[63] a substance that affects the functioning of the immune system, and thereby modulating the inflammatory response. (Merriam-Webster, Inc., retrieved August 12, 2020)

which mediates the Treg/Th17 equilibrium in the nervous system (Eisenstein and Williams, 2009).

As a consequence of that equilibrium, Th17 cells and their effector cytokines modulate autoimmune and inflammatory pathogenesis. The receptor for IL-17 is ubiquitously expressed in a variety of cell types, including nervous cells (Moseley et al., 2003). Therefore, IL-17 signaling mediates an overall crucial crosstalk between the immune system and the nervous system (Ouyang, Kolls and Zheng, 2008).

6.λ. Anti-/Pro-Inflammatory Cytokines Equilibrium

In the last years, more than forty cytokine major functions and disease association were described. In this study, we focus on some of them. The anti-/pro-inflammatory response is, as previously depicted, a balance between a functional network of chemokines.

For the purpose of this research, we used a *Bio-Plex Pro™ Human Th17 Cytokine Assay* (Geng, Ma and Roen, 2012) to study the cytokine dimension of the neuro-immune interface.

In this study, we wanted to understand Th17 cell type response in cognitive-immune coupling. Therefore, we proposed the following panel of 15 specific cytokines in the molecular weight range of [17;30]kDa, which we considered more relevant for the type of molecular response we were searching for (chapter 4; chapter 5).

We separated those cytokines or analytes into 3 distinct regulatory groups, depending on the disease and the model of study:

(i) anti-inflammatory: IL-4 (17,492 kDa, Uniprot: P05112), IL-10 (20,517 kDa, Uniprot: P22301), IL-25 (20,330 kDa, Uniprot: Q9H293);

(ii) pro-inflammatory: IL-1β (30,748 kDa, Uniprot: P01584), IL-6 (23,718 kDa, Uniprot: P05231), IL-17A (17,504 kDa, Uniprot: Q16552), IL-17F (18,045 kDa, Uniprot: Q96PD4), IL-23 (20,730 kDa, Uniprot: Q9NPF7), IL-31 (18,205 kDa, Uniprot: Q6EBC2), Interferon-γ (INF-γ) (19,348 kDa, Uniprot: P01579), sCD40L (29,274 kDa, Uniprot: P29965) and TNF-α (25,644 kDa, Uniprot: P01375);

(iii) anti&pro-inflammatory: IL-21 (17,923 kDa, Uniprot: Q9HBE4), IL-22 (20,011 kDa, Uniprot: Q9GZX6) and IL-33 (30,759 kDa, Uniprot: O95760).

Conceptualization via human validation and mechanistic studies, in which anti-/pro-inflammatory mediators (a) drive pathogenesis in chronic inflammatory diseases or, contrarily, (b) maintain the homeostatic balance, can be intrinsically complex and bewildering (Schett et al., 2013).

Notwithstanding, immune cells certainly release various and diverse cytokines, causing both pathologic and healthy *status*, thus, validating the importance of probing into this complexity.

6.λ.1. Anti-Inflammatory

The massive secretion of pro-inflammatory cytokines usually induces counter up-regulation of anti-inflammatory and regulatory cytokines (Volpin et al., 2014), generating a critical equilibrium for homeostasis.

IL-4 has been shown to be highly protective against delayed type hypersensitivity and organ-specific autoimmune and autoinflammatory reactions by the redirection of Th1/Th17 toward a Th2-type immune response. IL-4 selectively suppresses IL-23 transcription and secretion by cells of the innate immune system, sparing Th1 immune responses (Guenova et al., 2015).

IL-10 (Banchereau, Pascual and O'Garra, 2012) limits the immune response, preventing immune system–mediated damage to the host (Li and Flavell, 2008), involving several layers of regulation of: (a) IL-10 gene transcription (Saraiva and O'Garra, 2010) and (b) post-transcriptional mechanisms (Pandiyan et al., 2011). These mechanisms might explain the different duration and activation time of IL-10 expression (Murray and Smale, 2012).

IL-25, an epithelium-derived cytokine, is essential in initiating type 2 immunity through the induction of downstream IL-4 (Yang et al., 2013).

6.λ.2. Pro-Inflammatory

The inflammasome complex (Mishra et al., 2013) is a multiprotein oligomer, expressed in myeloid cells, and a component of the innate immune system. The inflammasome complex is responsible for the activation of inflammatory processes, pyroptosis and apoptosis. IL-1β is a central piece in this complex, and IL-6, via its classical receptor signaling and trans-signaling, represents a keystone cytokine in infection, autoimmunity, cancer and inflammation. They drive disease progression and support the maintenance of immunological reactions. Notably, the inflammatory context depends on the location and activation time of the sequential states of the immunity cascade (Hunter and Jones, 2015).

Th17 cells produce a variety of cytokines, such as IL-17A and IL-17F, that can be pathogenic or not, depending on their transcriptional and functional profile (Baeten and Kuchroo, 2016). IL-17A initiates the production of other pro-inflammatory mediators, such as IL-1, IL-6 and TNF-α, collectively resulting in an influx of a neutrophil lineage (Bosmann et al., 2012).

IL-23 was found to be produced by monocytes, macrophages, dendritic, T, B, and endothelial cells (Oppmann et al., 2000; Pirhonen et al., 2002). IL-23 heterodimeric receptor complex (Parham et al., 2002) expression on monocytes, memory T, natural killer T and dendritic cells correlates with cellular responsiveness to IL-23 (Belladonna et al., 2002; Parham et al., 2002), and has been linked to the pathogenesis of autoimmune inflammation (Di Cesare, Di Meglio, and Nestle, 2009).

IL-31 may be involved in promoting epithelial responses that characterize allergic and non-allergic diseases, involved in skin physiology and inflammatory cell recruitment (Dillon et al., 2004).

INF-γ is a cytokine that is critical for innate and adaptive immunity, specifically on Th1 differentiation, and upregulates major histocompatibility complex (MHC). It inhibits cell growth and has pro-apoptotic effects. Likewise, it controls activation-induced cell death, regulates local leukocyte endothelial interaction, enhances microbial killing ability (Sakaguchi, 2004) and intracellular pathogen and tumor regulation (Akdis, 2011). Nevertheless, it equally activates macrophages, induces MHC Class II, represses IL-33 and limits T regulator cell accumulation, acting as a regulator of the pro-inflammatory cascade (Molofsky et al., 2015).

The soluble form of CD40L (sCD40L, sCD154) (Jenabian et al., 2014) is a functional trimer that is shed from activated T cells (Matthies, 2006), establishing the communication with dendritic cells (Huang, 2012). sCD40L plays a key role in the co-stimulatory pathways involved in humoral B cell responses (Godin-Ethier, 2011). The interaction with its receptor (Ho et al., 2015) has been implicated to mediate inflammatory processes through the induction of various pro-inflammatory cytokines (IL-1β, TNF-α or INF-γ) and matrix metalloproteinases (Mach et al., 1997; Rizvi et al., 2008). It also activates the recruitment of leukocytes, in a feedback/feedforward loop

that contributes for the expansion of the inflammatory response (Suttles and Stout, 2009).

TNF-α is a critical regulatory cytokine, inducing the expression of other pro-inflammatory cytokines (Palladino, 2003). More importantly, TNF-α exerts both homeostatic and pathophysiological roles in the central nervous system. It leads to acute and chronic inflammatory responses, which are typically self-limiting and homeostatic. However, sustained TNF-α expression for protracted periods of time may result in cellular dysfunction and initiation of neurological disease (Montgomery and Bowers, 2012).

6.λ.3. Anti&Pro-Inflammatory

Svobodova and colleagues (2011) found the role of mesenchymal stem cells in the differentiation of naive T-cells into (i) anti-inflammatory Treg cells and/or (ii) pro-inflammatory Th17 cell populations. The following cytokines are, thus, characterized as chief supervisors and subtle regulators of the inflammatory warfare.

This dual nature in modulating tissue immune responses depends on the inflammatory context: duration, concentration levels, overall cytokine milieu, and the tissues involved.

IL-21 has such a function in amplifying Th17 differentiation that, in the absence of IL-21 signaling, Th17 response is compromised (Korn et al., 2007). After activation, CD4+ T cells differentiate into distinct effector subsets.

These effector subsets are characterized by their unique cytokine expression and immune-regulatory function. They may induce (i) anti-inflammatory regulation, promoting the maintenance of the immune system homeostatic Treg cells (Fantini et al., 2007); or (ii) pro-inflammatory regulation, through the production of INF-γ by Th1 and Th2 cells (Nurieva et al., 2007).

IL-22 also has a dual nature (Sanjabi, Zenewicz, Kamanaka, and Flavell, 2009) in modulating the responses of the tissue during an immune response: (i) protective, in acute inflammatory models, such as hepatitis, or chronic inflammation, such as inflammatory bowel disease; or (ii) inflammatory (Zheng et al., 2007), for instance, by the expression of S100A7 antimicrobial protein. S100A is a superfamily that may be altered by a mental health state (chapter 1).

IL-33 (Dinarello, 2009) has also been shown to induce the secretion of both (i) anti-inflammatory and (ii) pro-inflammatory cytokines and chemokines from mast cells, eosinophils, and Th2 lymphocytes (Espinassous et al., 2009). IL-33 belongs to a special subset of cytokines, e.g., IL-1α, which generates a double functionality in their (i) non-secreted (intracellular) and (ii) secreted forms. The (i) intracellular form acts as a precursor of a co-repressor of pro-inflammatory genes, i.e., a nuclear factor. The (ii) secreted from modulates systemic inflammatory responses and enhances lipopolysaccharide-induced inflammatory cytokine production, driving to allergic and Th2 responses (Sattler et al., 2014).

6.μ. Saliva, OralCard And The Neuropeptide and Neuroimmune Response

Saliva is composed of 99% water and 1% electrolytes, proteins, mucus, hormones and antibacterial components (Lim, Garssen and Sandalova, 2016). Its defense functions are due to the presence of immunoglobulins, inflammatory cytokines (Lim, Garssen and Sandalova, 2016), lysozyme, peroxydase, cystatins, lactoferrin and histatins that inhibit the growth of microorganisms (Lawrence, 2002).

Saliva can easily be collected noninvasively (Lawrence, 2002; Rosa et al., 2016), which is safer and more viable than drawing blood (Lim, Garssen and Sandalova, 2016). At present, saliva is used mostly for diagnosis of periodontal and other oral diseases (AlMoharib et al., 2014).

However, recent studies suggest that saliva could be used as an important tool (Chiappin, Antonelli, Gatti, and Elio, 2007) for clinical screening and/or diagnostic (Malamud, 2011) in somatic conditions like asthma (Little et al., 2014), cancer (Panneer and Sadaksharam, 2015) and diabetes (Lim, Garssen and Sandalova, 2016).

Likewise, saliva has also been proposed as an innovative sample for diagnosing neurological (Farah et al., 2018) and psychiatric conditions (Wormwood et al., 2015), and possibly for mental health profiling (Martins et al., 2016a) in neurotypical subjects. Shaila and colleagues, in 2013, showed that profiling salivary proteins can be used as markers for plasma protein leakage as a consequence of inflammation processes.

Indeed, and preceding this thesis, Arrais and colleagues (2013) have previously identified *in silico* certain neuropeptides and immunological proteins present in saliva and annotated them in the bioinformatics tool OralCard. Specifically, the OralCard database has annotated (i) all the neuropeptides present in the *HNPMAG-35K* assay and a significant sum of (ii) the cytokines present in the *Th17 Cytokine Assays*: IL-1β, IL-4, IL-6, IL-10, IL-17, IL-22, IL-23, INF-γ and TNF-α.

6.ξ. Multiplex-Biometric Immunoassay

Multiplex-biometric immunoassays have been widely used for different neuroscientific applications since 2014 (Hopp et al.,2014). An example of multiplex-biometric immunoassays is the Bio-Plex® MAGPIX™ Multiplex Reader. It is a system that employs low-cost light-emitting diodes and a charge-coupled device imager to illuminate and to image immobilized magnetic beads.

This design is not only very robust, but it reduces instrument preparation time, cost and sample volume. It was used in this thesis to complement the lack of the discriminative and quantification power of classical capillary electrophoresis, used in chapter 5 for preliminary stratification of the salivary samples. Magnetic systems have recently established new assay precision, specificity and sensitivity, when compared to traditional ELISA[64] or western blotting[65] (Peretz et al., 2013).

The Bio-Plex® system employs xMAP technology to permit the multiplexing of different analytes and it gives researchers the ability to look at them simultaneously. xMAP utilizes an antibody fluorescent sandwich for analyte detection, using differentially detectable bead sets as a substrate for capturing analytes in a solution. These bead sets identify the analytes and the detection antibodies are used to measure the quantity of analytes (Houser, 2012).

Hopp and colleagues (2014) started using xMAP technology to evaluate spatial memory deficits associated with aging. Ichinose and colleagues (2015) tested it with cerebrospinal fluid for profiling cytokines in neuropsychiatric conditions. Ma and

[64] enzyme-linked immunosorbent assay is an *in vitro* method for quantifying an antigen or antibody concentration in which the test material is immobilized on a surface and exposed either to a complex of an enzyme linked to an antibody specific for the antigen or an enzyme linked to an antigen specific for the antibody followed by reaction of the enzyme with a substrate to yield a colored product corresponding to the concentration of the test material (Merriam-Webster, Inc., retrieved August 12, 2020).

[65] a blot consisting of a sheet of nitrocellulose or nylon that contains spots of protein for identification by a suitable molecular probe and is used especially for the detection of antibodies (Merriam-Webster, Inc., retrieved August 12, 2020).

colleagues (2015) identified molecular biomarkers for profiling the cognitive status in the elderly. Miceli and colleagues (2016) evaluated the effect of cytokines in neuro-glial differentiation.

More recently, Park and colleagues (2017) used the Bio-Plex® system to assess cytokine levels in antidepressant response. Fernández-Paredes and colleagues (2017) identified Th17 cytokine biomarkers for multiple sclerosis stratification. Zingler and colleagues (2017) correlated the psychological and molecular aspects in post-orthognathic surgery. Martin and colleagues (2017) evaluated the well-being of IL-1 receptor deficient rats.

Later, after a spinal cord stimulation, Kriek and colleagues (2018) used a mixed magnetic bead set, similar to ours, with detection antibodies for both neuropeptides and cytokines to quantify their response.

Research Aims, Design And Tasks

The overall study was conducted as part of a doctoral program in neuroscience (Faculty of Medicine) under the Mind-Brain College, University of Lisbon, Portugal, and comprised two main sections; Section I: Mental Health Stratification and Section II: Molecular Stratification.

This quasi experiment is an exploratory, non-representative study. It was previously approved by the Ethics Committee of the Faculty of Medicine, University of Lisbon. The study was carried out by LIMMIT Lab, from the same university, as the centralizing research unit, and executed by SalivaTec (Molecular Cognition(G)), Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa, Viseu, Portugal. A collaboration was conducted with the Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal, for sample collection, storage and request.

As reviewed in the Introduction, the problematic of probing into mental health and its molecular correlates has been a trending topic in neuroscience. Mostly, the reason of interest for this topic lies on the technical difficulties inherent to studying subjective experience and objectivizing its molecular expression. Henceforth, the rationale of this dissertation resides predominantly on the study design and the definition of its research aims and tasks.

Consequently, the objectives of this dissertation are firstly introduced in Research Aims, Design and Tasks. Subsequently, the complexity of those objectives is further developed in Materials and Methods.

Section I: Mental Health Stratification

The research aims, design and tasks of the mental health stratification comprehend the first three chapters. The results of those chapters were obtained by a profiling method characterized by an induction of a mental health state and the analysis, by self-report measures, of that mental health state and trait.

1. Neurotypical Expression: Cognition And Proteins

The chapter 1 is the conceptual analysis of this PhD thesis and has the same research aims, design and tasks of chapters 2 and 3.

2. Mental Health Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults

A qualitative study is performed, in which a mental health behavioral intervention is used as the experimental procedure. In this theoretical background, the main outcome was to put forward a non-reductive explanation of subjective experience. Because subjective experience cannot be simply reduced to brain pictures and/or yes/no answers, this paradigm takes into consideration the rich first-person experience, in the attempt to combine it subsequently with third-person, quantitative, measurements.

In this experiment, two different groups were created: one (experimental group) practicing a demanding attentional and emotional task with autobiographical memory recalling and another (control group) attending a regular class. Both groups were subsequently asked to qualitatively evaluate their experience through a Psychological Assessment.

This Psychological Assessment was a standardized and structured clinical interview, which comprised the use of 4 psychological scales for the classical Mental Status Examination (MSE) (Trzepacz and Baker, 1993), but in a sample of neurotypical subjects. This allowed, first, to compare the experimental group and the control group, and, second, to probe the experience with regard to its psychological characteristics."(Hipólito and Martins, 2017)

3. Mental Health Profiling In Neurotypical Young Adults

As raised in the Introduction, the problematic of probing into the molecular expression of neurotypical subjects exists first in fully examining its clinical/psychological features (Hipólito and Martins, 2018). The chapter 3 research aims, design and tasks are better detailed subsequently and were applied in order to extract and present the mental health database of our neurotypical sample. They comprehended a profiling method characterized by an induction of a cognitive state and the analysis, by self-report measures, of that cognitive state and trait.

The objective is to understand (a), primarily, if mental health profiles of neurotypical subjects could be found; (b), secondly, if a psychological intervention, like a cognitive behavioral intervention, could generate these profiles; and (c), thirdly, if a set of self-report measures could stratify and clinically characterize the constructs of those profiles. To attain these objectives, the study includes a method composed of 2 parallel tasks running simultaneously, and represented in the flowchart (**Figure 6**):

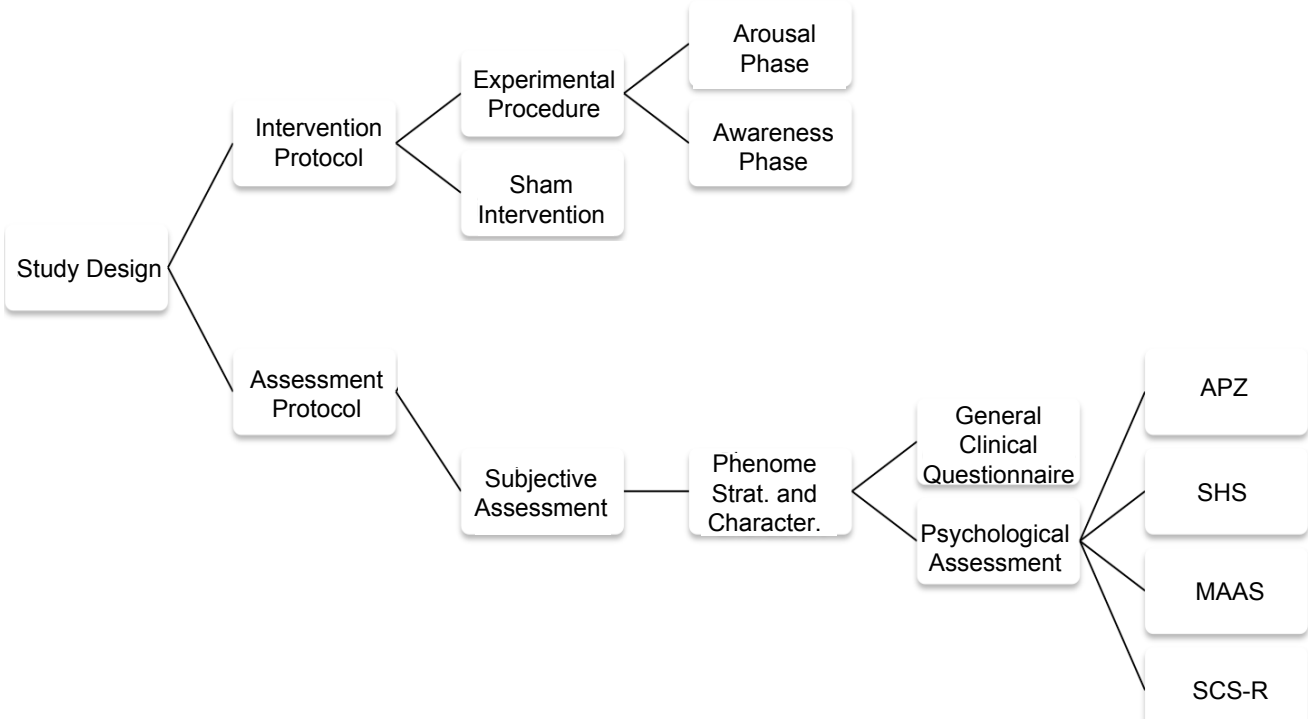


Figure 6. Fluxogram Of The Study Design
Review of the combined protocols used in the thesis. An Intervention Protocol comprised an experimental procedure or a sham intervention. The experimental procedure included an arousal and awareness phase. The Assessment Protocol was a proportionate stratified random sampling (Subjective Assessment) used for the phenotypic stratification and characterization. This comprised the use of a general clinical questionnaire and a psychological assessment with 4 clinical instruments: *Abnorme Psychischer Zustaende States of Consciousness* (APZ), *Subjective Happiness Scale* (SHS), *Mindful Attention Awareness Scale* (MAAS), *Self-consciousness Scale - Revised version* (SCS-R).

(i) the Intervention Protocol, which is used to isolate mental subphenomes in the sample. It comprised (a) the experimental procedure, i.e., a psychotherapeutic technique based on a cognitive behavioral intervention, and (b) the sham intervention, i.e., a simple cognitive task of maintaining attention, alertness and concentration during a class.

(ii) the Assessment Protocol, which is the clinical and phenomenological evaluation of those functional states and traits.

Both the (i) Intervention Protocol and (ii) Assessment Protocol were decisional processes or choices taken by the researcher based on the state of the art reviewed in Introduction and further explored in Materials and Methods.

Those choices were taken into account as a methodological strategy to validate the mental health stratification.

The hypotheses of this study are:

(i) the Intervention Protocol generates different mental profiles in neurotypical subjects;

(ii) the Assessment Protocol has the reliability to stratify and characterize them.

Accordingly, the outcome of this study is to screen, in a sample of neurotypical young adults, different subgroups with different cognitive and experiential characteristics.

Those subgroups should have high intergroup and low intragroup variability, given by the overall scores mean difference of the instruments used in the method to evaluate mental health.

Lastly, those different and reliable mental health profiles, with a consistency of constructs or features, will be proposed to follow a molecular screening strategy. The reason we established this methodology is these stratified subgroups may have a more homogeneous molecular response and, consequently, a possible specific biomarker signature.

Section II: Molecular Stratification

The molecular stratification comprehends the last three chapters. The results of those chapters were obtained by a profiling method characterized by an *in silico* characterization and bioinformatics analysis of the neuroproteome, and its molecular stratification, by Experion™ Automated Electrophoresis System (Biorad®) and Bio-Plex® MAGPIX™ Multiplex Reader.

The molecular stratification is a methodological approach using the three techniques explored in chapter 4, the chapter 5 and the chapter 6. This methodological approach combines both quantification and data analysis strategies.

The chapter 4, the chapter 5 and the chapter 6 are fully detailed subsequently.

4. Neuronal-Salivary Proteome: *In Silico* Characterization And Bioinformatics Analysis

This chapter comprises the study of the neuronal-salivary proteome by the generation of an embedded type database revising the state of the art in neuroproteomics. This study encompasses (i) an *in silico* characterization and (ii) a bioinformatics analysis.

This segment of the investigation is part of the mental health and molecular profiling of neurotypical young adults (Martins et al., 2016a, Hipólito and Martins, 2017). The main objective of this chapter is to offer a systematic bioinformatics tool for the salivary analysis of the molecular expression in neurotypical/atypical subjects.

The outcome of this specific analysis is to offer a neuronal-salivary database for neuroproteomics.

5. Protein Profiling Of Cognition And GUI Toolbox Development In Neurotypical Young Adults

This chapter involves a molecular analysis, through a simple molecular biology method, i.e., capillary electrophoresis, and a consequent data mining solution to better overcome the lack of the discriminative and quantification power offered by this classical technique.

This assessment aimed to find specific and characteristic molecular profiles in four previously determined subgroups (Hipólito and Martins, 2017, chapters 3 and 4). Thus, we investigated the molecular *strata* of the mental health subphenomes formerly identified in section I.

The neurotypical *strata* are characterized by total protein profiling, which used capillary electrophoresis to separate proteins according to molecular mass.

6. Functional Profiling Of Neurotypical Subjects: Neuropeptide Response And Anti/Pro T-Helper Cell Type 17 Equilibrium

This chapter involves a complex molecular analysis through multiplexed biometric immunodetection and a subsequent bioinformatic exploration.

This assessment aimed to explore the 4 previously identified molecular profiles.

The neurotypical *strata* characterized by total protein profiling were further studied to distinguish a particular neuropeptide and t-helper cell type 17 response. Simultaneous immunodetection was used for the quantification of this response.

Experimental Study

To conduct the overall study, a convenience sample of 128 healthy volunteers, neurotypical young adults (50% male), was recruited from the University of Lisbon (i. Faculty of Medicine, ii. Faculty of Economics, iii. Faculty of Sciences and iv. The School of Social and Political Sciences).

These neurotypical students were screened by the general questionnaire, concerning a) inclusion criteria: 1) 19-to-25-year-old young adults; Male/Female; 2) Cultural background: university students; 3) Normal body mass index (BMI) and 4) Non-medicated except for birth control pills; and b) exclusion criteria: 1) Serious physical illness or uncontrolled kidney, liver, lung, heart, musculoskeletal, rheumatologic, metabolic, neurological or psychiatric disorders; 2) Severe chronic or terminal disease, which might affect the Central Nervous System (CNS) or the Peripheral Nervous System (PNS); 3) Pregnant or breast-feeding women; 4) Abuse of alcohol or addictive substances, prior to the experience.

The allocation of the neurotypical young adults was based on the students that were attending the different lectures. Thus, the subjects were assigned to an experimental group or a control group arbitrarily, (i) according to the randomization of the weekly theoretical-practical classes for each course of each faculty, and (ii) depending on the students who were enrolled in one or another class. At any educational institution and by parallel assignment (1:1), students in Tuesday's class were allocated to the experimental group (experimental procedure), and students in Thursday's class were allocated to the control group (sham intervention).

Randomization was done mainly: (i) to balance the effect of internal conditions associated with the students attending courses at the various colleges of the University of Lisbon, and (ii) to program the logistics of collecting data.

Thus, the assignment was parallel, the allocation randomized, and both Experimental and Control group, with a 1:1 allocation, were strictly conserved. The blinding was maintained in the Intervention and Assessment protocols. As enrolled in the study, subjects received information and informed consent, approved by the Ethics Committee of the Faculty of Medicine, University of Lisbon. The confidentiality was maintained following the Biobanco-IMM and SalivaTec-CIIS defined confidentiality protocols.

A single blinding was maintained on the overall mental health and molecular stratification, as the researcher and statistician knew and used the sample screening. However, in the execution of the molecular assessments, a triple blind was guaranteed, as neither the researcher, the analyst, nor the statistician knew the sample screening.

The objectives of this dissertation were firstly introduced in Research Aims, Design and Tasks. Forthwith the intricacy and convolutedness of those objectives are systematized in two sections: Section I (Mental Health Stratification) and Section II (Molecular Stratification).

Both sections are divided in three chapters with a discrete experimental study. Each chapter has been published or is being submitted to peer-reviewed journals in neuroscience or molecular biology by the author of this thesis and the co-authors of the experiments. Every chapter starts with the formulation of the scientific question to be addressed, and a preamble conducting the reader to its answer. Afterwards, on each individual chapter, this question is fully answered, operated and detailed, by explicating its methods, describing its results and discussing its outcomes.

The research developed on the six chapters is sequential and consecutive, comprising the bulk of the dissertation developed in this work. The research outline (**Figure α**) systemizes the experimental study and helps the reader understanding the sequence of the chapters. The two sections are explained subsequently.

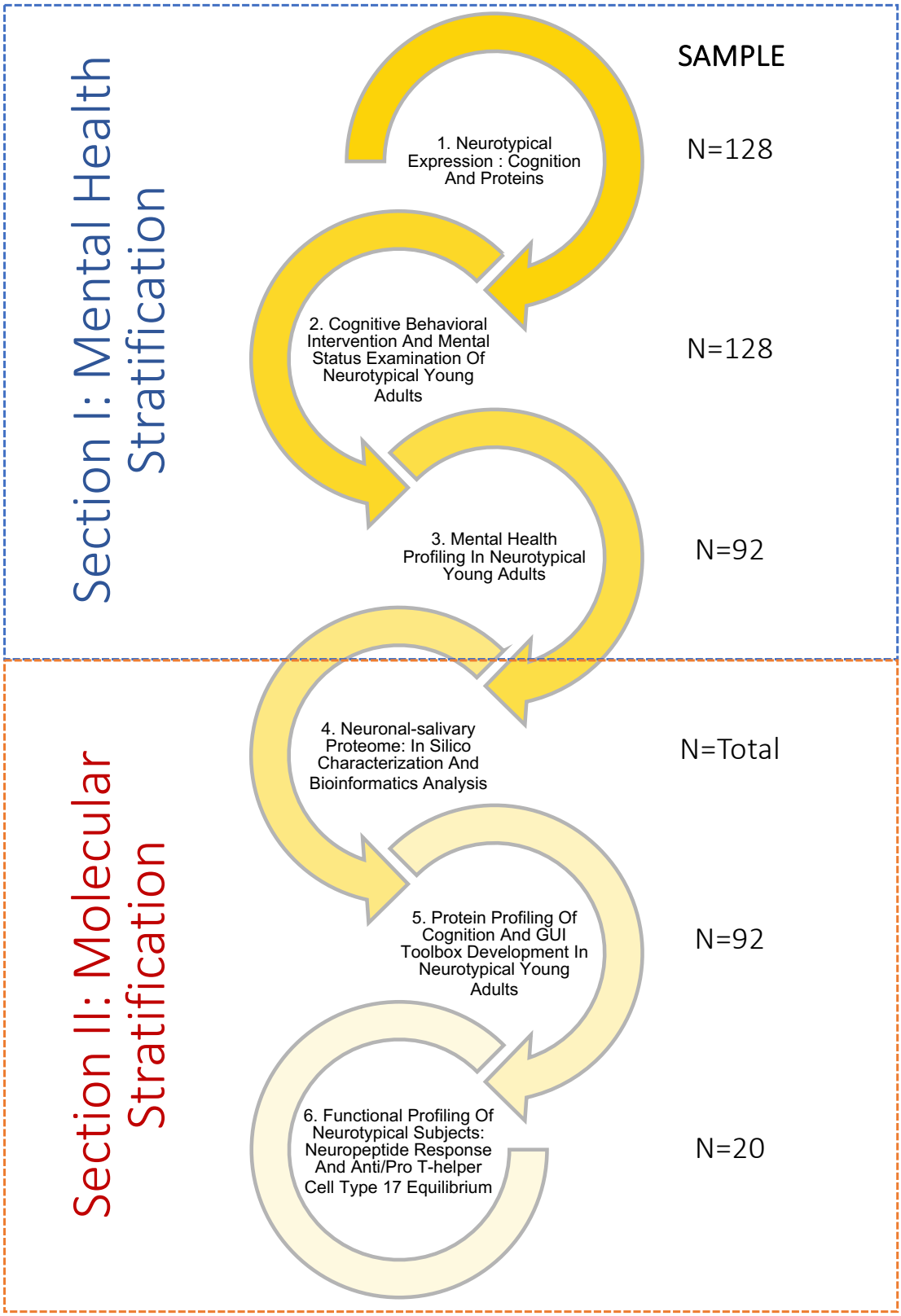


Figure α. Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults
The 6 different chapters of this PhD thesis are presented, each chapter corresponds to a stage of stratification. The N of subjects are equally presented for each stage.

Section I: Mental Health Stratification

The materials and methods, results and discussion of the mental health stratification comprehend the first three chapters and were designed to answer the research questions: “what was it done?”, “when were the saliva collections made?”, “what is the temporal relationship between the questionnaires and the saliva collection?”, and “what is the decalage of time between the beginning of the experiment, the collection of saliva and the collection of data related to the subject?”.

To answer this research questions a brief explanation of the experimental procedure (Martins et al., 2016a; Martins et al., 2016b; Hipólito and Martins, 2017) is presented subsequently. Those can also be consulted in the annexes (Annex C. Happiness as a self state and trait of consciousness: saliva molecular biomarkers - a brief revision; Annex D. Verbal protocol of the experimental procedure) and, moreover, in the publication (Hipólito and Martins, 2017) that integrates the chapter 2 of this dissertation, which is presented Facsimile in Annex A: Original Research as chapter 2 of the PhD Thesis.

In experimental terms, the Intervention Protocol, the Salivary Collection Protocol and the Assessment Protocol were carried out permanently during the mornings between 9:30 (a.m.) and 12:00 (p.m.). The salivary tests and scales used are, as well mentioned in literature, “possibly long and take time to apply”.

An initial saliva collection was consistently performed at 9:30 (a.m.). Then, at 9:45 (a.m.), the Intervention Protocol took place, which lasted 45 minutes. A second saliva collection started at 10:30 (a.m.). Finally, at 10:45 (a.m.), a General Clinical Questionnaire and four psychological scales were filled out, that is, in a standardized and structured clinical interview, which was used as an assessment tool. At 12:00 (a.m.) the experimental protocol was terminated and the collected salivary samples continued for processing at Biobanco-iMM, Centro Académico de Medicina de Lisboa, being processed at 12:15 (p.m.).

The time between the beginning of the experimental procedure (or sham intervention) and the second saliva collection, concomitantly with the acquisition of the data for each subject, was 45 minutes. This time interval corresponds to the time of exposure to the Intervention Protocol. The assessment protocol lasted between 45 and 75 minutes, depending on the subject.

Section II: Molecular Stratification

The materials and methods, results and discussion of the molecular stratification comprehend the last three chapters, and are supported by the methodological strategy developed in the SalivaTec Lab (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017).

The explanation of the rationale associated with the choice of the indicated markers is as follows. A pilot study was carried out with 5 individuals, applying the methodology used in the 128 young neurotypical adults. This pilot study demonstrated the constant presence of salivary protein clusters, in certain molecular weight ranges, after the application of the experimental procedure.

To verify that this profile was maintained in a significant neurotypical sample, the same procedure was performed, using capillary electrophoresis techniques to obtain

the total protein profile in saliva. Graphically confirming the results of the pilot study in the electrophoretic profile of the 128 young neurotypical adults, the individuals who had the highest protein concentration variation in certain molecular weight ranges were identified and selected.

Then, a data visualization and datamining of the protein profiles were performed through a graphical user interface (GUI) toolbox called MODeLING.Vis, and designed specifically for this study. This tool allowed, in a first step, to quantitatively identify two protein clusters as being the most likely to be altered in the respective molecular weight ranges, notwithstanding and bearing in mind the lack of the discrimination offered by the classical capillary electrophoresis.

Subsequently, in order to identify the possible proteins belonging to these clusters, a database literature review (Sousa, Martins, Rosa, Barros and Correia, 2016, *in* Annex I: Review) of the salivary neuroproteome was made. The neuroproteome described in the database showed the possible protein networks that could be altered after the experimental procedure. The exploration of this hypothesis allowed us to reason that these two previously identified protein clusters, in terms of molecular weights, could possibly correspond to small peptides that might pass the blood-brain barrier. These probable peptides that have small molecular weights between [9.1; 30]kDa, which were altered in the salivary samples, could correspond to possible molecules associated with the neuropeptide or neuroinflammatory response, as they present a characteristic electrophoretic profile of these specific responses. Please note that an error variable should be considered correspondent to the lack of accuracy offered by Experion^{TM} analysis, and that this consideration should take this inaccuracy in account but also the process of protein degradation observed and well documented in saliva.

As such and to better objectivize ad quantize, a combined multiplex panel was chosen to detect the peptides associated with these molecular weights: the HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay and the Bio-Plex ProTM Human Th17 Cytokine Assay. The kits (HNPMAG-35K and Bio-Plex ProTM Human Th17 Cytokine Assay) were used in multiplexed simultaneous immunodetection to verify our premise and quantify the following peptides: Orexin, β-Endorphin, Oxytocin, Substance P and the biochemical messengers of Th17 cells.

Finally, the MODeLING.Vis tool allowed the exploration and visualization of biomolecular data obtained by capillary electrophoresis and multiplexed simultaneous immunodetection mixed analysis and to perform the hypothesis test. This data treatment allowed the better visualization of the homogeneous separation of the salivary peptides that correspond to the studied mental health profiles. In conclusion:

(i) we explored better the assumption of probing molecular correlates in the molecular weight range of [9.1; 30]kDa;

(ii) we chose significantly Orexin, β-Endorphin, Oxytocin, Substance P and the biochemical messengers of Th17 cells, as possible biomarkers of the mental health states and traits present in the sample of 128 neurotypical young adults.

Chapter 1. Neurotypical Expression: Cognition And Proteins

Preamble ("Would it be achievable to have a molecular probe into cognition?")
The aim of this chapter is to show, as already published in *Progress in Biophysics and Molecular Biology* (Hipólito and Martins, 2017)[66], that there are reasons to doubt that cognitive life is only neurocentred. A living system remains alive by perceiving and relating with its environment through interactions that become meaningful in ways that are hardly and merely exclusive to the brain. The idea that cognitive life is completely reducible to brain function is usually suggested when one thinks that "there is a gap between the mind and the world" (Fodor, 2009, 14). This chapter starts by offering an overview of the debate on this matter, and moves to provide philosophical arguments and empirical evidence against such a hard reduction. Although brain representations are the dominant metaphor, it is claimed that there is no exclusive empirical support for it. In addition to this, as we attempt to show, systemic protein networks, brain and non-brain located, might play a role in cognitive life. More specifically, it is argued that evidence in molecular biology, and particularly in proteomics, offers reasons to think that cognition is not centered only in brain function. On this basis, the conceptual analysis offers an empirical method, combining phenomenal experience and molecular networks, that is consistent with a parallel and complement non-neurocentred view of cognition.

Materials And Methods

The chapter 1 is the conceptual analysis of this work and has the same materials and methods of chapters 2 and 3.

Results And Discussion

This chapter is a conceptual analysis and thus its results were presented in the introduction and discussed in the conclusion. The conceptual analysis of this chapter was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[67]: Martins, J. E.*[a,b,c], Hipólito, I.*[a,d,e], Barros, M.[c], Simões, M.[a,b].

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[66] Hipólito, I., & Martins, J. (2017). Mind-life continuity: A qualitative study of conscious experience. *Progress in Biophysics and Molecular Biology*. Volume 131, 432-444, doi: 10.1016/ j.pbiomolbio.2017.09.004.

[67]
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The excerpt of the research outline (**Figure β**) systemizes the Section I: Mental Health Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 1 in the overall experiment.

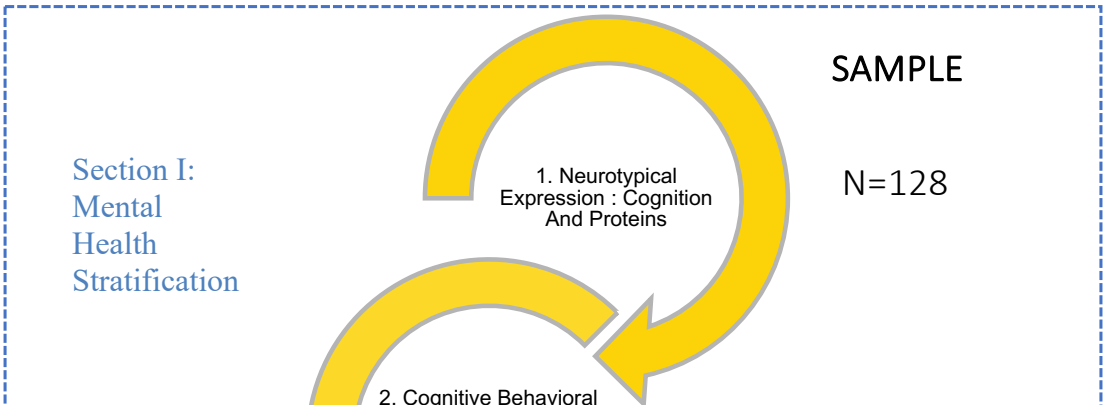


Figure β. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section I: Mental Health Stratification The chapter 1 (Neurotypical Expression: Cognition and Proteins) is displayed, which comprehended the conceptual analysis used for the characterization of the 128 subjects.

Chapter 2. Cognitive Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults

Preamble ("How would it be possible to study phenomenal data?")

There are two fundamental models to understanding the phenomenon of natural life. One is the computational model, which is based on the symbolic thinking paradigm. The other is the biological organism model. The common difficulty attributed to these paradigms is that their reductive tools allow the phenomenological aspects of experience to remain hidden behind yes/no responses (behavioral tests), or brain 'pictures' (neuroimaging). Hence, one of the problems regards how to overcome methodological difficulties towards a non-reductive investigation of conscious experience. It is our aim in this paper to show how cooperation between Eastern and Western traditions may shed light for a non-reductive study of mind and life. This study focuses on the first-person experience associated with cognitive and mental events. We studied phenomenal data as a crucial fact for the domain of living beings, which, we expect, can provide the ground for a subsequent third-person study. The intervention with a single cognitive behavioral intervention, and its psychological assessment, provided us with experiential profiles based upon subjects' evaluations of their own conscious experiences. The overall results should move towards an integrated or global perspective on mind where neither experience nor external mechanisms have the final word.

Materials And Methods

Chapter 2 comprises the methodology that led to the characterization of the neurotypical sample. Most significantly, this chapter presents the materials (Psychological Assessment), and the methods (Intervention Protocol) used in this thesis for the mental health stratification. The procedure included a mental status examination and a cognitive behavioral intervention in the sample of 128 neurotypical young adults.

2.1. Materials (Psychological Assessment)

The materials that were used in the Psychological Assessment are described below. These materials were applied in a standardized and structured clinical interview, which comprised the use of the following 4 psychological scales. Those materials were used for the classical Mental Status Examination (MSE) (Trzepacz and Baker, 1993), but in a sample of neurotypical subjects. This qualitative and quantitative psychological evaluation included subjective descriptions of the subjects and describes their mental state. This standardized and structured clinical interview is inspired on the MSE, which derives from an approach known as descriptive phenomenology (Taylor, 1967; Owen and Harland, 2006) developed by Karl Jaspers. As theorized by Jaspers, but here applied to a neurotypical sample of young adults, this standardized and structured clinical interview assumes that the only way to comprehend a subjects' experience is through his own description (Berrios, 1989).

(i) *Abnorme Psychischer Zustaende States of Consciousness*[68] (APZ) (Dittrich, 1998) was used to assess both the process and content of thought, as well as perception. The thinking process describes the stream and rate of thoughts, how they flow and are connected. Hence, thought process refers to the quantity, tempo (rate of flow) and form

[68] *Abnorme Psychischer Zustaende States of Consciousness*, termed in English: The Altered States of Consciousness Rating Scale

(or logical coherence) of thought (Sims, 1988). The content of thought refers to the themes that occupy the subject's thoughts and perceptions, i.e., the qualitative properties of thinking as well as its intensity, salience, and associated emotions (Fish, Casey and Kelly, 2007). A perception in the context of this evaluation is the organization, identification, and interpretation of sensory information in order to represent and understand the presented information or environment (Schacter et al., 2011). The items in the scale aim to evidence: alterations of thought; intense emotional response; bodily schema changes; perceptual changes; synesthesia; and meaning alterations, which are typically evoked during cognitive behavioral interventions.

(ii) *Subjective Happiness Scale* (SHS) (Lyubomirsky and Lepper, 1999) was used to assess mood and affect. Trzepacz and Baker (1993) described mood as "a person's predominant internal state at any one time" and affect as "the external and dynamic manifestations of a person's internal emotional state". Mood is evaluated as the prevalent subjective state, i.e., the emotional type (euthymic, dysphoric, euphoric), and affect as the congruency, intensity and mobility with the global subjective experience and thought content. Two items evaluated absolute and relative personal experience; two other items evaluated absolute and relative external experience. SHS measures accordingly the construct of subjective happiness and well-being and its stability. This scale aims to assess, in the context of this study, the self- and non-self perception of happiness, which can be stable or labile.

(iii) *Mindful Attention Awareness Scale* (MAAS) (Brown and Ryan, 2003) was used to assess cognition, and more specifically the level of consciousness, alertness, and attention, i.e., the awareness of, and responsiveness to the environment (Giannini, 1986). MAAS evaluates the ability to focus, sustain and appropriately shift mental attention. MAAS taps a unique quality of consciousness related to, and predictive of, pre-reflective self-experience. Thus, it evaluates the subjects' open awareness to the present, i.e., to the exercise of immediate and short-term memory.

(iv) *Self-consciousness Scale* - Revised version (SCS-R) (Scheier and Carver, 1985) was used to assess insight and judgment. Insight refers to the participant's understanding of his mental state and the awareness of his own situation (Erikson, 1994). Judgment refers to the ability to make reasoned and responsible decisions to preserve one's own and others' wellbeing (Martin and Hickerson, 2013). Hence, this assessment takes into account the participant's impulsiveness, social cognition and self-awareness (Quee et al., 2011). SCS-R evaluates thus private and public self-consciousness. This component or domain also evaluated in a classical MSE is considered relatively stable over time and arises from sustained consciousness, long-term memory and the capacity to abstraction and interpretation.

The contents and constructs evaluated by each scale are summarized in **Table 1**. For better understanding of the items evaluated on each psychological scale, an example of the questions posed to the participants is subsequently given for each self-report measure, and the complete materials are presented in Annex E. Case Report Form.

For instance, in the APZ scale, the main four constructs evaluated were (i) the content of thought and associated mood; (ii) cognitive processes; (iii) auditory-visual reformulations or changes in the meaning of percepts; and (iv) general consciousness alteration (Studerus, Gamma and Vollenweider, 2010). Dittrich (1996) determined three primary and one secondary etiology-independent dimensions of the APZ. The three primary dimensions were termed oceanic boundlessness, dread of ego dissolution and visionary restructuralization. These three primary dimensions were taken into account in the Psychological Assessment for the mental status examination. The

secondary scale, i.e. the global altered states of consciousness scale, was not only used in the Psychological Assessment, but additionally in chapter 3 of this work for the mental health profiling. APZ's secondary scale is composed of 23 binary items. An example of a variable assessed was: "Time passed faster than usually". In our neurotypical sample, APZ was used to evaluate sensory gating, its relation to cognitive alterations, e.g. concentration, (Gouzoulis-Mayfrank et al., 1998; Vollenweider et al., 2007) and the individual's openness to a variety of cognitive, perceptual, and imagistic experiences by vivid imagery (Vollenweider and Geyer, 2001; Geyer and Vollenweider, 2008).

For the SHS scale, the main construct evaluated was that of self and non-self experiences of happiness, composed of six items on a [0; 7] scaling. SHS assesses not only the balance of positive and negative emotions experienced over a particular period of time, but also the capacity to make a judgment of the overall life quality. This judgment is likely not equivalent to a simple sum of the subject's recent levels of affect and satisfaction with life, but indicative of an overall affective and emotional perception (Lyubomirsky and Lepper, 1999). An example of a variable assessed was: "Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?".

Moreover, for the MAAS scale, the main construct evaluated was open-awareness experiences, composed of 15 items on a [1; 6] scaling. MAAS measures constructs of private self-monitoring and cognition, and is targeted for cognitive behavioral interventions and designed for nonclinical samples, the case (intervention and sample) of this study. This assessment probes into the effective regulation of emotionality and the capacity of attention to present internal state experiences (Osman et al., 2016). An example of a variable assessed was: "I find it difficult to stay focused on what's happening in the present".

Finally, for the SCS-R scale, the main construct evaluated was private and public self-consciousness, composed of 23 items on five-point Likert scale. SCS-R scale evaluates the constructs of self-reflection and self-concept (Hart, Tortoriello and Richardson, 2019). This assessment probes into the effective regulation internal state awareness and the capacity of self-presentation and introspection, i.e., insight and judgment. An example of a variable assessed was: "I often daydream about myself."

Table 1. Self-report Measurement Of The Psychological Assessment
Example of the variables assessed in the Psychological Assessment, for each of the 4 clinical instruments. The constructs evaluated, as well the number of items and the type of scaling is presented.

Subjective Experience Assesment				
Scale	Constructs	N. Items	Scaling	Variables (items) example
APZ	Self-boundaries alteration	23	binary	"Sounds and noises seemed different from what they usually are." "Time passed faster than usually." "My body seemed bigger/longer than before."
SHS	Self and non-self experiences of happiness	4	[0; 7]	"Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?" "Some people are generally not very happy. Although they are not depressed, they never seem as happy as they might be. To what extent does this characterization describe you?"
MAAS	Open-awareness experiences	15	[1; 6]	"I find it difficult to stay focused on what's happening in the present." "I get so focused on the goal I want to achieve that I lose touch with what I'm doing right now to get there." "I tend not to notice feelings of physical tension or discomfort until they really grab my attention."
SCS-R	Private & public Self-consciousness	23	five-point Likert scale	"I generally pay attention to my inner feelings." "I often daydream about myself." "I know the way my mind works when I work through a problem."

2.2. Methods (Intervention Protocol)

The method, or Intervention Protocol, comprised an experimental procedure with a cognitive behavioral intervention. Following the paradigm of Kavanagh and colleagues (2009), the objective was to explore the impact of preventive interventions on inequalities in young people's mental health (Pacheco, Martins and Simões, 2019). In this study, this method was applied to study neurotypical young adults by the application of a combined relaxation and suggestion intervention evoking positive past memories.

It is well established that a variety of cognitive-behavior interventions, as shown by the Dunning and colleagues (2019) meta-analysis of randomized controlled trials, ultimately improves the reduction of neuropsychiatric symptomatology in subjects with clinical risk factors or existing symptoms. These interventions include mental imagery and are not only effective at addressing symptoms such as pain, anxiety, nausea, and insomnia, but also known to improve the mental health and wellbeing (Culbert, 2017).

2.2.1. Cognitive Behavioral Intervention

Trait mindfulness refers to 'absorption' into a focused and stable state of concentration (Khema et al., 2001). It is nowadays amenable to greater scientific understanding and used in cognitive behavioral interventions.

Cognitive behavioral interventions have been shown to play a physiological role in inducing neuroplastic changes in amygdala activity (Leung et al., 2017), as well as preventing age-related changes in cognitive functioning (Cotieret et al., 2017). Cognitive behavioral interventions have been revealed as an effective alternative to pharmacology on blood pressure response (Park and Han, 2017), and as a successful cognitive behavioral therapy for epilepsy (Leeman-Markowski and Schachter, 2016).

In addition, these interventions appear to promote a change in attentional focus from the internal to the present-moment external world, thereby having important implications for the treatment of anxiety (Xu et al., 2017), and generalized anxiety disorders (Hoge et al., 2017).

Physiologically, cognitive behavioral interventions may be directed to the short-term goal of stimulating the reward dopaminergic system. If the reward is greater than expected, dopaminergic neurons in the Ventral Tegmental Area (VTA) in the brain stem are activated. The VTA in turn innervates the nucleus accumbens (NAc) in the ventral striatum, which leads to higher centers in the orbital frontal cortex (OFC). Human studies have shown that activity in the medial OFC at the time of a reward correlates with subjective reports (Hagerty et al., 2013). According to Hagerty et al. (2013), these interventions have also been associated with specific brain correlates, which may characterize trait mindfulness:

(1) external awareness diminishing;
(2) internal verbalizations fading;
(3) self-boundaries alteration;
(4) increase of focused attention;
(5) well-being.

The brain correlates, obtained by fMRI (BOLD signal) and qEEG (bands power), consisted in:

(1) decreased activation compared to the rest state in the visual (BA 17e19) and auditory (BA 41e42) processing areas;

(2) decreased activation compared to the rest state in Broca's area (BA 44.45) and in Wernicke's area (BA 39.40);

(3) decreased activation compared to the rest state in the orientation area (BA5);

(4) increased activation compared to the rest state in the Anterior Cingulate Cortex (ACC) (BA 32.33);

(5) increased activation compared to the rest state in the dopamine reward system of the brain (NAc in the ventral striatum and medial OFC).

Reciprocal connections between the NAc and the medial OFC were found (D'ardenne et al., 2008). The authors suggest the possibility of activating a feedback loop between the two (Hagerty et al., 2013). In this study, a cognitive behavioral intervention was adopted in the experimental group to promote this possible feedback loop, by switching attentional focus.

2.3. Study Design

This is a randomized, controlled, multicentered and single blinded study. The study, approved by the ethics committee of the Faculty of Medicine, University of Lisbon, was carried out in LIMMIT Lab at the same university as the centralizing research unit, and in collaboration with SalivaTec (Molecular Cognition(G)), Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa, Viseu, Portugal, and the Mind-Brain Doctoral College, University of Lisbon.

The study consisted of two groups: the intervention group exposed to a cognitive behavioral intervention that aimed to promote a more conscious experience, and a control group attending a regular class. After the Intervention Protocol, all the participants (experimental procedure and sham intervention) were asked to qualitatively evaluate (posttest design) the experience in relation to:

1) process and content of thought, and perception;

2) mood and affect;

3) cognitive awareness and attention;

4) insight and judgment.

Participants were asked to conduct this qualitative evaluation using the four psychological scales outlined above.

2.3.1. Sampling And Population Of The Study

For this study, students from the University of Lisbon (Faculty of Medicine, Faculty of Economy, Faculty of Sciences, and The School of Social and Political Sciences) were recruited. A convenience sample of 128 healthy volunteers, students at the University of Lisbon, were recruited and screened by the inclusion criteria, in order to achieve a proportion of 50% men. Subjects received information and gave informed consent as they enrolled in the study.

2.3.2. Sample Screening And Group Characterization

Sample screening was conducted, concerning the following (a) inclusion criteria:

1) 19-25-year-old young adults; male/female;

2) cultural background: university students;

3) normal body mass index (BMI);

4) non-medicated excepting birth control pills;

Parallelly, the following (b) exclusion criteria were strictly followed:

1) serious physical illness or uncontrolled disorders of kidney liver, lungs, heart, musculoskeletal, rheumatologic, metabolic, neurological or psychiatric;

2) severe chronic or terminal disease, which might affect the CNS or PNS;
3) pregnant or breastfeeding women;
4) abuse of alcohol or addictive substances, prior to the experience.

The allocation was randomized and parallel assigned, based on the students that were attending different lectures. Both experimental and control arms, with a 1:1 allocation, was conserved. The blinding was maintained in the interventions protocol and partially in the assessment protocol.

2.4. Statistical Analysis

The following software was used for (i) statistical and data management: IBM SPSS Statistics 22-significance level 5%; and (ii) laboratory information management: LabWare™ LIMS.

2.4.1. Descriptive And Exploratory Analysis

Frequency tables (simple/double entries) were used. Scale variables were summarized as mean, standard deviation, median, quartiles, minimum, maximum and/or others order statistics, when the sample distribution justified it. Categorical variables were summarized using frequency and percentages. When the scale of measure was ordinal, order statistics were considered, e.g., tercile. Boxplots were used as a graphic exploratory tool. Moreover, Spearman's rank correlation coefficient was applied.

2.5. Power Analysis And Sample Size Study

The main outcome of this work is to observe if there is a molecular correlation to the mental health stratification. Hence, power analysis (Zhang et al., 2019) was used to calculate the minimum sample size required so that one can be reasonably likely to detect an effect of a given size. Moreover, power analysis was also used to calculate the minimum effect size that is likely to be detected in this study using a given sample size.

The full sample size study is supported by the previous preliminary results of the experimental group (Martins et al., 2016). Sample size determination (Dahiru, Aliyu and Kene, 2006) was done to choose the number of observations to include in our statistical sample.

The sample size was an important feature of this empirical study in which the goal was to make limited inferences about a population from a neurotypical sample. In practice, the sample size used in this exploratory type of study was ultimately determined by the highly cost of the molecular analysis and the need to still offer sufficient and adequate statistical power.

Bearing in mind that the main objective was to test the research hypothesis that led to full study (i.e., the evidence that there is a significant change in the protein profile in the subphenomes characterized in chapters 2 and 3), the following approach was designed. It was, for that purpose, considered a potency (T-Student) of 80%, a significance level of 5%, and an effect size corresponding to 40% of the estimated value for the standard deviation of the variation of the molecular weights in the preliminary study, executed before proposing the mental health stratification. Taking into account this option, that corresponds to a medium Cohen's effect size, an appropriate sample size for each group (experimental and control) of 64 elements was found.

2.6. Limitations Of Psychological Assessment And Reliability Analysis

The reliability, temporal consistency of the psychological measures and the test and retest stability, was considered in the protocol elaboration and the methodology used, as well as in the definition of the objectives and the expected results.

This is a descriptive study, where the intention is to observe the correlation between cognitive and physiological variables and not a causal analysis, or clinical trial, where the intention is to objectify the effect of an intervention. It is intended, therefore, to determine the relationship between a set of psychological measures and a profile of biological markers.

Regarding the test-retest reliability of the psychological measures, or repeatability, it should be noted that, being a descriptive study, the priority objective is not to show causality between psychological measures, the Intervention Protocol, and molecular measures. Certainly, a mental state is in permanent change and, above all, the measures "state".

The expected results are just to show that the applied molecular methodology is valid for measuring psychological states. For a better understanding of the psychological measures used in the assessment, they are briefly described subsequently, but the statistical strategy is fully detailed in the results as it is part or the observed outcomes. Henceforth, to ensure the stability of the data, i.e., the reliability and temporal consistency of the psychological measures obtained by the standardized and structured assessment of the mental states and traits, two types of questionnaires were used: (i) those that measured states and (ii) those that measured traits.

In the author's previous publications (Martins et al., 2016a, Hipólito and Martins, 2017) and further in chapter 3, the instruments used are fully analyzed, as well as the strategy for the stratification of the subjects; having in mind the problematic of the stability of mental states in healthy subjects.

In chapter 3, and in the description of the instruments used, reference is made to the reliability of all questionnaires, in their original publication, as well as in their application to this sample. It should be noted that the questionnaires for measuring a state and a trait were used and validated in the Portuguese population, for that same mental state or trait, by the authors in their original publications in the Portuguese population: APZ (Simões et al., 1986), SHS (Pais-Ribeiro, 2012), MAAS (Gregório and Gouveia, 2011) and SCS-R (Neto, 1986).

The scales were then applied in this thesis with the author's consent concerning and respecting the internal consistency of the original publication. Furthermore, the instruments used in the standardized and structured clinical interview are fully detailed in Annex E. Case Report Form.

Considering the reliability tests, internal consistency measures, obtained by statistical tools such as Cronbach's α, are usually intended to study the capacity for sample separation through the instrument used. The specific Cronbach's α was studied, in chapter 3, and will be demonstrated that the 4 scales used present an adequate internal consistency in the sample of neurotypical young adults, when compared with the internal consistency if applied to the Portuguese population.

In view of this, the reliability test and internal consistency measures are conveniently calculated in chapter 3 and proposed as the test-retest reliability. However, the collection of psychological measures was done in a single time and the study design did not include a further exploration of test-retest reliability.

Results And Discussion

The results and discussion of this chapter were published as original research by the author in first co-authorship and is presented *Facsimile* in Annex A: Original Research as chapter 2 of the PhD Thesis. The referred article (Hipólito and Martins, 2017) was published in *Progress in Biophysics and Molecular Biology* with an impact factor of 3,427. This set of results was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[69]: Martins J. E.*[a], Hipólito, I.*[b].

2.A. Stratification Of The Neurotypical Sample

Through a cognitive behavioral intervention, subjects were led to evoke a more conscious experience, and later asked to evaluate the experience. We tested the qualitative relation between this experience and the:

1) process and content of thought, and perception;
2) mood and affect;
3) cognitive awareness and attention;
4) insight and judgment.

The sample was separated into independent groups:

(a) experimental group (experimental procedure) (64 subjects);
(b) control group (sham intervention) (64 subjects).

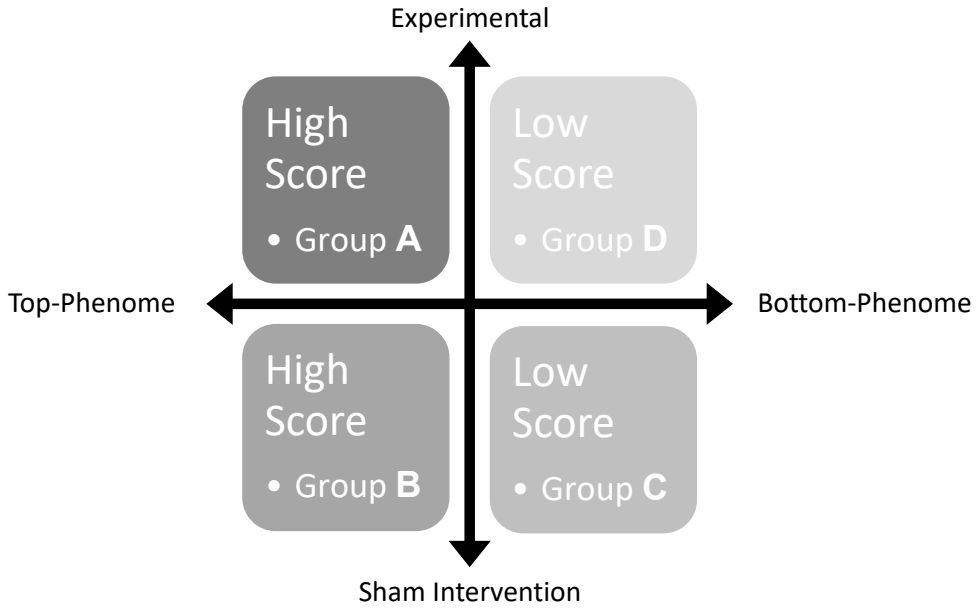


Figure 7. Stratification Of The Neurotypical Sample
Qualitative examination and quantitative scoring of the 128 subjects. A 2-axis representation is presented. In the horizontal axis, the sample was statistically divided in two: the Top-Phenome (higher score) and Bottom-Phenome (lower-score). In the vertical axis, the sample was separated by the Intervention Protocol (experimental procedure and sham intervention) in two.

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Then, these 2 groups were stratified by the subjective assessment, further detailed in chapter 3, into Top and Bottom Phenomes, by a statistical strategy, which will further inform the interpretation of this pre-molecular clinical stratification.

This first clinical subtyping permitted a stratification resulting in four subgroups, as shown in **Figure 7**:

(i) the Experimental Top Phenome;

(ii) the Control Top Phenome;

(iii) the Experimental Bottom Phenome,

(iv) the Control Bottom Phenome,

The four subphenomes (subgroups) were tested for comparability statistics and external biometric data, possibly confounding variables, and were considered comparable for age, gender and BMI. This qualitative study, with a quantitative analysis, allowed us not only to characterize the subjective experience of the whole neurotypical sample (**Table 2**), but also to look at the differences between the mental *status* of the experimental group in the cognitive behavioral intervention versus the control group attending a class.

Table 2. Descriptive Analysis Of The Psychological Assessment and Biometric Data
A descriptive analysis (mean, median, SD, minimum, maximum, P25, P50 and P75) is presented for each of the clinical instruments used in the 92 stratified neurotypical adults. Biometric data (age and BMI) is also presented.

Descriptive Analysis		Instrument	Biometric Data		Abnormer Psychischer Zustand Scale	Subjectiove Happiness Scale	Mindful Attention Awarness Scale	Self-Consciousness Scale (Total)	Self-Consciousness Scale (Private)	Self-Consciousness Scale (Public)
	N	Variable	Age	BMI						
Total	92	Mean	21,67	21,6627	21,79%	79,43%	66,45%	69,33%	71,38%	77,69%
		Median	21,36	21,0937	17,39%	78,57%	65,56%	69,70%	74,07%	76,19%
		SD	1538	2,68483	25,10%	8,64%	10,12%	25,10%	25,10%	25,10%
		Minimum	19	17,57	0,00%	54,00%	46,00%	26,00%	19,00%	24,00%
		Maximum	26	32,64	70,00%	100,00%	94,00%	89,00%	100,00%	100,00%
		P25	20,46	19,8177	4,35%	75,00%	59,17%	60,98%	62,96%	66,67%
		P50	21,36	21,0937	17,39%	78,57%	65,56%	69,70%	74,07%	76,19%
		P75	22,53%	23,0518%	34,78%	85,71%	73,33%	75,76%	81,48%	90,48%

As displayed in **Figure 8**, there is a clear distinction between the experimental subgroups and the control subgroups.

The qualitative examination and quantitative scoring of standardized and structured clinical interview (four psychological scales, i.e., APZ, SHS, MAAS and SCS-R) allowed us to identify four subgroups:

(1) Experimental Top subgroup (the higher tercile) constituting the sample of subjects with the highest scores during the intervention;

(2) the Control Top subgroup (the higher tercile) indicating the subjects with the highest scores on the sham intervention, i.e., attentional focus during the class;

(3) the Experimental Bottom subgroup (the lower tercile) constituting the group of subjects that had the lowest scores during the intervention;

(4) the Control Bottom group (lower tercile) constituting the group of subjects that had the lowest scores on the sham intervention, i.e., lack of attentional focus during the class.

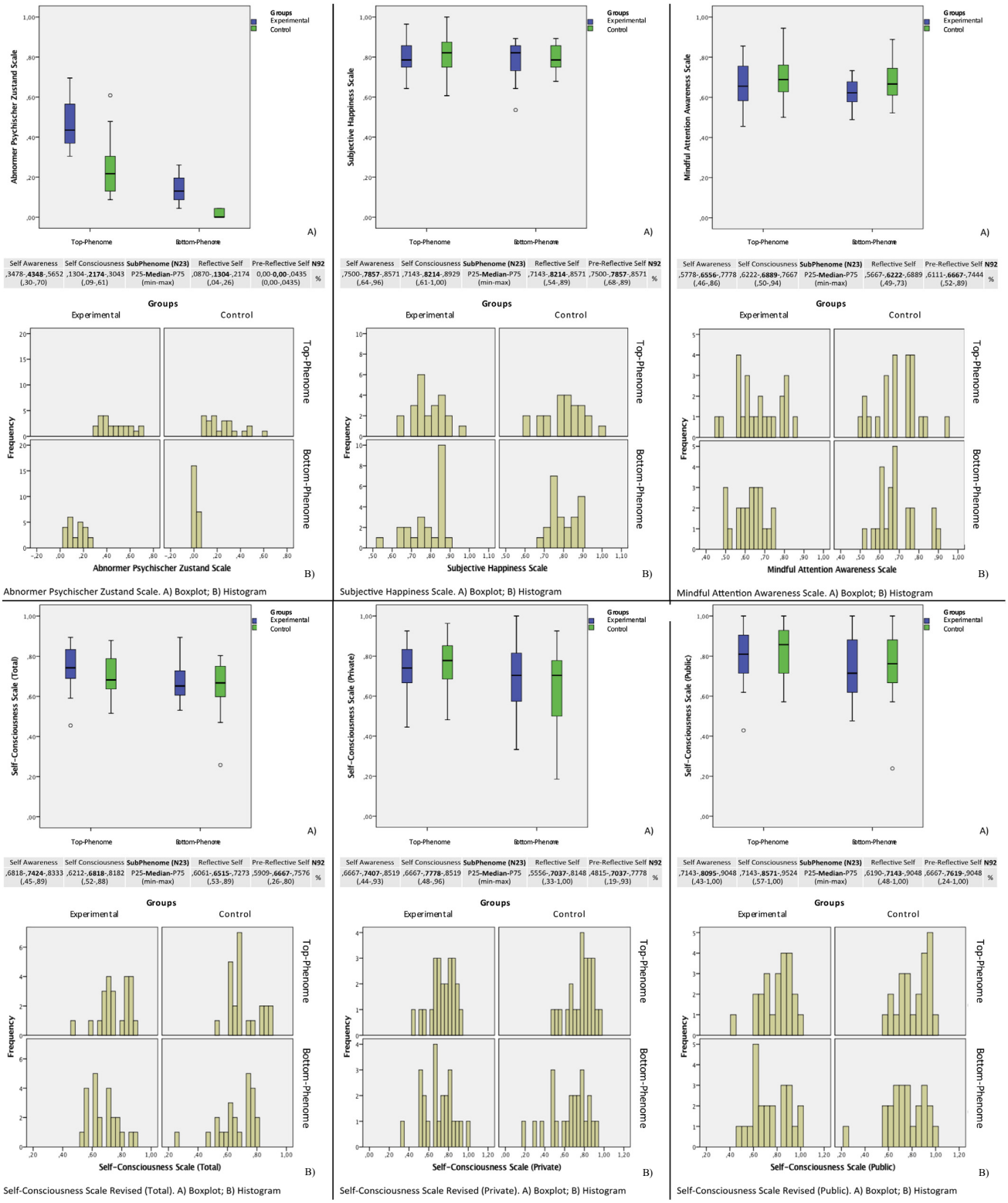


Figure 8. Standardized And Structured Clinical Interview

Qualitative examination and quantitative scoring of the neurotypical sample used in the Psychological Assessment. In A), for each of the instruments a boxplot is presented. In the boxplot, the P25, median, P75, minimum and maximum is shown and calculated by the instrument's total percentage score. Blue corresponds to the experimental groups and green to the control groups. In B), for each of the instruments a histogram is presented. The density of the underlying distribution of the data for each instrument is shown, estimating the probability density function of the underlying variable.

2.B. Discussion Of The Mental Status Examination

A standardized and structured clinical interview was made, which comprised the use of the following 4 psychological scales. This classical mental status examination in a sample of neurotypical subjects allowed the systematic characterization of the 1) Experimental Top subgroup; the (2) Control Top subgroup; the (3) Experimental Bottom subgroup; and the (4) Control Bottom subgroup.

This psychological evaluation estimated the 1) process and content of thought, and perception (APZ); the 2) mood and affect (SHS); the 3) cognitive awareness and attention (MAAS); and the 4) insight and judgment (SCS-R), of the 4 healthy subgroups.

The 1) Experimental Top subgroup is defined by no alterations on the process and content of thought, and with a conserved perception. The stream and rate of thoughts are adequate (quantity and tempo), as well as their flowing and rational connection or logical coherence. There are no exhibited loosening of associations or flight of ideas. Parallelly, the qualitative properties of thinking as well as its intensity, salience, and associated emotions are optimal. No delusions or hallucinations are present. The organization, identification, and interpretation of sensory information is preserved in order to represent correctly and understand accurately the experimental procedure.

Henceforth, there are no negative alterations of thought or negative perceptual changes; but there is an intense emotional response, particular bodily schema changes and meaning alterations, which are typically evoked during cognitive behavioral interventions.

The mood is reported as “happy”, viz. the predominant and prevalent internal/subjective state at any one time. The emotional type is characterized as euphoric and associated with a deeply felt associated mood. The affect is characterized by neither a depressive nor anxious dimension. The external and dynamic manifestation of the subjects internal emotional state is described as intense, not labile, and congruent to the global content of the descriptions after the experimental procedure. Absolute and relative emotions of well-being are pronounced and defined as stable. Self- and non-self perception of subjective happiness is evoked. Thus, the subjects in this subgroup balance positive and negative emotions, but also have the capacity to make a judgment of the overall life quality. This judgment is likely not equivalent to a simple sum of the subjects recent levels of affect and satisfaction with life, but indicative of an overall affective and emotional perception.

Cognition, and more specifically the level of consciousness, alertness, and attention is ideal, i.e., the awareness and responsiveness to the experimental procedure. This subgroup is characterized by the ability to maintain focus, but also sustain and appropriately shift mental attention. Subjects in this subgroup have a higher open awareness to the present, i.e., to the exercise of immediate and short-term memory. The subjects in this subgroup show elevated private self-monitoring and cognition, which was targeted with the cognitive behavioral intervention. Hence, they show an effective regulation of emotionality and the capacity of attention to present internal state experiences.

The insight and judgment is highly maintained, as well as a high availability and cooperation with the experiment. The subjects in this subgroup understand their mental state and have a high level of awareness of their own situation. Moreover, they have the ability to make reasoned and responsible decisions to preserve their own and others’ wellbeing. It is characterized by participants with low impulsiveness, and high

social cognition and self-awareness. Thus, private and public self-consciousness is maintained, and is stable over time. This mental status arises from sustained conceptual thought, long-term memory and the capacity to abstraction and interpretation. Hereafter, they present highly developed constructs of self-reflection and self-concept and an effective regulation of internal state awareness and the capacity of self-presentation and introspection.

This subgroup is thus interpreted as having a highly developed self-awareness, which is characterized by the progression from the pre-reflective self, to reflective phenomenology, and then to the narrative autobiographical interpretation (De Jaegher, Pieper, Clénin and Fuchs, 2016) and high-level cognition (Klein and Loftus, 2015). Phenomenologically, this subgroup described the experiment as an oceanic boundlessness, i.e., experiencing a deeply-felt positive mood and self-understanding.

The 2) Control Top subgroup is also characterized by no alterations on the process and content of thought, and with a preserved perception. The stream and rate of thoughts are adequate (quantity and tempo), as well as their flowing, rational connection, logical coherence, and associations of ideas. Parallelly, the qualitative properties of thinking as well as its intensity, salience, and associated emotions are ideal. No delusions or hallucinations are present. The organization, identification, and interpretation of sensory information is preserved in order to represent correctly and understand accurately the content of the university class, i.e., the sham intervention.

Henceforth, there are no negative alterations of thought or negative perceptual changes. Though, there is no intense emotional response, bodily schema changes or meaning alterations evoked during the cognitive task of attending a class, as expected.

The mood is also reported as "happy", viz. the predominant and prevalent internal/subjective state at any one time. The emotional type is characterized as euthymic. The affect is characterized by neither a depressive nor anxious dimension. The external and dynamic manifestation of the subjects internal emotional state is described as mild, not labile, and congruent to the global content of the lesson. Absolute and relative emotions of well-being are designated and described as stable. Self- and non-self perception of subjective happiness is evoked. Thus, the subjects in this subgroup also balance positive and negative emotions and have the capacity to make a judgment of the overall life quality.

Cognition, and more specifically the level of consciousness, alertness, and attention is standard, i.e., the awareness and responsiveness to focusing at the class. This subgroup is also characterized by the ability to maintain focus and appropriately shift mental attention, as well as to the exercise of immediate and short-term memory.

The insight and judgment are maintained, as well as the interest in the contents of the class, i.e., the sham intervention. The subjects in this subgroup understand their mental state and situation, having a high social cognition. Moreover, they also have the ability to make reasoned and responsible decisions to preserve their own and others' wellbeing. Thus, private and public self-consciousness is maintained, and is relatively stable over time. Likewise, they present highly developed constructs of self-reflection and self-concept and an effective regulation of internal state awareness and the capacity of self-presentation and introspection.

This subgroup is interpreted as having an elevated self-consciousness, which can be characterized as having pre-reflective bodily sensations and emotional states, in a 1st or 2nd person interaction with the environment, that can be described as being-in-the-world or an in a natural attitude (Husserl, 1952; Merleau-Ponty, 1976; Varela, Thompson and Rosch, 1991).

The 3) Experimental Bottom subgroup is also characterized by no alterations on the process and content of thought, and with a still preserved perception. The train of thought is preserved but the rate of negative thoughts is slightly increased (quantity and tempo), fairly affecting the flowing and rational connection or logical coherence. Still, there are no pathological loosening of associations or flight of ideas. The qualitative properties of thinking and the associated emotions are less congruent. Hence, the organization and interpretation of sensory information is less preserved and somewhat affecting the adequate representation and understanding of the experimental procedure. Still, no delusions or hallucinations are present. Henceforth, there are no negative alterations of thought or negative perceptual changes, neither an emotional response evoked during the cognitive behavioral intervention.

The mood is less reported as "happy". The predominant and prevalent internal/subjective state is characterized as slightly apathetic. The affect is characterized by not pathological, but intermittent and minor anxious dimension. The external and dynamic manifestation of the subjects internal emotional state is described as marginally labile, and somewhat not completely congruent with the personal descriptions after the experimental procedure. Scarce absolute and relative emotions of well-being are described, and limited self perception of subjective happiness is evoked.

The level of consciousness, alertness, and attention is ordinary, i.e., the awareness and responsiveness to the experimental procedure. This subgroup is characterized by some tendency to inattention and to slight difficulties in sustaining concentration. Subjects in this subgroup have a lower open awareness to the present.

The insight and judgment are maintained, but the subjects in this subgroup understand less their mental state and have a lower level of awareness of their own situation. It is characterized by participants with some propensity to impulsiveness, showing lower social cognition and self-awareness. Private and public self-consciousness is maintained, but rather unstable.

This subgroup is interpreted as having a highly reflective self-experience. Reflective self-experience is a procedural cognitive process that takes place whenever one inspects or reflectively introspects one's experience, or recognizes one's specular image in the mirror, or refers to oneself with the use of the first-person pronoun, or constructs a self-narrative. Therefore, this precedes the content to which self-focus is addressed. In other words, it is a reflective experience intentionally directed towards conscious thought (Gallagher and Zahavi, 2015).

The 4) Control Bottom subgroup is also characterized by no alterations on the process and content of thought, and with a still preserved perception. There are no pathological loosening of associations or flight of ideas, but the qualitative properties of thinking and the associated emotions are less congruent. Hence, the organization and interpretation of sensory information is less preserved and affecting the adequate representation and understanding of the contents of the university lesson, i.e., the sham intervention. However, as in the other subgroups, no delusions or hallucinations are present. Henceforth, there are no negative alterations of thought or negative perceptual changes, neither an emotional response evoked during the cognitive task of attending a class.

The mood is less reported as "happy". The predominant and prevalent internal/subjective state is characterized as dysphoric. The affect is defined by not pathological, but intermittent and minor depressive dimensions. The external and dynamic manifestation of the subjects internal emotional state is described as slightly labile, and somewhat not completely congruent with the global content of the lesson.

Few absolute and relative emotions of well-being are described, and reduced distinctive self perception of subjective happiness is evoked.

The level of consciousness, alertness, and attention is average, i.e., the awareness and responsiveness to focusing at the class. This subgroup is also characterized by some propensity to inattention and to slight difficulties in sustaining concentration. Likewise, subjects in this subgroup have a lower open awareness to the present.

The insight and judgment are kept, but the subjects in this subgroup understand less their mental state and have a lower level of awareness of their own situation. It is characterized by participants with some predisposition to impulsiveness, presenting a lower social cognition and self-awareness. Private and public self-consciousness is maintained, but slightly less stable.

This subgroup is interpreted as having a pre-reflective self-experience, which is an embodied and situated cognition. Pre-reflective in the sense that (1) it is an awareness occurring before any reflecting on the experience; (2) it is implicit. An explicit reflective self-consciousness is possible only because there is a pre-reflective self-awareness that is an ongoing and more primary self-consciousness. In other words, the experiential dimension always involves a significant implicit pre-reflective self-awareness (Gallagher and Zahavi, 2015). In agreement with Husserl (1952), the notion of pre-reflective self-awareness is the idea that experiences have a subjective 'feel', a certain phenomenal quality of 'what is like' (a perceptual experience) or 'what it 'feels' like to have them (bodily sensation). Such first-person experience presents us as with an immediate and non-observational access to ourselves in a (minimal) form of self-consciousness (Gallagher and Zahavi, 2015).

From an epistemological perspective, there are two different epistemological self-experiences to be distinguished: pre-reflective and reflective (Gallagher and Zahavi, 2015). The dynamics between pre-reflective and reflective experience, and the progression to self-conscious/aware experiences may result in a healthy self-experience. This dynamic will be further explored in chapter 3 and chapter 6 of this thesis, and is summarized in the **Supplementary Figure 1.A**. This dynamic is exposed, thus, in cognition of the neurotypical self, but also in chronic pathologies (Osborn and Smith, 2006; Mozo-Dutton, Simpson, and Boot, 2012) or in neuropsychiatric syndromes, e.g., schizophrenia and autism (Hipólito and Martins, 2018).

Ultimately, self-awareness can be considered as a progression (**Supplementary Figure 1.B**) in a 2-dimensional axis: a) unconscious to conscious experience and b) ill-being to well-being. This progression will also be further explored in chapter 3 and chapter 6 of this thesis. The self-consciousness trait is a prerequisite to that progression (**Supplementary Figure 1.C**). It is postulated that well-being or *eudaemonia*, in neurotypical subjects (**Supplementary Figure 1.D**), may result from that progression.

2.C. Neurophenomenological Data And Method

Figure 9 presents the comparative results of the phenomenological descriptions of each of the four subgroups. As illustrated in **Figure 8**, the four subgroups performed differently on the overall scores of self-report measures. This suggests specific constructions of subjective experience, which resulted in the four subgroups: 1) Experimental Top subgroup; (2) Control Top subgroup; (3) Experimental Bottom subgroup; and (4) Control Bottom subgroup. These specific psychological characteristics can further be correlated to a specific molecular expression.

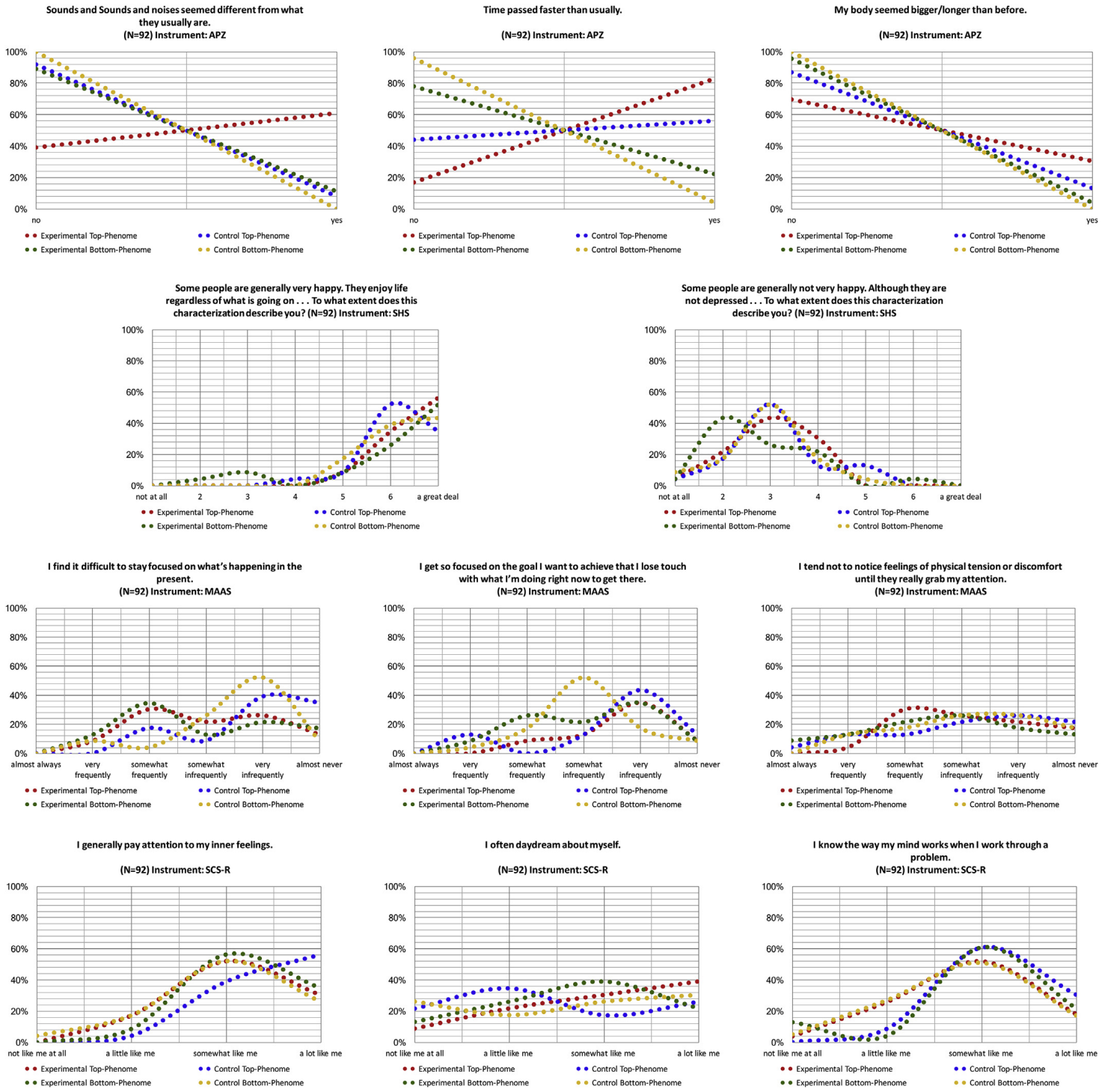


Figure 9. Comparative Results Of The Phenomenological Reports For each of the 4 instruments used in the Psychological Assessment, a sample of the variables evaluated and type of scoring is provided. Multimodal histograms are presented and each color is representative of a subgroup/subphenome: red (Experimental Top subgroup), blue (Control Top subgroup), green (Experimental Bottom subgroup) and yellow (Control Bottom subgroup). In the APZ, a sample of 3 variables is shown with yes/no answers. In the SHS, a sample of 2 variables is shown with a score ranging from "not at all" to "a great deal". In the MAAS, a sample of 3 variables is shown with a score ranging from "almost always" to "almost never". In the SCS-R, a sample of 3 variables is shown with a scoring from "not like me at all" to "a lot like me".

In **Figure 9**, as a proof of concept, we describe the same variables as in **Table 1**. These variables, or questions posed to the participants in the Psychological Assessment, are dissected discretely and ubiquitously for all items of the self-report measures.

For instance, in the APZ scale the (1) Experimental Top subgroup (0.60) reported that "Sounds and noises seemed different from what they usually are"; while (2), the Control Top subgroup, and (3), the Experimental Bottom subgroup, did not report this (0.91); while (4), the Control Bottom subgroup, did not consider the question at all (0).

On the SHS scale, the (1) Experimental Top subgroup appeared frequently with the statement: "Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?". This was less true of (2), the Control Top subgroup; even less so of (3), the Experimental Bottom subgroup; and only moderately true of (4), the Control Bottom subgroup.

On the MAAS scale, (1), the Experimental Top subgroup, somewhat infrequently " … found it difficult to stay focused on what's happening in the present", while for (2), the Control Top subgroup, this was almost never reported; for (3), the Experimental Bottom subgroup, somewhat frequently reported; and for (4), the Control Bottom subgroup, this was infrequently reported.

On the SCS-R scale, (1), the Experimental Top subgroup, report that "daydreaming about themselves" pertains greatly to them; while (2), the Control Top subgroup, report that the statement pertains only a little to them; (3), the Experimental Bottom subgroup, report that the statement somewhat pertains to them; and (4), the Control Bottom subgroup, reported that the statement does not pertain to them at all.

The method and materials used in this study permitted thus to:

(i) Identify experiential profiles based on the subjects' evaluations of their own conscious experience;

(ii) Clinically and phenomenologically understand the groups, and their subtypes,

(iii) Prompt the investigation of protein correspondence (i.e., quantifying the differences in their proteomic profiles).

The model presented here bears resemblance to the neurophenomenological method, which proposes to introduce, into brain research on subjective consciousness, first-personal methods of examining experience. A point of difference regards the Psychological Assessment: the participants are not trained in the phenomenological method, that is, they complete the intervention naively.

This method, however, constitutes a variation insofar as the quantitative part of the study does not consist only in a brain-centered methodology. The quantitative method, developed in the chapters 4, 5 and 6 of this thesis, consists instead in a functional protein profile (proteomics), that is, dynamic protein information that is distributed along the organism itself. The aim of this study is the understanding of molecular interactions induced by the Intervention Protocol. For that matter a focus is given, in chapters 4 and 5, to the protein profile, referring to the entire set of proteins produced or modified by an organism or system.

2.D. Outcomes And Limitations

Commonly, there are two biochemical methodologies used to study a biological system:

a) directed studies (classical biochemical studies), and b) enlarged studies (the omics biochemical studies). This work is not intended as a directed analysis into a specific or exact candidate neural pathway or phosphorylation process. The sensibility and specificity necessary to conduct such a study are virtually impossible, and would fail power analysis.

Rather, it is proposed a study with a different magnitude, i.e., an enlarged, non-directed perspective, or an omics study. The intent is thus to probe physical interactions between molecules, as an example of "top-down" molecular biology, taking an overhead -as well as overall- view of a biosystem (Bruggeman and Westerhoff, 2007; Kiemer and Cesareni, 2007).

The work is proposed as an omics paradigm, usually defined by contrast to a reductionist biological organization. As Denis (2006) notes, "Omics […] is about putting together rather than taking apart". As a method, omics comprises high-throughput analytic techniques (Romualdi et al., 2009). These techniques include, for example, transcriptomics, metabolomics or proteomics, to propose specific testable hypotheses (Alberghina and Westerhoff, 2005); for instance, the result of an experiment, i.e., in this work the Intervention Protocol.

Normally, the output of an omics study is a multi-database generation (Baitaluk, 2009). Specifically, in chapter 4, 5 and 6 of this thesis, it will be generated layered results of *in silico* characterization (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017), using bioinformatics tools, like Cytoscape® and PANTHER® (chapter 4), total proteins profiles (chapter 5), and simultaneous immunodetection and quantification of the neuropeptide and neuroimmune response.

Top-down molecular biology like these could in principle identify molecular interaction networks (Bruggeman et al., 2007). Although there are still today explanatory limitations, the interaction between molecular networks can be correlated to observed behavior of subjective experience, evaluated through self-report measures. Explanatory limitations include, for instance, the incapability of connecting a specific neural pathway to a specific self-reported behavior. Indeed, such an explanatory error resides in the inability to rule out preliminary reasoning, suggesting that such behavioral-molecular correlations were present in the first place.

Subjective experience has been studied as the macroscopic cartography of synaptic connections in a macro-network, but could also be probed on an intra-cellular scale: a micro-network. Those neural networks are regulated upwards or downwards via intracellular regulatory networks of the transcriptome interfaces (Foster, 2015). Insufficient findings describe specific molecular networks that can be elicited through inducting a specific subjective experience. This scientific gap motivates setting up the approach further developed in the section II of this thesis, i.e., the molecular stratification. The object of this chapter is then the interplay between mental and biological characteristics. In specific, this chapter concerns how subjective experience modulates objective biological measurements (Ulvestad, 2012).

Omics methodologies have been recently gathering a complex and extensive quantity of data enabling biomedical sciences. Methodologies such as the one proposed by Ritchie and colleagues (2015), assesses biological systems via multi-omics, i.e., from transcriptome to phenome. Omics studies began to characterize different biological substrates. Nevertheless, such studies have only recently looked into neuronal structures (English et al., 2012), giving access to solid neuroproteomics databases (Collins et al., 2006; Behan et al., 2009; Hahn et al., 2009; Ishii et al., 2009; Baye's et al., 2011).

Henceforward, this chapter points that omics studies advance towards insights into the biomolecular underpinnings of subjective experience, for instance, the physiological mechanisms involved in strong emotional states (Chiappelli et al., 2006). Such an advancement in omics studies would offer a neuroscientific approach to the *hard problem of consciousness* (Susan et al., 2005).

Nevertheless, no publication yet has used analytical proteomic techniques, like those that will be used in chapter 4, 5 and 6, to evaluate physiological states and traits of neurotypical young adults (Martins et al., 2016). Indeed, the difficulty surrounding such research consists in the fact that self-conscious experience, in a neurotypical population, varies both with time and with the external stimuli, if not properly controlled. The question resides if there are cognitive, as well as are physiologic, states and traits that maintain *continuity* and remain stable over time.

As referred before, the reliability of the cognitive states and traits will be tested by the measures used in chapter 3. The mental status of the participants is maintained over time, and remain similar, since it's a fundamental nature of a mental state if no external stimulation is given. Again, to note, that the psychological variable studied is traces of autobiographical memory, which is a measure that remains constant in neurotypical individuals. The stability of self-conscious experience is therefore maintained because there were no external stimuli, not properly controlled or monitored.

The excerpt of the research outline (**Figure γ**) systemizes the Section I: Mental Health Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 2 in the overall experiment.

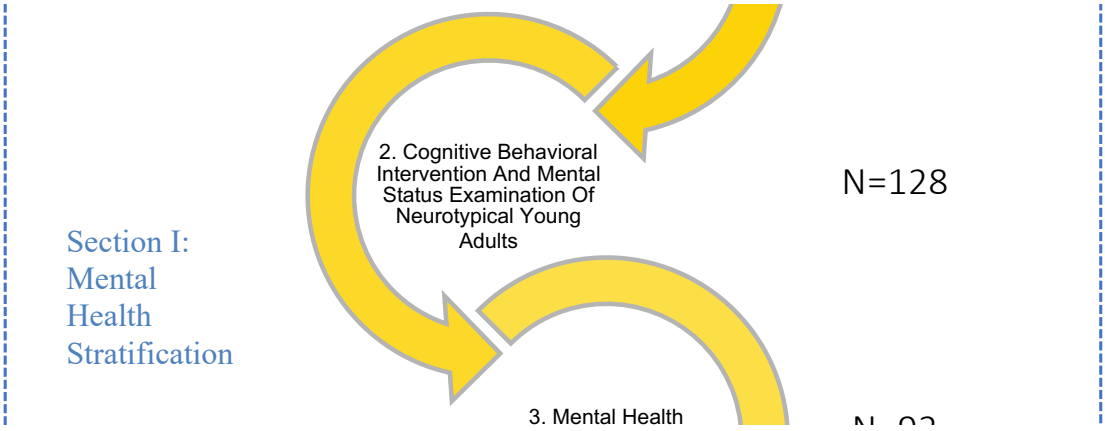


Figure γ. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section I: Mental Health Stratification
The chapter 2 (Cognitive Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults) is displayed, which comprehended the Psychological Assessment and Intervention Protocol used for the characterization of the 128 subjects.

Chapter 3. Mental Health Profiling In Neurotypical Young Adults

Preamble ("Would it be viable to stratify mental health in neurotypical subjects?")

Well-being is related to healthy cognitive life. However, how such relation is established, in neurotypical subjects, requires further evidence. Do cognitive life influence or modulate well-being? How does subjective experience modulate mental health (Pacheco, Martins and Simões, 2019) in neurotypical subjects? This chapter focuses on a phenomenological assessment used for a mental health profiling. The assessment comprised a psychological intervention, i.e., a cognitive behavioral intervention, and evaluation protocols. Both have been used to generate cognitive profiles of mental health functioning, although, this profiling method needed further explanation and exploration. Consisting of phenomenological descriptions of well-being and self-constructs, it clinically portrays phenotypic states and traits of young neurotypical adults. 128 (19-to-25-year-old) healthy university students, non-medicated and with no serious, uncontrolled or chronic diseases were stratified and characterized according to their cognitive functioning. Four different subgroups, i.e., mental health strata, were screened. We expect that each of these different mental health profiles may have a homogeneous molecular response and, consequently, a possible specific biomarker signature. This study fulfills an *a priori* pre-molecular assessment for a subsequent molecular typing.

Materials And Methods

Chapter 3 comprises the methodology that led to the stratification of the neurotypical sample. Most specifically, this chapter presents the clinical and statistical strategy for the adequate profiling of the mental status. The procedure includes a psychological intervention and a clinical assessment. This strategy executes a proportionate stratified random sampling of the 128 subjects in this study. The psychological intervention extends the use of the cognitive behavioral intervention, already discussed in chapter 2. The clinical assessment comprehends the application of (i) a General Clinical Questionnaire, and extends the use of the (ii) Psychological Assessment scales, already introduced in chapter 2.

3.1. Study Design

This chapter of the study is an exploratory, non-representative, randomized descriptive study with a two--grouped, four setting, single-blinded, post-test design. The study design (Intervention and Assessment Protocols) is considered a descriptive neurophenomenological type and was approved by the Ethics Committee of the Faculty of Medicine, University of Lisbon.

The method comprehends a descriptive research strategy (Best and Kahn, 2014) combining:

(i) psychological intervention, i.e., the Intervention Protocol;

(ii) clinical assessment, i.e., the Assessment Protocol.

This strategy executes a proportionate stratified random sampling (Aoyama, 1954) for the classification of the subjects (González et al., 2017).

In this chapter, we advanced with the previously published methodology (chapter 2), using the same participants, basing our methodology in authors that have recently stratified neurotypical and neuropsychiatric subjects into subclinical groups

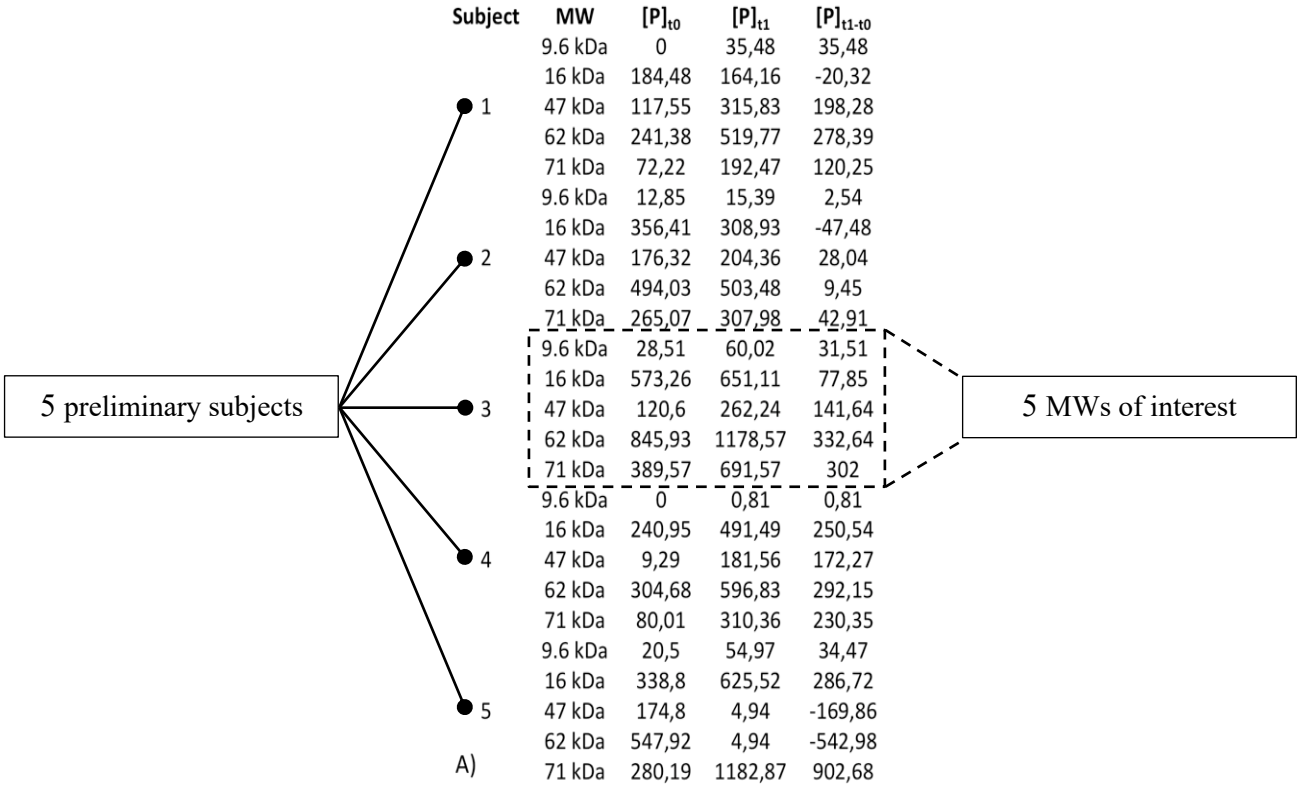
(Biundo et al., 2014; Britton, Lindahl, Cahn, Davis, and Goldman, 2014; Hanley and Garland, 2014; Matthews et al., 2014; Schumann et al., 2014; DaSilveira, DeSouza and Gomes, 2015; Shafer, 2015; Bearden et al., 2016).

3.2. Sample Screening And Sample Size Study

A power analysis (Zhang et al., 2019) was used to calculate the sample size required to detect an adequate molecular effect size.

The sample size study is thus supported by the previous preliminary results of the experimental group (Martins et al., 2016). These results were obtained before advancing with the mental health stratification. Consequently, it was aimed to test if there was a significant change in the protein profile in a neurotypical sample.

Figure 10 shows the quantitative data of the tested MWs that were used to evaluate significant changes in the protein profile, by capillary electrophoresis. Capillary electrophoresis method allowed to measure the concentration (ng/µl) of the five most significative ranges of MWs in the preliminary study: 9.6 kDa, 16 kDa, 47 kDa, 62 kDa and 71 kDa, before and after the experimental procedure.



Subject	MW	$[P]_{t0}$	$[P]_{t1}$	$[P]_{t1-t0}$
1	9.6 kDa	0	35,48	35,48
	16 kDa	184,48	164,16	-20,32
	47 kDa	117,55	315,83	198,28
	62 kDa	241,38	519,77	278,39
	71 kDa	72,22	192,47	120,25
2	9.6 kDa	12,85	15,39	2,54
	16 kDa	356,41	308,93	-47,48
	47 kDa	176,32	204,36	28,04
	62 kDa	494,03	503,48	9,45
	71 kDa	265,07	307,98	42,91
3	9.6 kDa	28,51	60,02	31,51
	16 kDa	573,26	651,11	77,85
	47 kDa	120,6	262,24	141,64
	62 kDa	845,93	1178,57	332,64
	71 kDa	389,57	691,57	302
4	9.6 kDa	0	0,81	0,81
	16 kDa	240,95	491,49	250,54
	47 kDa	9,29	181,56	172,27
	62 kDa	304,68	596,83	292,15
	71 kDa	80,01	310,36	230,35
5	9.6 kDa	20,5	54,97	34,47
	16 kDa	338,8	625,52	286,72
	47 kDa	174,8	4,94	-169,86
	62 kDa	547,92	4,94	-542,98
	71 kDa	280,19	1182,87	902,68

Figure 10. Power Analysis And Sample Size Study, Using The $\Delta[P]_{\text{after-before (experimental procedure)}}$ Protein concentration and change/variability (ng/µl) of the five preliminary subjects, in the five MWs of interest used to estimate a potency (T-Student) of 80%, a significance level of 5%, and an effect size corresponding to 40%.

The following approach was designed and used to calculate the adequate sample size for this thesis:

First, the difference $\Delta[P]_{\text{after-before (experimental procedure)}}$ of the protein concentration (ng/µl) was calculated, i.e., the protein change/variability (ng/µl). Secondly, based on the results of the change/variability (ng/µl) it was then considered a potency (T-

Student) of 80% and a significance level of 5%. Thirdly, it was chosen an effect size corresponding to 40% of the estimated value for the standard deviation of the variation of the MWs.

Taking into account this statistical option, that corresponds to a medium Cohen's effect size, an appropriate sample size for each group (experimental and control) of 64 elements was found. Consequently, the sample was initially separated into two independent groups: (a) Experimental group (experimental procedure) (64 subjects); and (b) Control group (sham intervention) (64 subjects).

Afterwards, the Statistical Analysis presented in this chapter permitted that those groups were further stratified into the top and bottom phenomes, resulting in 4 subgroups: (i) the top phenome of the Experimental group; (ii) the top phenome of the Control group; (iii) the bottom phenome of the Experimental group; and (iv) the bottom phenome of the Control group. This stratification assigned 92 subjects, as it comprehended the use of the higher (Top) and lower (Bottom) tercile of the sample (further explained in Results and Discussion), and an additional 10% of subjects.

These final 4 subgroups (profiles), with an N=92, were tested for comparability statistics and external biometric data, possibly confounding variables, and were considered comparable for: a) age (**Figure 11.A**) (P25; median; P75) and overall (N=92) mean = 21,495; b) gender mean = 50% males and SD=0; and c) BMI (kg/m^2) (**Figure 11.B**) (P25; median; P75).

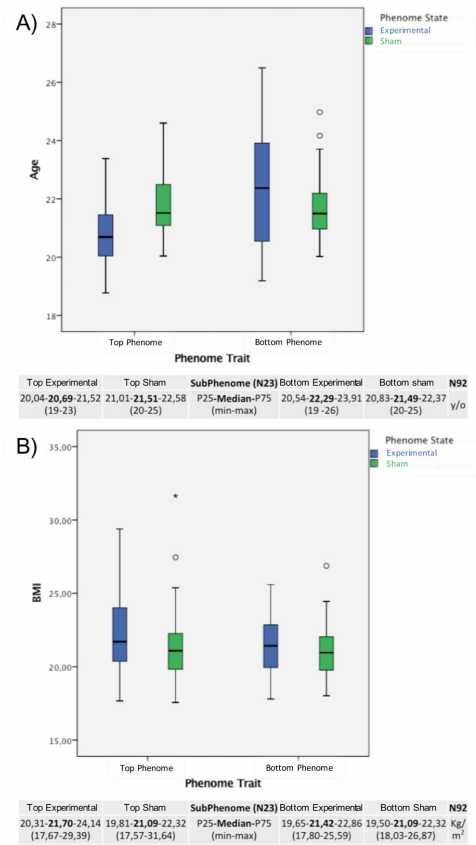


Figure 11. Biometric Data Of The Neurotypical Sample
In A), a boxplot (P25, median, P75, minimum and maximum) of the sample's age in the 4 subphenomes (Experimental Top subgroup, Control Top subgroup, Experimental Bottom subgroup and Control Bottom subgroup) is presented.
In B), a boxplot (P25, median, P75, minimum and maximum) of the sample's BMI (Body Mass Index), in Kg/m^2, for the same 4 subphenomes is presented.

3.3. Intervention Protocol

The Intervention Protocol (experimental procedure and sham intervention) was carried out in one single session, on Tuesdays or Thursdays, performed between 09:45 (a.m.) and 10:30 (a.m.). The experimental procedure is a structured psychological intervention, i.e., a single cognitive behavioral intervention (Segal and Teasdale, 2018), and was fully detailed in chapter 2.

It was applied in a psychotherapeutic setting (Simões, 1998; Sobrinho, 2003; Simões et al., 2013; Martins et al., 2016a) with a total duration of 45 min. The research aim was to increase awareness and wakefulness (**Figure 12**), by a cognitive exercise of psychophysical training and autobiographical-self memory-recalling (Martins et al., 2016b). The script of intervention is published in the Annex D: Verbal protocol of the experimental procedure and is based on the publications of the PhD supervisor (Simões et al., 1998; Simões, 2002; Simões et al., 2013). It was initially tested in the pilot study of this dissertation (Martins et al., 2016a) and as the experimental procedure of this work.

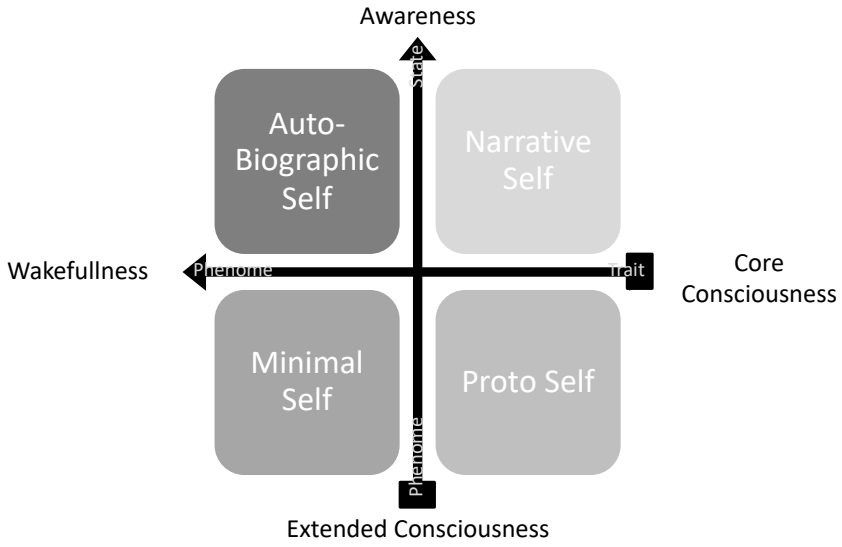


Figure 12. Neurophenomenological Sampling and Subgroup Identification: Constructs of Self
A two-axis plot is presented for the graphical representation of the Intervention Protocol and the types of self (Damasio and Meyer, 2009; Proto Self, Minimal Self, Narrative Self and Autobiographic Self) stimulated in the two phases of the experimental procedure. On the horizontal axis, the progression of the mental trait is plotted, stimulating core consciousness to a more wakefulness trait. On the vertical axis, the progression of the mental state is plotted, stimulating extended consciousness to a more awareness state.

This exercise helped in stratifying the mental health state and trait of the neurotypical subjects in the study, by somatic, emotional and cognitive regulation. The procedure was divided into 2 main phases (**Figure 6**) and the outcomes are further described in the flow chart in **Figure 13**.

The 1st phase, the *Arousal Phase* (Britton et al., 2014) is a conditioning procedure (Wager and Atlas, 2015). With the duration of 15 min, it aims to modulate arousal, or the level of consciousness.

The exercise consisted of a serious of directed verbal cues to enhance somatic focus, i.e., on breathing patterns (e.g., “Concentrate on your inhalation.”) and postural positioning (e.g., “Feel your right forearm.”).

According to Gallagher (2005), this phase may help the subject being aware of his bodily self and body schema. The somatic focus, used in this Intervention Protocol, is defined, by Gallagher, as (i) physiological self-awareness and (ii) sensory-motor self-awareness, as illustrated in **Figure 13**.

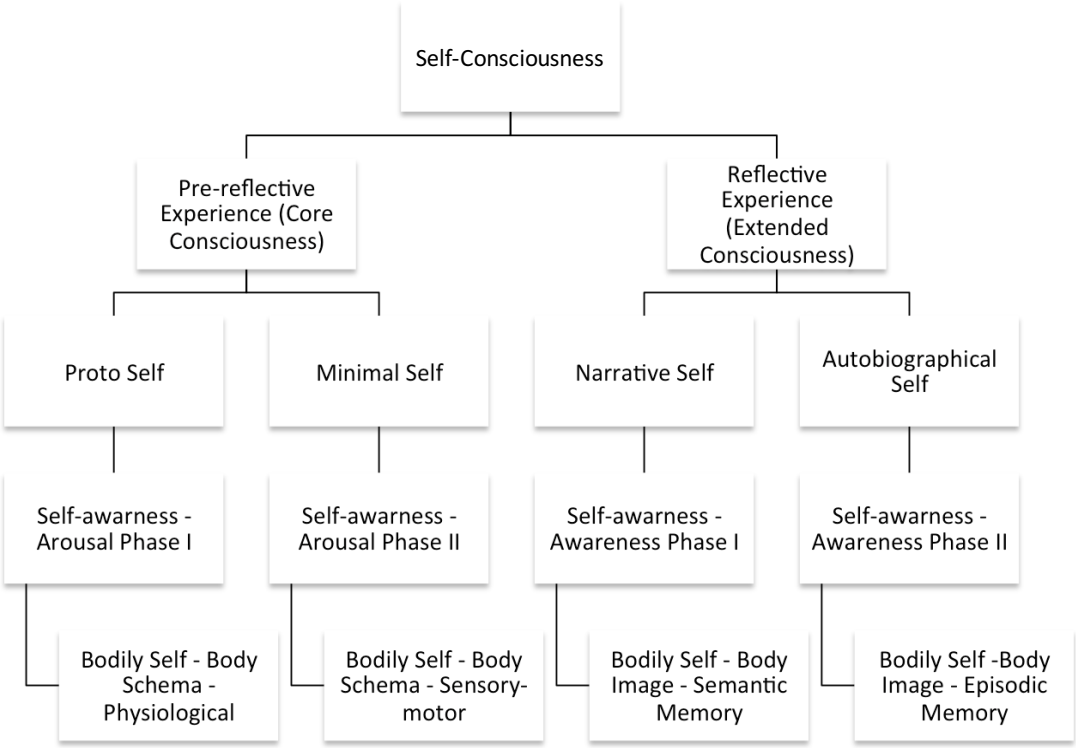


Figure 13. Fluxogram of the Neurophenomenological Method
A fluxogram is presented to systemize the Intervention Protocol and the two phases of the experimental procedure: Arousal Phase and Awareness Phase. The correspondence to the bodily self and body schema (Gallagher, 2005) induced in each phase is represented.

The outcome of this preliminary 15-minute procedure is stimulating a pre-reflective self-experience (Martins et al., 2016b), modulating what Damasio and Meyer (2009) described as core consciousness and/or the proto and minimal self, as illustrated in **Figure 14.A**.

The 2nd phase, the *Awareness Phase* (Laureys, Gosseries and Tononi, 2015), is an expectancy procedure (Kirsch et al., 2014). With the duration of 30 min, it aims to modulate awareness, or the content of consciousness. The exercise consisted of a serious of directed verbal cues to enhance a memory focus, i.e., sequential memory recalling (e.g., "Remember your first day of school."; "Remember your favorite holiday,", "Remember your last birthday."). These were linked to meaning making and reflexive emotional verbal prompts (e.g., "How good was it?").

According to Gallagher (2005), this phase may help the subject being aware of his self-body image. The memory focus used in this Intervention Protocol is defined, by Gallagher, as (i) semantic memory self-awareness and (ii) episodic memory self-awareness, as illustrated in **Figure 13**.

The outcome of this 30-minute exercise is stimulating a reflective self-experience (Martins et al., 2016b), modulating what Damasio and Meyer (2009)

described as extended consciousness and/or the narrative and autobiographical self, as illustrated in **Figure 14.B**.

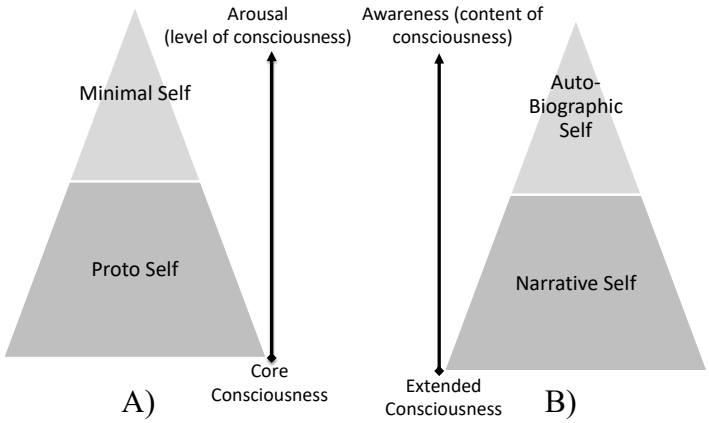


Figure 14. Conceptual Analysis Of The Intervention Protocol: A) Arousal Phase And B) Awareness Phase A pyramidal hierarchization is represented for each phase. In A) Arousal phase, the level of consciousness is plotted. In B) Awareness phase, the content of consciousness is plotted.

Therefore, based on this Intervention Protocol, two separate cognitive tasks, with the duration of 45 min, were established: (a) the experimental procedure and (b) the sham intervention.

On the one hand, in the experimental procedure (a), the Intervention Protocol was used in the Experimental group and comprised both arousal and awareness phases. It comprehended a structured script for:

(i) the arousal phase, suggesting successive body awareness;

(ii) the awareness phase, evoking consecutive and uninterrupted autobiographical memory contents from childhood to the present moment.

The mutual aim of both arousal and awareness phases, in the Experimental group, was to increase self-awareness. In healthy individuals, this is a cognitive phenomenon associated with well-being or *eudaemonia* (Veenhoven, 2009).

On the other hand, sham intervention (b) was used as a control study in the Control group. Sham intervention controls memory recalling, emotion regulation, and learning in a vigil state of consciousness. It consisted of listening to the same lecture in a classroom, i.e., without any other external stimuli. The sham intervention did not comprise either the focus on arousal or awareness phases, of the Intervention Protocol.

The outcome of the Intervention Protocol is, hence, generating different subphenomes or subtypes of subjective experience, in neurotypical subjects. Those 2 types of intervention generated distinct cognitive functioning.

The flow chart in **Figure 13** systematizes the self-constructs, as experienced in trait mindfulness (Brown and Ryan, 2003). Those constructs were aroused by this Intervention Protocol as a process for a neurophenomenological analysis.

Finally, for a detailed description of the protocol, i.e. “how it was”, “what was done”, “how was it done?”, and “what was intended to compare?”, adequate information is now available on the annexes, fully presenting the author’s previous work on this subject (Martins et al., 2016a; Martins et al., 2016b; Hipólito and Martins, 2017).

The methodology of the three referred articles, such as the verbal protocol of the experimental procedure, i.e., the single cognitive behavioral intervention, is entirely presented on the annexes of the thesis dissertation (Annex C. Happiness as a self state and trait of consciousness: saliva molecular biomarkers - a brief revision; Annex D. Verbal protocol of the experimental procedure).

3.4. Assessment Protocol

The Assessment Protocol (**Figure 6**) is a clinical assessment used for psychological scaling and classification of the neurotypical subjects. For that purpose, the subjects completed a case report form (Annex E: Case Report Form; Bellary, Krishnankutty, Latha, 2014).

In the protocol, from 10:45 (a.m.) to 12:00 (a.m.), on Tuesdays or Thursdays, a standardized and structured clinical interview was conducted, as part of the assessment protocol, and subjects filled out (i) a General Clinical Questionnaire and (ii) Psychological Assessment scales. The assessment protocol lasted between 45 and 75 minutes, depending on the subject. These (i) General Clinical Questionnaire and (ii) Psychological Assessment, were then compiled in case report forms (Annex E: Case Report Form) and originated a knowledge base of cognitive processes (Bodick and Marquis, 1990).

We published this database in a secured and protected online access: https://tinyurl.com/NeurotypicalDB (retrieved July 26, 2020; *in* Annex K: External Support), granted by private invitation only.

We then performed knowledge discovery (Pazzani, Mani and Shankle, 1997) with the acquired and stored data. We named the database: Neurotypical Database (Neurotypical DB). Neurotypical DB characterizes our sample and stratifies their mental health subphenomes or profiles. For better visualization of the variables used to generate the database, we created a Prezi infographic (Krum, 2013). It can be accessed online in: https://tinyurl.com/NeurotypicalDBPrezi (retrieved July 27, 2020; *in* Annex K: External Support). This infographic clearly shows the instruments that we used to acquire our mental health data.

The following instruments were used with the author's consent: (i) a General Clinical Questionnaire; and (ii) a Psychological Assessment, with psychological scales validated for the Portuguese population.

3.4.1. (i) General Clinical Questionnaire

The General Clinical Questionnaire is an instrument used in chapter 3 for different research purposes, and is part of the standardized and structured clinical interview already introduced in chapter 2. Not only, the General Clinical Questionnaire permits the sample screening in order to respect the inclusion/exclusion criteria, but also allows to collect clinical data to characterize the neurotypical sample. This questionnaire is based on the Biobanco-iMM and SalivaTec clinical questionnaires (Rosa et al., 2016) for saliva sampling, and is gold standard for the adequate clinical data collection. In the questionnaires a research section is dedicated to the specific research questions of a study. Thus, the author complemented the questionnaire with interest research question to address mental status variables discussed in this thesis. Data then was input to LabWare™ LIMS in order to systemize the clinical information of the 128 subjects.

The objective of the General Clinical Questionnaire is then to complement the four psychological scales used in the Assessment Protocol and to obtain a

comprehensive cross-sectional description of the participant's mental state. This objective clinical evaluation assesses the subject's medical history including education, cultural and social factors.

Thus, and in the Neurotypical DB, (i) the General Clinical Questionnaire evaluates the neurotypical subjects':

(a) Biometric data, physical activity, alcohol and smoking habits, and clinical anamnesis (diagnosis and therapeutics). These variables evaluate a phenome trait.

(b) Research data. It comprehends 20 items, or variables, designed to evaluate a phenome state.

Trait variables (a), such as age, gender and body-mass index, were mainly used to help characterizing the sample for future studies. However, state variables (b) comprehended a mental health state assessment dataset. This dataset was obtained by questions used to assess the self-experience induced by the Intervention Protocol.

The 20 binary variables, of (b) the Research data, are clustered (**Table 3**) in a psychological scale with the following domains:

Table 3. Assessment Protocol: (i) General Clinical Questionnaire - (b) Research data
The General Clinical Questionnaire evaluated specific (b) Research data in the neurotypical sample. The domains ($N_{total} = 8$), and subdomains ($N_{total} = 17$), created by the author for this study are synthesized in this table.

Domain	Subdomains
Altered State of Consciousness	understanding the meaning
	by personal experience
	frequency
	composition
Self-consciousness	understanding the meaning
	relation to the Intervention Protocol
Happiness Trait	understanding the meaning
	relation to the Intervention Protocol
	relation of the trait to the self
Happiness State	understanding the meaning
	by personal experience
	relation to the Intervention Protocol
Eudaimonia	sum of happiness trait and happiness state
Self-awareness	sum of Altered States of Consciousness, Self-consciousness and Eudaimonia
Doctor-patient Relationship	understanding the meaning
	relation to the Intervention Protocol
Self-awareness medicine	sum of all items of the General Questionnaire

(a) Altered State of Consciousness (4 subdomains): understanding the meaning, by personal experience, frequency and composition;

(b) Self-consciousness (2 subdomains): understanding the meaning, and the relation to the Intervention Protocol;

(c) Happiness Trait (3 subdomains): understanding the meaning, the relation to the Intervention Protocol, and the relation of the trait to the self;

(d) Happiness State (3 subdomains): understanding the meaning, by personal experience, and the relation to the Intervention Protocol;

(e) *Eudaemonia* (1 subdomain): sum of the happiness trait and the happiness state;

(f) Self-awareness (1 subdomain): sum of the Altered States of Consciousness, Self-consciousness and *Eudaemonia*;

(g) Doctor-patient Relationship (2 subdomains): understanding the meaning and relation to the Intervention Protocol;

(h) Self-awareness medicine (1 subdomain): sum of all the items of the General Clinical Questionnaire.

The consistency of those domains is further explored in Methods: Statistical Analysis.

3.4.2. (ii) Psychological Assessment

In the Neurotypical DB, (ii) the Psychological Assessment comprehends the use of a set of self-report measures, as already published in chapter 2.

The self-report measures are the following psychological validated scales:

(i) *Abnorme Psychischer Zustände* States of Consciousness (APZ) (Simões et al., 1986);

(ii) Subjective Happiness Scale (SHS) (Pais-Ribeiro, 2012);

(iii) Mindful Attention Awareness Scale (MAAS) (Gregório and Gouveia, 2011);

(iv) Self-consciousness Scale – Revised version (SCS-R) (Neto, 1986).

The *Abnorme Psychischer Zustände* States of Consciousness scale (APZ) (Dittrich, 1998), secondary scale: altered states of consciousness (Simões et al., 1986), is composed of 23 items and is used to assess a consciousness or cognitive state.

The items bring into evidence a (primary) modification of thought, the basic subjective experience (self and environmental), bodily scheme and perceptual changes, intense emotional response and meaning-making alterations.

This scale is used to evaluate a state of consciousness or the state of mind, natural or induced. This scale is usually proposed to evaluate a more focused and aware cognitive state, when compared to waking or resting states (the ordinary cognition).

It is, therefore, a psychological assessment of the cognitive and perceptual characteristics related to specific states of the mind, also described as a phenome state in this study. These states are usually stimulated during single cognitive behavioral interventions, but also in ordinary cognitive processes.

With this scale, we evaluated awareness and arousal states and their specificities (Duval and Wicklund, 1972).

The Subjective Happiness Scale (SHS) (Lyubomirsky and Lepper, 1999) is used to assess a mental health trait and it is a 4-item scale, which uses ratings on a 7-point rating measure of global subjective happiness. Two items evaluate absolute and relative personal happiness; two others evaluate absolute and relative external happiness. SHS measures, not only, the construct of subjective happiness, but also well-being and/or *Eudaemonia*.

Therefore, this scale aims to assess self- and non self-perception of happiness. It also aims to address the eudemonic (McMahon DM, 2004) perspective, opposing it to hedonic pleasure:

(1) man should aspire higher than a life of mere pleasure (McDowell, 2010);

(2) happiness is predicted by well-being, personality (Costa and McCrae, 1980), and life events.

Concluding, the SHS (Lyubomirsky S, Lepper, 1999) offers to examine the subjective mental health processes, or a subphenome trait of happiness (Diener et al., 1985).

The Mindful Attention Awareness Scale (MAAS) (Brown and Ryan, 2003) is used to assess a cognitive trait and is a 15-item scale, designed to assess a core

characteristic of dispositional mindfulness, named awareness at that moment to the present.

MAAS evaluates a construct of cognition designated pre-reflective self-experience. Conscious experience has 2 components: arousal and awareness, which have both interpersonal and personal variability. These inherent and sustained cognitive traits, denominated trait mindfulness (Brown and Ryan, 2003), can be enhanced.

Trait mindfulness (Brown and Ryan, 2003) is correlated to human health, indicating its incremental validity as a unique mental health construct (Pacheco, Martins and Simões, 2019). Therefore, MAAS constructs:

(1) are significantly and inversely associated with a variety of mental health indicators, e.g., depression (Fetterman et al., 2010);

(2) are positively associated with mental and physical health or well-being. For example, life satisfaction, perceived general health, physical functioning, and attention and self-regulation (Fetterman et al., 2010; Thompson & Waltz, 2007; Zvolensky et al., 2006).

The Self-Consciousness Scale – Revised version (SCS-R) (Scheier and Carver, 1985) is used to assess a mental health trait and consists of 22 items, measured on a five-point Likert scale, which are divided into two dimensions: Private Self-Consciousness and Public Self-Consciousness. It understands self-consciousness as an adaptive trait of the reflective self-experience (Duval and Wicklund, 1972; Wiley, 1994). Its private/public aspects can distinguish its adaptive/maladaptive characteristics in self-consciousness (DaSilveira, DeSouza and Gomes, 2015), and also its pre-reflective and reflective experiences.

Private Self-Consciousness is related to the inward direction of one's thoughts and feelings – the reflective self (Anderson et al., 1996; Creed and Funder, 1998; Silvia, 1999), whereas Public Self-Consciousness, or self-monitoring (Franzoi et al., 1990; Grant, 2001; Eichstaedt and Silvia, 2003), is related to the outward direction of one's thoughts, i.e., an awareness that the others have a perceptive awareness towards me, – a pre-reflective experience of the self (DaSilveira, 2011, 2012a,b). Both are considered as sustained mental health traits that are relatively stable over time.

Moreover, adaptive and maladaptive aspects of self-consciousness can be seen as a healthy self-experience (Trudeau and Reich, 1995), or as psychological distress, respectively (Trapnell and Campbell, 1999; McKenzie and Hoyle, 2008; Simsek, 2013).

In this chapter, we bring forth the hypothesis of a possible balance between pre-reflective and reflective experience for maintaining well-being, and therefore as an indicator of health.

Psychological Assessment clinically evaluates the neurotypical subjects in the domains and constructs that we considered characteristic of neuropsychiatric health.

3.5. Statistical Analysis

For the descriptive and exploratory analysis, frequency tables (simple/double entries) and boxplots were used as analytical/graphic exploratory tools.

Scale variables were summarized as mean, standard deviation, median, quartiles, minimum, maximum and/or other order statistics, when the sample distribution justified it.

Categorical variables were summarized using frequency and percentages. When the scale of measure was ordinal, order statistics were considered, e.g., tercile. Moreover, Spearman's rank correlation coefficient was applied.

For the inferential analysis, T-student for two independent samples and T-student for two paired samples were used to compare medians. One-way ANOVA, parametric and non-parametric, was used when comparing more than two means, i.e., the mean difference of the 4 subgroups in study, for each instrument.

Furthermore, correlations between scores on the subscales of the five instruments were calculated. To test the conditions of application of T-student test and one-way ANOVA, the following tests were used: Normality, Kolmogorov, with Lilliefors correction, $n \geq 50$, or Shapiro Wilks, $n < 50$, and the Levene's equality of variances test.

When the conditions were not met, non-parametric tests were used. When appropriate, results were complemented with confidence intervals at 95% and a significance level = 5%.

The sample size study is supported by the previous preliminary results of the Experimental group (Martins et al., 2016a). A potency (T-Student) of 80%, a significance level of 5%, and an effect size corresponding to 40% of the estimated value for the standard deviation of the variation of the molecular weights of the protein profile in the preliminary study were considered.

Taking this option in the account, it corresponds to a medium Cohen's effect size, an appropriate sample size of 64 elements for each group (Experimental and Control) was found.

3.6. Software

The following pieces of software were used:

(i) IBM SPSS Statistics 22 (with a significance level = 5%) for statistical and data management;

(ii) LabWare™ LIMS and Microsoft Excel for laboratory information management and the Neurotypical DB generation;

(iii) Prezi Inc. infographic for data visualization.

Results And Discussion

This set of results was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[70]: Martins, J. E.[a,b,c,d], Hipólito, I.[a,d,f], Rodrigues, T.[e], Rosa, N.[b], Correia, M. J.[b], Barros, M.[b], Simões, M.[a,c].

The results comprehend the data acquired in: (3.A.) the Neurotypical DB, (3.B.) the Subjective Assessment, (3.C.) the Descriptive Analysis of the Assessment Protocol, and (3.D.) the Inferential Analysis of the Assessment Protocol.

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[c]Mind-Brain College, University of Lisbon, Lisbon, Portugal
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[e]IMM, Faculty of Medicine, University of Lisbon, Lisbon, Portugal
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The (3.A.) Neurotypical DB data is the knowledge base of the 128 healthy volunteers.

The (3.B.) Subjective Assessment is the statistical strategy to stratify the 128 neurotypical subjects into subgroups and the study of the adequacy and reliability of the instruments used for the stratification (i.e., the Assessment Protocol).

The (3.C.) descriptive and (3.D.) inferential analysis data are the results of the Assessment Protocol.

3.A. Neurotypical DB

The dataset comprehended the use of 138 variables. Those included independent variables and 20% of internal variables for function calculation.

The following variables were acquired to compile the knowledge base (https://tinyurl.com/NeurotypicalDB, retrieved July 28, 2020):

three variables were used for procedural noting;

Sixty-one variables were used for (i) the General Clinical Questionnaire.

Those included (a) trait variables:

(i) biometric data (8 variables);

(ii) physical activity (2 variables);

(iii) alcohol habits (4 variables);

(iv) smoking habits (4 variables)

(v) anamnesis (diagnosis, three variables; therapeutics, four variables).

And also included (b) state variables: 36 research variables.

Finally, 74 variables were used for (ii) the Psychological Assessment.

Those included:

(i) 24 variables for the *Abnorme Psychischer Zustände* States of Consciousness scale;

(ii) 6 variables for the Subjective Happiness Scale;

(iii) 17 variables for the Mindful Attention Awareness Scale;

(iv) 27 variables for the Self-consciousness Scale.

3.B. The Subjective Assessment

The Subjective Assessment is a statistical strategy to stratify the neurotypical sample into subgroups. Moreover, we studied if the instruments used in the Assessment Protocol (i) were adequate for the probe into our sample; and (ii) had the reliability necessary to discriminate the 4 subgroups found.

3.B.1. Sample Stratification Strategy

The sample stratification strategy, or phenome stratification, intended to find different subgroups or profiles with distinct mental health signatures.

The subphenomes were identified through weighted analysis of the Assessment Protocol, which was hierarchically structured.

Firstly, 2 groups were identified:

(i) Experimental group;

(ii) Control group.

Afterwards, these 2 groups were stratified by the following statistical strategy (**Figure 15**).

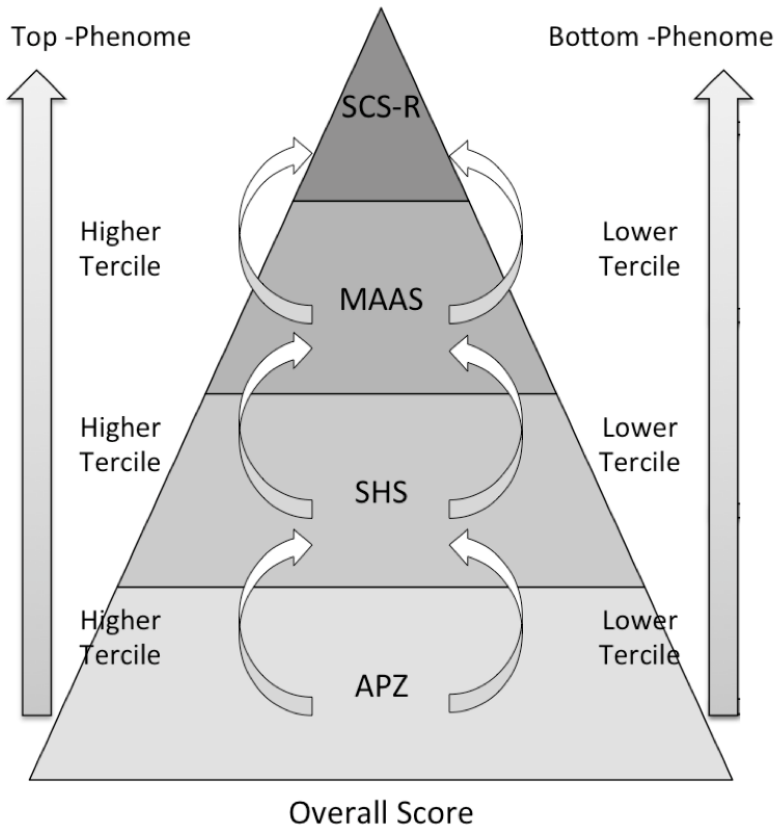


Figure 15. Subjective Assessment Of The Phenome: Hierarquization By Psychological Scaling
The pyramid chart represents the strategy used for the proportionate stratified random sampling of the 128 subjects. Two vertical stratification procedures were used for both the Top Phenomes and Bottom Phenomes. For each instrument used, the higher or lower tercile was used correspondingly, in each step of the statistical stratification.

The top phenome (subgroup A) is constituted by the higher tercile of the sample. This tercile was given by the sum of the scores of the instruments (Self-report Measures) of the Psychological Assessment, but in a sequentially pondered order:

i. APZ;
ii. SHS;
iii. MAAS;
iv. SCS-R.

Simultaneously, the bottom phenome (subgroup B) is constituted by the lower tercile of the sample, established on the same weighing. The middle tercile was not used.

Consequently, the sample was stratified into 4 distinguished subgroups, as illustrated in **Figure 16**:

(i) the Experimental Top Phenome;
(ii) the Control Top Phenome;
(iii) the Experimental Bottom Phenome;
(iv) the Control Bottom Phenome.

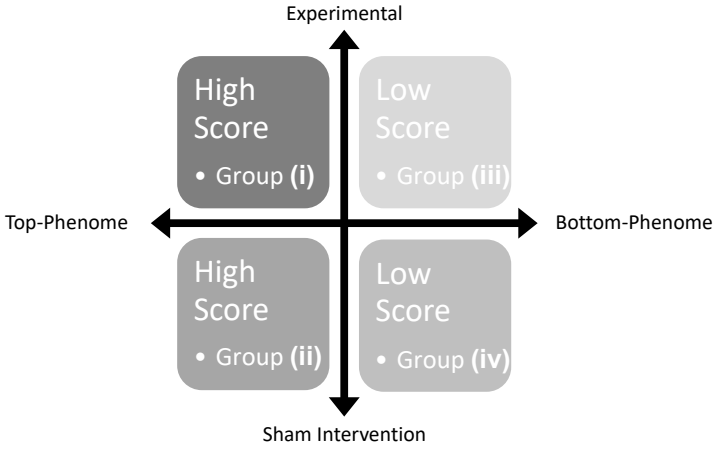


Figure 16. Neurophenomenological Sampling And Subgroup Identification: Score
A two-axis plot is presented to represent the identification of the 4 subgroups with distinct mental phenotypes. The vertical axis represents the separation by experimental procedure and sham intervention. The horizontal axis represents the stratification by the overall score in the Subjective Assessment.

The *rationale* (**Figure 17**) of this strategy followed a cost/efficiency approach.
This strategy aimed:

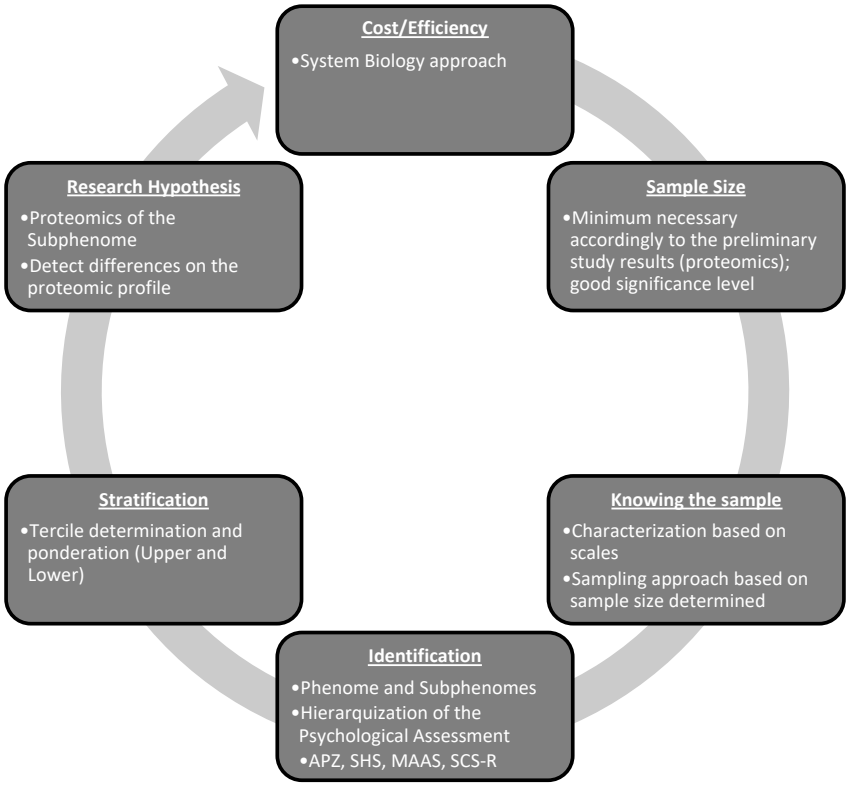


Figure 17. Conceptual Analysis: Stratification Of Phenomes Into Subphenomes
A circular plot is presented to represent the guidelines used for the clinical and statistical stratification of the 128 neurotypical subjects in the first section of this PhD Thesis.

(i) to match a minimum necessary sample size, according to the preliminary total protein profile study results (Martins et al., 2016a);

(ii) to foresee a good significance for the molecular screening that follows this clinical evaluation.

The major concern was to have optimal knowledge of the sample for a future biomarker research, which involved a full clinical and psychological characterization of neurotypical subjects.

Consequently, the sampling consisted of the assignment of 92 subjects, from the initial sample of 128, who *a priori* have the most characteristic and specific mental health features.

3.B.2. Adequacy And Reliability Statistics

The study of the adequacy and reliability of instruments used in the Assessment Protocol, i.e., (i) the General Clinical Questionnaire and (ii) the Psychological Assessment were given by the internal consistency, with the inferior limit given by Cronbach's α coefficient, of each instrument.

The first step (i) was to understand if the General Clinical Questionnaire (**Table 1)** was adequate for the sample and if all items used in the instrument were necessary.

The General Clinical Questionnaire had neither item homogeneity nor redundancy, with an initial general Cronbach's α=0.735. Nevertheless, after a *posteriori* statistical treatment, the General Clinical Questionnaire was optimized: unnecessary items/subdomains were removed, and dimensions/domains were restructured.

This optimization was performed with principal component analysis (PCA) extraction through a Varimax rotation method with Kaiser Normalization, respecting the following criteria.

Some subdomains (**Table 1;** https://tinyurl.com/NeurotypicalDBPrezi, retrieved July 23, 2020) had poor internal consistency and diminished reliability when using the instrument in our sample, because:

(x) they were not correctly applied or found to be out of the scope;

(y) the limit threshold of more than 50% on rotated component matrix of PCA was applied;

(z) they did not have discriminative capacity, i.e., Cronbach's α increases (if items were deleted).

Consequently, the following subdomains were removed/extracted from:

(a) Altered State of Consciousness: subdomains - frequency (x - because it is a binary variable); and quality (z) (the specific items: physical activity, clinical hypnosis, reading, playing instruments, sightseeing, watching movies, sleeping, studying, being in silence, contacting with nature and ethnomedicines);

(b) Self-consciousness: subdomain - understanding the meaning (y);

(c) Happiness Trait: subdomain - understanding the meaning (z);

(g) Doctor-patient Relationship: subdomain - understanding the meaning (y,z) and relation to the Intervention Protocol (x).

According to the previous extraction, and to improve internal consistency and comprehension, the General Clinical Questionnaire was, subsequently, optimized and divided in only 2 sections (**Table 4**).

The following dimensions and subdomains, or components extracted by PCA through a Varimax rotation method with Kaiser Normalization, were found.

Section 1 is constituted by the dimension Altered State of Consciousness and has 2 subdomains: i. understanding meaning with personal experience (PC1 = 0.72) and

ii. composition of experience (items chosen to remain: meditation, arts, lucid dreaming and yoga) (PC2 = 0.73).

Section 2 is constituted by the dimensions Self-consciousness, Happiness Trait, Happiness State, *Eudaemonia* and Self-awareness and has 2 subdomains:

i. theoretical understanding of the concept (PC1 = 0.77);

ii. practical experience of the concept in the Intervention Protocol (PC2 = 0.77).

Table 4. General Clinical Questionnaire After Optimization By The Adequacy And Reliability Study
The final General Clinical Questionnaire created by the author to address certain supplementary research variables is synthesized in two main sections with 4 Principal Components ($PC_{1,2,3,4}$).

	Dimension	**Subdomains**	
Section 1	Altered State of Consciousness	understanding the meaning with personal experience PC1=0.666	composition of experience (items chosen to remain: meditation, arts, lucid dreaming and yoga) PC2=0.862
Section 2	Self-consciousness	theoretical understanding of the concept PC3=0.730	practical experience of the concept in the Intervention Protocol PC4=0.983
	Happiness Trait		
	Happiness State		
	Eudaimonia		
	Self-awareness		

Therefore, the General Clinical Questionnaire was conclusively structured in only 12 items versus the original 20 items. Furthermore, its internal consistency, given by a final Cronbach's α, was 0,757, set by 0.73 (Section 1) and 0.77 (Section 2).

The General Clinical Questionnaire was, thus, categorized in only 4 clustered subdomains versus the original non-clustered 17 subdomains. Those subdomains were extracted by a final PCA (two from Section 1 and two from Section 2) with:

PC1 = 0.666;

PC2 = 0.862;

PC3 = 0.730;

PC4 = 0.983.

The second step (ii) was to understand if the 4 psychological scales of the Psychological Assessment were adequate for the sample.

As the 4 psychological scales had already been validated for the Portuguese population, and in order to evaluate their adequacy to our sample, we tested their reliability in our sample, comparing it to the one published by the authors who validated the scale.

Hence, the estimate of the reliability of the measures/instruments, in our neurotypical sample, was calculated using the lower limit of the internal consistency given by the Cronbach's α of the standardized scale items.

The Cronbach's α of each of the 4 psychological scales individually, by subgroup and by total overall, are provided by the following scores (**Table 5**).

(a) APZ, 23 items, i. total overall (N=92) equals to 0,853, and ii. partial (N=46) Experimental equals to 0,808, Control (Sham) equals to 0,846, Top phenome equals to 0,724, and Bottom phenome equals to 0.588;

(b) SHS, 4 items, i. total overall (N=91) equals to 0,491, and ii. partial Experimental (N=45) equals to 0,361, Control (N=46) equals to 0,605, Top phenome (N=46) equals to 0,683, and Bottom phenome (N=45) equals to 0.2;

(c) MAAS, 15 items, i. total overall (N=92) equals to 0,76, and ii. partial (N=46)

Experimental equals to 0,698, Control equals to 0,793, Top phenome equals to 0,79, and Bottom phenome equals to 0.723;

(d) SCS-R, 22 items, i. total overall (N=92) equals to 0,621, and ii. partial (N=46) Experimental equals to 0,477, Control equals to 0,713, Top phenome equals to 0,456, and Bottom phenome equals to 0.685.

Table 5. Internal Consistency Given By The Lower Limit Of The Cronbach's α
The adequacy and reliability statistics is given by the Cronbach's α for each of the instruments used in the Psychological Assessment in the overall 92 stratified subjects. The statistics are given for the total sample of 92 neurotypical young adults, but also for Experimental (N = 46) Vs Control (N = 46) Vs Top (N = 46) Vs Bottom groups (N = 46).

Scale	Nº Items	Total Overall N=92	Experimental Group	Sham Group	Top Phenome N=46	Bottom Phenome
APZ	23	0.853	0.808	0.846	0.724	0.588
SHS	4	0.491	0.361	0.605	0.683	0.2
MAAS	15	0.76	0.698	0.793	0.79	0.723
SCS-R	22	0.621	0.477	0.713	0.456	0.685

Comparatively, the internal consistency (Cronbach's α) of the 4 validated scales for the Portuguese population, already published by the authors, is as following:

(a) APZ (Simões et al., 1986) equals to [0.83;0.95];

(b) SHS (Pais-Ribeiro, 2012) equals to 0.76;

(c) MAAS (Gregório and Gouveia, 2011) equals to 0.87;

(d) SCS-R (Neto, 1986) equals to [0.78;0.82].

Therefore, by comparing our scores to the validated scale scores, we concluded that the scale's item correlation is acceptable to good.

Consequently, it was also concluded that the four measurement instruments, APZ, SHS, MAAS and SCS-R, have an acceptable to good reliability (Spearman-Brown) to evaluate the domains or constructs they propose to classify, and more importantly, to be applied specifically to the neurotypical sample of this study.

The individual subphenomes or subgroups reliability were not calculated due to lack of interest.

To conclude, the reliabilities were adequate to good, and some measures clearly had greater internal reliability than others (e.g., General Clinical Questionnaire, APZ and MAAS).

The assessment of the results permitted the creation of 4 subgroups or strata. Each of those 4 subgroups or mental health profiles was constituted by 23 subjects. Likewise, from this assessment, we asserted that N=92 subjects were adequately stratified and chosen to follow a successive molecular screening.

3.C. Descriptive Analysis Of The Assessment Protocol

For the descriptive analysis of the Assessment Protocol, the variables analyzed by the instruments were quantitative/discrete, as they were the final scores of:

(i) the General Clinical Questionnaire;

(ii) of each psychological scale of the Psychological Assessment.

The results of the descriptive analysis are shown both in **Figure 18** and **Table 6**, for all the Assessment Protocol.

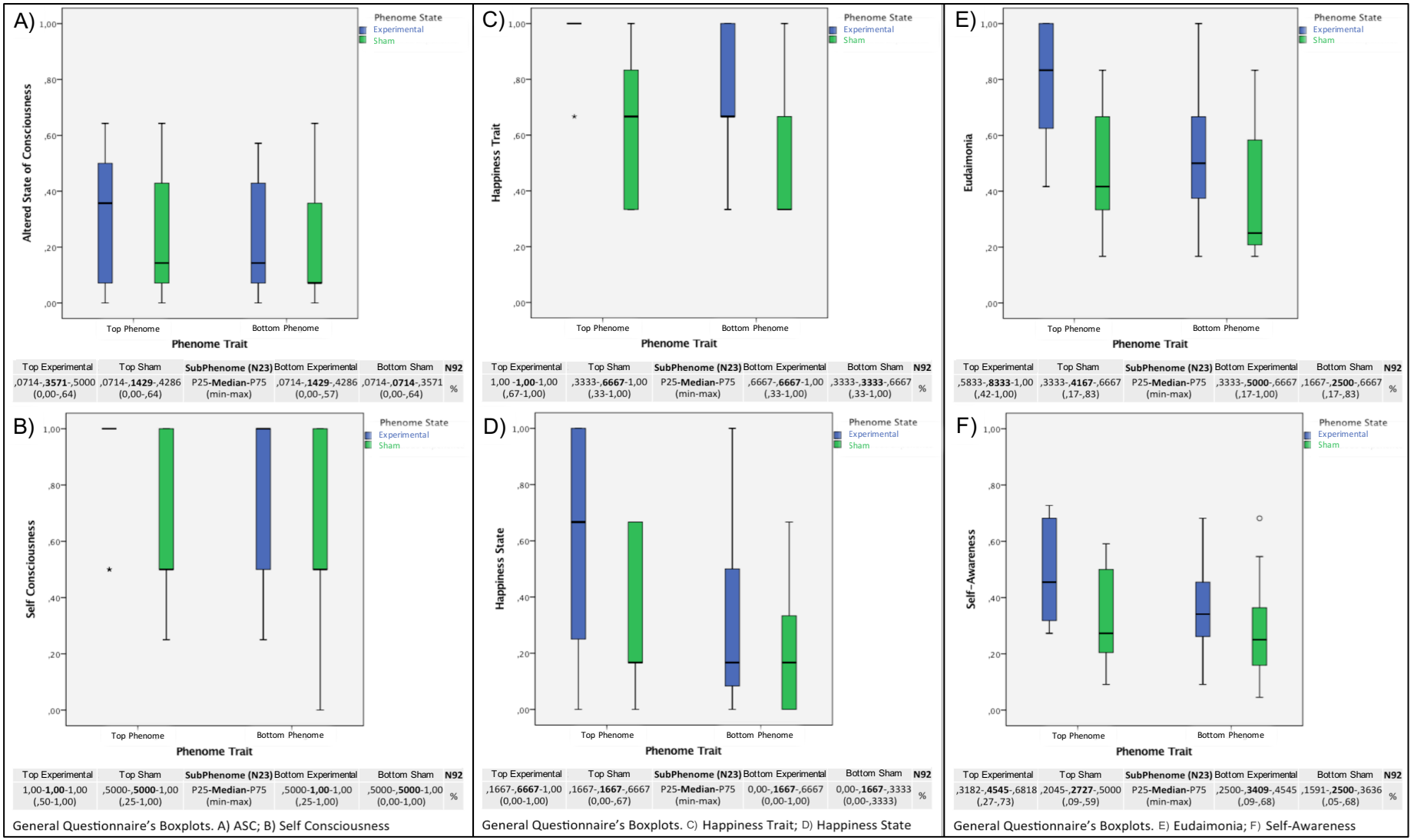


Figure 18. Descriptive Analysis Of The General Clinical Questionnaire

Shows overall score of the different variables of the General Clinical Questionnaire ($p<0.05$; see Table 6). In A), a boxplot (P25, median, P75, minimum and maximum) of the variable Altered State of Consciousness in the 4 subphenomes (Experimental Top subgroup, Control Top subgroup, Experimental Bottom subgroup and Control Bottom subgroup) is presented. In B), a boxplot for the Self Consciousness. In C), a boxplot for the Happiness Trait. In D), a box plot for the Happiness State. In E), a boxplot for the Eudaimonia. In F), a boxplot for the Self-Awareness.

Table 6. Descriptive Analysis Of The Subjective Assessment
The means and standard deviations for all the measures used in the section I (Mental Health Stratification) are represented, in each of the 4 subphenomes (N = 23) (Experimental Top subgroup, Control Top subgroup, Experimental Bottom subgroup and Control Bottom subgroup).

Descriptive Analysis	Instrument	Biometric Data		General Questionnaire												
SubPhenomes (Groups)	N	Variable	Age	BMI	Altered State of Consciousness	Self-Consciousness	Happiness Trait	Happiness State	Eudaimonia	Self-Awareness	Abnormer Psychischer Zustand Scale	Subjective Happiness Scale	Mindful Attention Awareness Scale	Self-Consciousness Scale (Total)	Self-Consciousness Scale (Private)	Self-Consciousness Scale (Public)
Top Experimental	23	Mean	20,84	22,43	30,75%	93,48%	98,55%	59,42%	78,99%	49,60%	47,07%	78,88%	66,28%	74,44%	73,91%	79,50%
		SD	1,17	3,08	21,37%	17,22%	6,95%	39,51%	20,85%	17,57%	12,41%	8,05%	10,89%	10,30%	12,41%	13,58%
Top Sham	23	Mean	21,77	21,59	22,67%	66,30%	65,22%	30,43%	47,83%	33,50%	24,76%	80,59%	68,89%	70,62%	76,65%	81,78%
		SD	1,16	3,17	20,28%	28,81%	25,58%	25,94%	21,05%	16,12%	14,45%	10,21%	11,08%	9,96%	12,95%	12,81%
Bottom Experimental	23	Mean	22,33	21,43	25,16%	75,00%	73,91%	31,16%	52,54%	37,15%	13,99%	78,26%	62,03%	66,93%	70,05%	74,74%
		SD	2,07	2,24	20,87%	27,18%	24,53%	34,20%	22,95%	15,99%	7,18%	9,51%	7,38%	10,31%	15,75%	15,56%
Bottom Sham	23	Mean	21,74	21,19	18,63%	61,96%	50,72%	24,64%	37,68%	27,77%	1,32%	79,97%	68,60%	65,35%	64,90%	74,74%
		SD	1,27	2,06	18,37%	31,86%	26,34%	25,06%	22,87%	15,79%	2,05%	6,79%	9,83%	13,08%	19,53%	16,89%

Figure 18 shows the boxplots [P25-Median-P75; (min-max)], of the 4 subgroups exclusively for the variables of the General Clinical Questionnaire: a) Altered States of Consciousness (**Figure 18.A**), b) Self-consciousness (**Figure 18.B**), c) Happiness Trait (**Figure 18.C**), d) Happiness State (**Figure 18.D**), e) *Eudaemonia* (**Figure 18.E**), and f) Self-awareness (**Figure 18.F**).

Table 6 presents means and SDs for all the measures (variables) used in the study, in the 4 subphenomes: Experimental Top, Control Top, Experimental Bottom and Control Bottom.

3.C.1. Intergroup And Intragroup Variability

In the first phase of the analysis, in **Figure 18** and **Table 6**, we wanted to test (i) intergroup variability, assumed by the variable mean, and (ii) intragroup variability, given by the variable SD. For the confounding variables, i.e., Age and BMI, low intergroup and intragroup variability were found (as analyzed in Methods: Sample screening).

From all the variables used to characterize the 4 subgroups, some variables had higher discriminative power than others, as described below.

In the instrument General Clinical Questionnaire, all the variables had high intergroup variability, and some had low intragroup variability, like Self-consciousness, Happiness trait, *Eudaemonia* and Self-awareness; whilst, other variables had medium intragroup variability, such as Altered state of consciousness and Happiness state.

The instrument *Abnorme Psychischer Zustände* States of Consciousness scale had high intergroup variability and low intragroup variability.

The instrument Subjective Happiness Scale had low intergroup variability and low intragroup variability.

The instrument Mindful Attention Awareness Scale had medium-low intergroup variability and medium-low intragroup variability.

In the instrument Self-consciousness Scale – Revised version, (i) the variable Total had high intergroup variability and low intragroup variability, (ii) the variable Private had high intergroup variability and medium-low intragroup variability, and (iii) the variable Public had medium-high intergroup variability and medium-low intragroup variability. The analysis of the intergroup and intragroup variability is further explored in the Inferential Analysis.

3.C.2. Strata Characterization

In the second phase of the analysis of **Figure 18** and **Table 6**, we wanted to perform group, or strata, characterization, given by the investigation of each variable. The objective was to test if there was a constant tendency present in all variables.

In the instrument General Clinical Questionnaire, for the variable:

(i) Altered state of consciousness, the score of the Experimental Top subgroup is the highest, followed by the Experimental Bottom group, and then by the Control Top group, and finally by the Control Bottom group; (ii) Self-consciousness, the score of the Experimental Top subgroup is the highest, which is much higher than the Experimental Bottom group. Thirdly is the Control Top group, which, ultimately, is higher than the Control Bottom group; (iii) Happiness Trait, the score of the Experimental Top subgroup is the highest, which is much higher than the Experimental Bottom group, which is much higher than the Control Top group, which is much higher than the Control Bottom group; (iv) Happiness state, the score of the Experimental Top subgroup is the highest, which is much higher the Experimental Bottom and the Control Top subgroups, which have similar scores. The Control Bottom subgroup is the lowest; (v) *Eudaemonia*, the score of the Experimental Top subgroup is the highest, which is much higher than the Experimental Bottom group. Thirdly is the Control Top subgroup and, ultimately, the Control Bottom group; (vi) Self-awareness, the score of the Experimental Top subgroup is the highest, then the Experimental Bottom group, then the Control Top subgroup and, lastly, the Control Bottom group.

In the instrument *Abnorme Psychischer Zustände* States of Consciousness scale, the variable total score of the Experimental Top subgroup is the highest, which is much higher than the Control Top group. The score of the Experimental Bottom subgroup is much lower. Finally, the Control Bottom subgroup has the lowest score.

In the instrument Subjective Happiness Scale, the variable total score of the Control Top subgroup is higher than the Control Bottom, the Experimental Top and the Experimental Bottom subgroups, which have similar scores.

In the instrument Mindful Attention Awareness Scale, the variable total score of the Control Top subgroup is similar to the Control Bottom group. Those are higher than the Experimental Top group. The Experimental Bottom subgroup is the lowest.

In the instrument Self-consciousness Scale – Revised version, for the variable:

(i) Total, the score of the Experimental Top subgroup is higher than the Control Top group. Thirdly, the Experimental Bottom and the Control Bottom subgroups have equal scores; (ii) Private, the score of the Control Top subgroup is the highest, followed by the Experimental Top group, and then by the Experimental Bottom group, and finally by the Control Bottom group; (iii) Public, the score of the Control Top subgroup is similar to the Experimental Top group. Those are higher than both the Control Bottom and the Experimental Bottom subgroups.

This strata analysis showed the presence of a statistical tendency. Generally, in all the variables in the Assessment Protocol, the score of Experimental Top Subgroups the highest, then the Experimental Bottom Group, then the Control Top Subgroup and, finally, the Control Bottom Group.

3.D. Inferential Analysis Of The Assessment Protocol

The Inferential analysis examined the same variables in the descriptive analysis. The results of the inferential analysis were obtained by parametric and/or non-parametric hypothesis testing and are summarized in **Table 7**.

Table 7. Inferential Analysis Of The Subjective Protocol

Parametric and non-parametric, with multiple comparisons, testing used in instruments of the subjective assessment, in the 4 subphenomes (Experimental Top subgroup, Control Top subgroup, Experimental Bottom subgroup and Control Bottom subgroup).

Psychological Scale	Dependent Variable	Pre-testing			Parametric		Non Parametric		Multiple Comparisons (I and J)				Reject H0
* The mean difference is significant at the 0.276 level. Not showing inferences results that have a sig > 0,273, exept for #		Test of Homogeneity of Variances: Levene Test p-value	Test of Homogeneity of Variances: α = 0.05 Homoscedasticity	Robust Tests of Equality of Means: Brown-Forsythe Test (α < 0,05)	One-Way ANOVA * p-value	Between All Groups (Score) α = 0.273	Independent Samples Kruskal-Walis Test Sig.	Between All Groups (Score) α = 0.05	*I* - Phenome State and Trait (ANOVA α =0.05 and Bonferroni correction; KW asymtoptic sig.= 0.05)	*J* - Phenome State and Trait	ANOVA p-value	Kruskal - Wallis p-value	
General Questionnaire	Altered State of Consciousness	0,539	Ñ Rejected	Confirmed	0,237	Rejected equality			*Top Experimental*	*Bottom Sham*	0.273		S/N
									Bottom Sham	*Top Experimental*	0.273		S/N
	Self-Consciousness	< α	Rejected				0,001	Rejected equality	*Distribuition is the same across all multiple comparisons*			0.001	S
	Happiness Trait	< α	Rejected				<0,001	Rejected equality	*Distribuition is the same across all multiple comparisons*			<0,001	S
	Happiness State	< α	Rejected				0,005	Rejected equality	*Distribuition is the same across all multiple comparisons*			0.005	S
	Eudaimonia	0,944	Ñ Rejected	Confirmed	<0,001	Rejected equality			*Bottom Experimental*	*Top Experimental*	0.001		S
										Bottom Sham	0.145		S/N
									Top Experimental	*Bottom Experimental*	0.001		S
										Bottom Sham	<0,001		S
										Top Sham	<0,001		S
									Bottom Sham	*Bottom Experimental*	0.145		S/N
										Top Experimental	<0,001		S
									Top Sham	*Top Experimental*	<0,001		S
	Self-Awareness	0,436	Ñ Rejected	Confirmed	<0,001	Rejected equality			*Bottom Experimental*	*Top Experimental*	0.07		S
										Bottom Experimental	0.07		S
									Top Experimental	*Bottom Sham*	<0,001		S
										Top Sham	0.008		S
									Bottom Sham	*Top Experimental*	<0,001		S
									Top Sham	*Top Experimental*	0.008		S
Abnormer Psychischer Zustände Scale		<0,001	Rejected				<0,001	Rejected equality	*Distribuition is the same across all multiple comparisons*			<0,001	S
Subjective Happiness Scale		0,411	Ñ Rejected	Confirmed	0,802	Ñ rejected equality			*Distribuition is the same across all multiple comparisons*		1		N #
Mindful Attention Awareness Scale		0,248	Ñ Rejected	Confirmed (Welch = 0,034)	0,077	Rejected equality			*Bottom Experimental*	*Bottom Sham*	0.162		S/N
										Top Sham	0.126		S/N
									Bottom Sham	*Bottom Experimental*	0.162		S/N
									Top Sham	*Bottom Experimental*	0.126		S/N
Self-Consciousness Scale	Total	0,648	Ñ Rejected	Confirmed (Brown-Forsythe)	0,029	Rejected equality			*Bottom Experimental*	*Top Experimental*	0.137		S/N
										Bottom Experimental	0.137		S/N
									Top Experimental	*Bottom Sham*	0.037		S
									Bottom Sham	*Top Experimental*	0.037		S
	Private	0,106	Ñ Rejected		0,063	Rejected equality			*Bottom Sham*	*Top Sham*	0.068		S
									Top Sham	*Bottom Sham*	0.068		S
	Public	0,677	Ñ Rejected		0,276	Ñ rejected equality	0,287	Ñ rejected equality	*Distribuition is the same across all multiple comparisons*		0.662		N #
									Distribuition is the same across all multiple comparisons			0.287	S/N

The results are based on the percentage of the maximum score for each variable or scale, and the mean difference was considered significant when $p < \alpha = 0.273$ (inferences with higher significance level are not considered, nor presented).

The normality, the homogeneity of variances, the asymmetries in the distribution, the sample dimension and the scale of the variables determined the type of testing.

Subsequently, the *rationale* of the inferential analysis is explained and the results are described for each scale used in the Assessment Protocol.

Primarily, it comprehended (i) the rejection ($\alpha = 0.05$) of homoscedasticity and (ii) the confirmation of Welch and Brown-Forsythe equality of means.

Consecutively, a) One-way ANOVA or b) Independent-Samples Kruskal-Wallis tests were performed, to reject ($\alpha = 0.05$ mean difference) intergroup scale score equality (in (i) the Experimental Top Phenome group; (ii) the Control Top Phenome group; (iii) the Experimental Bottom Phenome subgroup and (iv) the Control Bottom Phenome group).

Afterwards, if rejected, a) ANOVA or b) non-parametric multiple comparisons were also applied, accordingly, to understand which subgroups were responsible for the score mean difference.

Therefore, in the General Clinical Questionnaire, the subscales:

a) Altered states of consciousness (higher α), b) Self-consciousness, c) Happiness trait, d) Happiness state, e) *Eudaemonia*, and f) Self-awareness rejected equality ($p<0.05$) of scale score means between subgroups and in multiple comparisons.

And specifically, for the variable:

(i) Altered state of consciousness, the Experimental Top & Control Bottom subgroups were considered distinct, but with a low significance ($p = 0.273$ (ANOVA));

(ii) Self-consciousness, the Experimental Top & Control Top & Experimental Bottom & Control Bottom subgroups were considered distinct, with a high significance (the distribution was the same across all multiple comparisons $p = 0.001$ (Kruskal - Wallis));

(iii) Happiness Trait, the Experimental Top & Control Top & Experimental Bottom & Control Bottom subgroups were considered distinct, with a high significance (the distribution was the same across all multiple comparisons $p<0.001$ (Kruskal - Wallis));

(iv) Happiness state, the Experimental Top & Control Top & Experimental Bottom & Control Bottom subgroups were considered distinct, with a high significance (the distribution was the same across all multiple comparisons $p = 0.005$ (Kruskal - Wallis));

(v) *Eudaemonia*, (a) the Experimental Bottom & Experimental Top subgroups were considered distinct, with a high significance ($p = 0.001$ (ANOVA)), whereas the Experimental Bottom & Control Bottom were considered distinct, with a medium-low significance ($p = 0.145$ (ANOVA)). (b) the Experimental Top & Control Bottom subgroups and the Experimental Top & Control Top subgroups were considered distinct, with a high significance ($p<0.001$ (ANOVA));

(vi) Self-awareness, (a) the Experimental Bottom & Control Top subgroups were considered distinct, with a medium significance ($p = 0.07$ (ANOVA)). Whereas (b) the Control Top & Control Bottom subgroups and the Control Top & Control Top subgroups were considered distinct, with a high significance ($p<0.001$ and $p = 0.008$ (ANOVA), respectively).

Similarly, in the instrument *Abnorme Psychischer Zustände* States of Consciousness scale, it was rejected ($p<0.05$) that the score of the scale was equal between subgroups (Kruskal-Wallis Test Sig. $\alpha = 0.05$) and in multiple comparisons.

Specifically, the Experimental Top & Control Top & Experimental Bottom & Control Bottom subgroups were considered distinct, with a high significance (the distribution was the same across all multiple comparisons $p<0.001$ (Kruskal-Wallis).

Equally, in the instrument Mindful Attention Awareness Scale, it was rejected (but with a $p<0.162$) that the score of the scale was equal between subgroups (One-Way ANOVA $\alpha = 0.077$) and in multiple comparisons.

Specifically, the Experimental Bottom & Control Bottom subgroups and the Experimental Bottom & Control Top subgroups were considered distinct, but with a low significance ($p<0.162$ and $p<0.126$ (ANOVA), respectively).

Likewise, in the instrument Self-consciousness Scale – Revised version, and for the subscales:

a) Total and b) Private), it was rejected ($p<0.137$ and $p<0,068$, respectively) that the score of the subscale a) Total (One-Way ANOVA $\alpha = 0.029$) and b) Private (One-Way ANOVA $\alpha = 0.063$) were equal between subgroups and in multiple comparisons.

Hence, for the variable:

(i) Total, the Experimental Top & Experimental Bottom subgroups were considered distinct, but also with a low significance ($p<0.137$ (ANOVA)). Whereas the Experimental Top & Control Bottom subgroups were considered distinct, with a high significance ($p = 0.037$ (ANOVA);

(ii) Private, the Control Bottom & Control Top subgroups were considered distinct, with a medium-high significance ($p<0.068$ (ANOVA)).

On the other hand, and in contrast with the results of the instruments previously described, the instrument Subjective Happiness Scale did not reject equality ($p>0.05$) of means between subgroups or in multiple comparisons.

Similarly, subscale Public, of the instrument Self-consciousness Scale – Revised version, had the lowest significance ($\alpha = 0.276$ One-Way ANOVA), being in the limit of rejecting that the score of the subscale was equal between subgroups and in multiple comparisons.

Specifically, on that variable, the Experimental Top & Control Top & Experimental Bottom & Control Bottom subgroups were not considered distinct (the distribution was the same across all multiple comparisons with a $p = 0.287$ (Independent-Samples Kruskal-Wallis).

3.E. Discussion Of The Mental Health Profiling

Considering the complex relationships found among the constructs measured by the Assessment Protocol, our expectations for this research were solely the following:

(i) to better understand self-regulatory cognitive faculties, like self-awareness;

(ii) to deliver a more comprehensive in-depth analysis about mental health functioning (Pacheco, Martins and Simões, 2019);

(iii) to transfer this analysis into the neurobiological study of neuropsychiatric disorders.

Thus, the results of this work permitted a general phenomenological evaluation of the mental functioning of neurotypical subjects. Accordingly, the study proposed the characterization of distinct profiles, or mental health subphenomes, presented by that population.

Firstly, and in what refers the Intervention Protocol, we used a cognitive

stimulation (Hipólito and Martins, 2017) to stratify 4 distinct profiles, or subphenomes, in terms of the constructs of self and bodily representations. Specific cognitive states were induced, and specific cognitive functions were recruited; e.g., declarative memory. The Control Top subgroup exercised semantic memory during the task, whereas the Experimental Top subgroup had to exercise cognitive functions associated with episodic memory.

As an intended outcome, the result of this cognitive stimulation was to generate a more focused, awake and aware mental state (Bayne, Cleeremans and Wilken, 2014). Secondly, and in what concerns the Assessment Protocol, which aimed at the mental health profiling, our neurotypical sample was later studied by a statistical strategy and 4 self-report measures. The results of this strategy permitted the adequate profiling of the sample into 4 different subtypes or strata. With this assessment, 4 subgroups of discrete mental health functioning were significantly and relevantly identified and characterized.

This profiling of the neurotypical young adults was assured by a hierarchical ranking of the self-report measures (**Figure 15**) of the Psychological Assessment. This hierarchical ranking, which led to the mental health strata, was based on the pertinence of each psychological scale, to evaluate the importance and degree of the mental health constructs we wanted to assess (**Figure 19**).

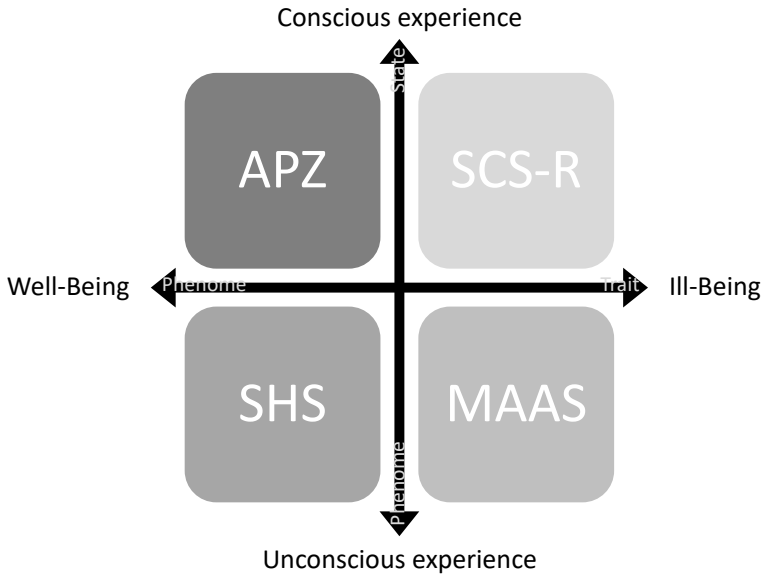


Figure 19. Neurophenomenological Sampling And Subgroup Identification: Psychological Scales
A two-axis plot is used to represent the mental health profiling and the 4 instruments used for the clinical stratification. The vertical axis represents the constructs of a more self-conscious experience vs. a less self-conscious experience. The horizontal axis represents the complementary constructs (postulated in this thesis) of a higher tendency to well-being vs. a lower tendency to well-being.

APZ has the highest weighing, since, in our neurotypical sample, it was first intended to identify a state of self-awareness.

Likewise, APZ is the only psychological evaluation in our set of psychological scales of a state phenome, and therefore able to characterize the cognitive functions stimulated by the Intervention Protocol.

SHS comes second, as it was intended, in the identification of neurotypical subjects that tend towards a well-being experience with self-consciousness traits. For

this purpose, SHS tries to address, on the one hand, the personal and the external happiness trait; and on the other hand, the absolute and relative happiness trait. Therefore, SHS evaluates a phenome trait.

These 2 scales (APZ, SHS) best characterized constructs present in the top-phenomes, in both Experimental and Control groups (**Figure 19**), which have scored higher in the Assessment Protocol.

MAAS comes third, specifically to identify pre-reflective self-experience. This type of self-experience is essential for a passable state of self-awareness and a stable self-consciousness trait.

Awareness, in a pre-reflective self, seems to be related to a cognitive construct associated with present experiences, which one is aware of, without any further judgment or symbolization. MAAS evaluates a phenome state.

Finally, SCS-R (DaSilveira, DeSouza and Gomes, 2015) identifies, in our neurotypical sample, a core reflective self-experience and a core trait of cognition necessary for high-level reasoning and logic. The reflective self tends to tap into past experiences, rationalizing concepts that were already processed. As such, SCS-R evaluates a phenome trait.

These 2 scales (MAAS; SCS-R) best characterize constructs present in the bottom phonemes, in both Experimental and Control groups, which have scored lower in the Assessment Protocol (**Figure 19**).

Finally, after this classification, given by the statistical strategy and the self-report measures of the Psychological Assessment, N=92 neurotypical young adults were finally stratified into 4 subgroups.

Those young adults were also evaluated by the General Clinical Questionnaire in order to understand the effectiveness of separating those 4 mental health subgroups. It was compelling to find that the General Clinical Questionnaire's scores *per se* have a moderate consistency to differentiate them, prefatorily.

The excerpt of the research outline (**Figure δ**) systemizes the Section I: Mental Health Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 3 in the overall experiment.

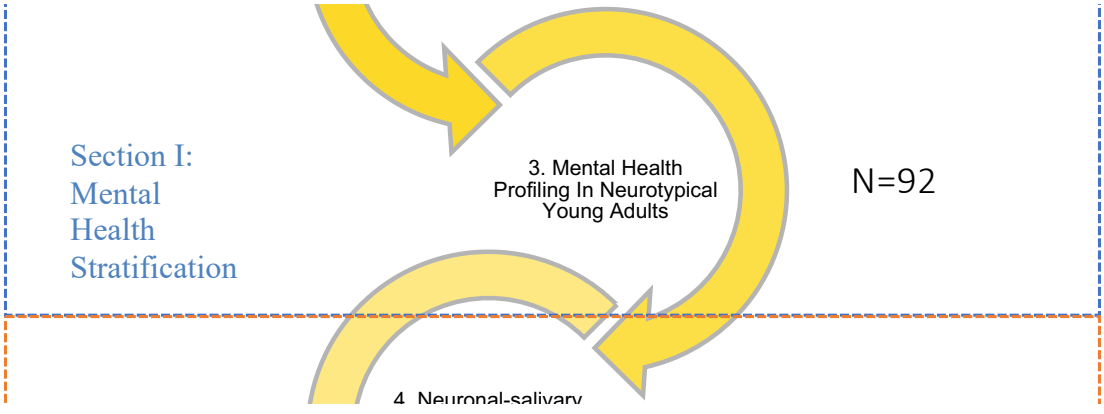


Figure δ. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section I: Mental Health Stratification The chapter 3 (Mental Health Profiling In Neurotypical Young Adults) is displayed, which comprehended the proportionate stratified random sampling of the 128 subjects.

Chapter 4. Neuronal-Salivary Proteome: *In silico* Characterization And Bioinformatics Analysis

Preamble ("How to study a protein database of neuropsychiatric conditions?")
The neuronal proteome can be probed in salivary samples. While neuropsychiatric states result from an altered neuroproteome, neurotypical conditions also have a characteristic molecular signature. Most of the molecular correlates to those conditions are identified in brain cerebrospinal fluid or blood samples. We suggest characterizing the neuronal-salivary proteome, in order to produce knowledge in biomarker research of neuropsychiatric conditions probed in saliva. This study focuses on a database literature review (Sousa, Martins, Rosa, Barros and Correia, 2016) of the neuronal proteome found both in neurotypical subjects and in the ICD-10 chapters 'Diseases of the Nervous System' and "Mental and Behavior Disorders", the Neuro.OralCard. This database offers an update of the OralCard (Arrais et al., 2013), revising 2416 neuronal proteins identified in neuroproteomics, from which 765 are present, detected and identifiable in the saliva proteome. To evaluate this proteome, we executed an *in silico* characterization and a bioinformatics analysis (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017) by PANTHER and Cytoscape functional clustering. We concluded that 85% of the neuronal proteins were initially correlated with a neuropsychophysiological condition. From those, only 13% were in fact identified, quantified and proposed as biomarkers of susceptibility. A functional enrichment, in comparison to the *Homo sapiens* (Human - taxon 9606) proteome, determined that proteins in brain circuits are predominantly involved in metabolic functions, immune system processes and neuropeptide transmission. As a functional network milestone, we developed a molecular biology atlas for a better understanding of the neuronal proteome functions, the MolecularCog(G) Atlas. We expect that the characterization of the neuronal-salivary proteome may be used (i) as a theoretical revision of susceptibility neuronal-salivary biomarkers, (ii) as an *in silico* proteomic biobank for the clinical study of the nervous system, and (iii) as a molecular reference for further correlation with the mental health profiles of 128 neurotypical university students, non-medicated and with no serious, uncontrolled or chronic diseases affecting the nervous system.

Materials And Methods

Chapter 4 comprises the methodology that led to the construction neuroproteome database and annotation of the neuronal proteins present in salivary samples as reviewed from the literature at 15.08.2020. Most significantly, this chapter presents the readers the strategy on how to use this database when studying neurotypical subjects, but also the advantages to use it other molecular biology-based neuropsychiatric studies.

4.1. Salivary Sampling

An exploratory study (Hipólito and Martins, 2017) was conducted to collect the salivary samples of our participants. The samples were collected following a method established in the SalivaTec lab (Rosa et al., 2016). Participants consented to the collection and storage of the samples and associated data by signing an informed consent approved by the Ethics Commission of the Centro Hospitalar Lisboa Norte – Hospital de Santa Maria. The clinical database was authorized by the National Commission for Data Protection (Fonseca, 2013).

Afterwards, saliva aliquots were stored in the Biobanco-iMM databank (Afonso, Protereira and Dias, 2014).Henceforth, in experimental days and permanently on Tuesday or Thursday's mornings, the protocol was as follows: (T0) an initial saliva collection was consistently performed at 9:30 (a.m.); (T1) a second saliva collection was consistently performed at 10:30 (a.m.).

The time Δ[T0;T1] between the beginning of the experimental procedure (or sham intervention) and the second saliva collection was 45 minutes. This time interval corresponds to the time of exposure to the Intervention Protocol.

At 12:00 (a.m.) the experimental procedure was terminated and the collected salivary samples, immediately stored and transferred at -4 °C, started preprocessing at 12:15 (p.m.), in Biobanco-iMM, Centro Académico de Medicina de Lisboa. This time interval of 15 minutes was planned and always respected to avoid proteolysis and degradation of the protein quality.

A Δ_T[collection; preprocessing] was calculated and registered in order to respect literature and the Biobanco-iMM and SalivaTec Standard Operation Protocol (SOP) (Rosa et al., 2016) for salivary sampling and transportation. A $\Delta_T[\text{collection; preprocessing}]_{\text{marginal}}$ at -4 °C was defined at 180 minutes.

For all the 256 samples (128 subjects, T0 and T1) the SOP was always respected and protein quality guaranteed. The SOP (Rosa et al., 2016) is reviewed subsequently.

4.1.1. Collection Methods

Unstimulated whole saliva was collected from all the healthy neurotypical subjects. Subjects were asked to refrain from eating, drinking or have oral hygiene procedures 1 hour prior to saliva collection. A sublingual cotton-roll based method was used.

Sublingual saliva was collected with two cotton rolls placed under the tongue area, for 2 minutes. Collection and transport process of the sample aliquots were made on -4ºC. The cotton rolls were placed inside a 15 mL sterile plastic falcon tube with a sterilized 100 µl pipette tip in the bottom to facilitate saliva collection by centrifugation process at 10,000 x g's for 10 minutes at 4ºC. The total volume collected and the protein concentration were measured and after re-suspended by vortex. The total sample volume is aliquoted and stored at –80ºC.

4.1.2. Saliva Volume, Protein Concentration, Circadian Variability And Inter-Sample Variability.

Both, circadian and circannual rhythms influence the unstimulated salivary flow rates (Dawes, 1975) and the baseline collection time should be set at nearly the same time every day for all individuals in order to minimize variability (Swenson, 2007).

Rosa and colleagues (2016) assessed if there was circadian effect on saliva volume and protein concentration with the SOP used in this thesis, by a statistical analysis performed by Paired t-test. By this method, an average of 1,460 ± 0.522 ml of saliva is collected in 2 minutes and an average of 2,051 ± 1,004 mg/ml of total protein is obtained, viz. as in the 256 salivary samples of the 128 neurotypical young adults.

Rosa and colleagues (2016) showed that the total volume is different in the morning and afternoon ($p=0.0119$) and although the volume of morning collections is lower, inter-sample variability is smaller. Regarding total protein, there seems to be no circadian effect. For these reasons, in this thesis, the Salivary Collection Protocol was maintained strictly and took place during the mornings of Tuesdays and Thursdays between 09:30 (a.m.) and 10:45 (a.m.).

4.1.3. Inter-Individual And Intra-Individual Variability Of The Protein Profile

Salivary bioscience technologies such as electrophoresis are beginning to be applied for diagnosing systemic health status, proposing potential substitutes for serum protein biomarkers.

However, the optimal protocol for collecting saliva has not yet been established and sampling and statistical errors often occur when handling samples from healthy volunteers. These errors can be due to collection methods, and/or sample processing (Hsiao et al., 2018).

It is strongly advised that only one type of collection device be used throughout a given study (Golatowski et al., 2013). The cotton-roll method is considered to be the most promising method and large volumes of saliva should be collected in a short time, minimizing these potential sources of error (Shirtcliff et al., 2001) (as used in this thesis).

In the original study by Rosa and colleagues (2016), to evaluate if intra individual variability undermines the use of saliva as a marker of individual physiological status, samples from healthy donors were collected at 11 different times for 5 months.

The analysis of the variability of total volume of saliva collected and total protein between and within individuals was performed on a total of 88 samples. The results on both variables showed a higher statistically significant difference between subjects (inter-individual) ($p<0.0001$) and lower between the same subject (intra-individual variability).

Hsiao and colleagues (2018) applied multiplexed LC-MRM-MS to precisely quantify 90 disease-related proteins and assess their intra- and interindividual variability in saliva samples from healthy donors. Saliva samples were collected from 10 healthy volunteers at three different time points (day 1 morning and afternoon, and day 2 morning).

The coefficient of variation for assay reproducibility was 13%, and for the intraday variability was 38%. This study confirms Rosa and colleagues (2016), and the protocol used in this thesis, and that morning collections show lower inter-sample variability.

In Rosa and colleagues (2016) the results showed that even within healthy individuals and in spite of similar electrophoretic protein profiles, there are proteins which are different between individuals even though all the individuals are considered healthy. In this study with 128 neurotypical young adults, it is thus expected that there are also different profiles in the electrophoretic patterns.

In conclusion, in this thesis, proper collection of saliva from healthy volunteers was maintained to minimize sampling error. Furthermore, the General Clinical Questionnaire obtained extensive clinical information for selecting the eligible individuals, and application of the exclusion criteria. The application of these criteria reduced the risk of inaccurate measurement of salivary analytes from healthy individuals.

For example, the use of medications and nicotine/alcohol/drugs was fully documented (Salimetrics and SalivaBio, 2011).

Henceforward, as shown in different studies, if whole saliva is handled wisely, salivary biomarkers such as enzymes and proteins, e.g., alpha-amylase (Giusti et al., 2010) and stress proteins, e.g., GRP78 (Giusti et al., 2010) or HSP70 (Fábián et al., 2007), can be effectively assessed and taking into account the limitations of inter-sample variability.

4.1.4. Presence Of Degradation Products And Post-Translational Modifications

As referred, one responsible factor for sampling error (Malamud, 2011; Takagi et al., 2013), which was sufficiently controlled in thesis study, was the collection method and the sample storage prior to analysis. However, another factor to be extremely cautious in salivary sample analysis (Malamud, 2011) is the presence of degradation products and existence of post-translational modifications.

In neurotypical young adults, viz. n healthy participants, two types of analysis were performed in this thesis. In chapter 5, a full protein profile was conducted. In chapter 6, a simultaneous immune detection and quantification of the neuropeptide and neuroimmune response was executed.

In SELDI-TOF-MS studies (Esser et al., 2008), degradation products were found mostly in the lower mass range of 1 to 5 kDa. 3 peptides with masses 2937 Da, 3370 Da and 4132 Da were found as stable breakdown products of larger proteins.

The degradation of complex substrates, like salivary mucins, requires an arsenal of glycosidases and proteases to sequentially degrade the oligosaccharides and polypeptide backbone. In Western blot studies (Takehara et al., 2013), two types of mucins were analyzed, MUC7 and MUC5B, i.e., the major salivary glycoproteins. Their metabolic turnover was elucidated by focusing on the relationship between their deglycosylation and proteolysis. MUC7 was highly susceptible to specific proteolysis in saliva, though major part of MUC5B was more resistant to degradation. MUC5B is a complex oligomeric glycoprotein and only certain bacteria in supragingival dental plaque can cooperate to proteolyze human salivary MUC5B and hydrolyze glycosides (Wickström et al., 2009). Through capillary electrophoresis studies it was shown that whole saliva contains 8 cationic proteins (Xu et al., 1992). These proteins include lysozyme, histatin 6 and the 6 salivary histidine-rich polypeptides (HRPs 1–6). Neither histatin 2 nor histatin 4 are present in native undegraded whole saliva but appear only after autoproteolytic degradation of the saliva. Histatin 2 appears to arise through slow degradation of HRP-1, and histatin 4 is mainly produced as a rapid breakdown product of HRP-3. As a rule of thumb (Salimetrics, 2011), to minimize degradation and to prevent bacterial growth, all salivary samples should be refrigerated at 4°C immediately after collection and maintained at this temperature for no longer than 2 hours before freezing below -20ºC. This aspect was rigorously followed in the protocol of this thesis. Likewise, to maintain analyte integrity, it was used only high-quality polypropylene collection tubes and vials to store samples. Polystyrene or other non-validated plastic tubes can adversely affect the measured values of analytes.

In chapter 6, post-translational modifications were taken into account in two multiplex-biometric immunoassays: (i) neuropeptides (Orexin, β-Endorphin, Oxytocin and Substance P), as described in **Complementary Data 1**; and (ii) cytokines (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α), as described in **Complementary Data 2**. Both assays analyzed molecules with a molecular weight range of approximately [9.1;30]kDa.

In neuropeptides (**Complementary Data 1**), certain proteolytic processing is accompanied by covalent post-translational modifications (Hook et al., 2018). They usually comprise C-terminal amidation, N-terminal pyroglutamate, disulfide bonds, phosphorylation, sulfation, acetylation, glycosylation, and others. The post-translational modifications together with proteolytic processing of pro-neuropeptides generate bioactive peptides.

In Th17 cytokines (**Complementary Data 2**), toll/interleukin (TIR) domains play a critical role in the innate immune response, which is characterized by an extracellular leucine-rich repeat region. The post-translational modification of these

domains adds an additional level of control over these pathways (Dunne and O'Neill, 2005).

4.1.5. Saliva Protein Stability And Degradation Profile

Since saliva is a non-sterile biofluid, it becomes important to evaluate saliva stability at room temperature to identify how different protein bands are affected by degradation. In the original study by Rosa and colleagues (2016), to assess the profile of salivary proteins degradation aliquots of saliva, samples were maintained for 0, 24, 48 and 72 hours at room temperature. It was shown that regardless the degradation profile of some protein MW bands, the degradation at room temperature is only significant after 24 hours. Henceforth, as in this thesis the same SOP was used, a Δ_T[collection; preprocessing] was calculated and registered in order to respect literature and the Biobanco-iMM and SalivaTec Standard Operation Protocol (SOP) (Rosa et al., 2016) for salivary sampling and transportation. The collected salivary samples, immediately stored and transferred at -4 °C, started preprocessing no longer than 180 minutes after collection. This time interval was planned and always respected to avoid proteolysis and degradation of the protein quality.

4.1.6. Limitations Of The SOP

This SOP is simple, patient friendly and an easy method to perform, requiring inexpensive materials. However, there are important considerations to take in a large sample like the one in this study. One aspect to consider during collection is the circadian variation in protein concentration and content. Likewise, an important concern of saliva as a diagnostic fluid is the fact that within the same individual its composition varies throughout time. However, Rosa and colleagues (2016) demonstrate that the variation between individuals is larger than the variation within the same individual for a period of 5 months, which was also confirmed by other studies (Hardt et al., 2005; Harmon et al., 2008; Neyraud et al., 2012). This is why the time for sample collection in this study was strictly standardized.

Regarding the protein profile, Rosa and colleagues (2016) showed that the proteins which are less variable, and more interesting as biomarkers, are involved in the immune or stress response (for instance, stress-related neuropeptides). Furthermore, these protein bands more conserved between individuals seem to be less prone to degradation, which makes them especially suitable for physiological markers.

Hence, a clustering analysis of electrophoretic protein profiles in healthy neurotypical young adults should show different protein profiles, as in Rosa and colleagues (2016), and may be used as long as certain variability factors are controlled. In this thesis, variability factors were controlled, strict inclusion and exclusion criteria were applied, and more specific and detailed data was acquired. The SOP for this collection and storage is not covered by a patent neither a commercial disclosure.

4.2. Data Analysis

We performed the data analysis of an embedded type database, using the following bioinformatics tools: clustering analysis, PANTHER and Cytoscape. In clustering analysis, a Venn diagram was used for data representation. In PANTHER, a Fisher's Exact with FDR multiple test correction was applied for the comparison of the datasets. In Cytoscape, the network specificity was defined to "detailed", showing only specific pathways (terms were correlated with only a few associated genes) with pV≤0,05, with gene/term = 50%. Mapped genes represented more than 50% of the

associated genes. This analysis permitted data visualization and exploration of the neuronal-salivary proteome. To better visualize the extensive information gathered in this study, different and variate graphic and visual exploratory tools were used and created: organigrams, distribution and hierarchical charts, double and triple entries tables, online accessible databases and a medical atlas with molecular biology maps.

4.3. Bioinformatics Analysis

The following pieces of software were used:

(A) PUBMED, Google Scholar and a spreadsheet platform, for the systematic revision of the neuronal-salivary proteome and the generation of the embedded type databases;

(B) Clustering Analysis (with VENNY v.2.1), PANTHER v.15.0 and Cytoscape v.3.6.0, as bioinformatics tools used for *in silico* characterization;

(C) Shockwave Flash, Macromedia platform (Adobe Flash), for the creation of the medical atlas.

4.4. Neuronal-Salivary Proteome Database

The following methods were used for the database literature review (Sousa, Martins, Rosa, Barros and Correia, 2016) of the neuronal-salivary proteome and the generation of the embedded type database. To synthesize the Neuronal-Salivary proteome (**Figure 20)**, the Neuro.OralCard has 51 variables, which are represented in 6 separate categories.

4.4.1. Study Selection And Bibliography Research

A systematic bibliographic search was performed using the search tool at PubMed's portal, complemented by Google scholar searching utilities (Falagas et al., 2008). The following keywords were used as filters: "MeSH (Medical Subject Headings)", "neurological diseases", "psychiatric diseases" and "salivary diagnosis".

In order to perform an ordered review based on the publications on molecular biology and the nervous system, additional keyword correlations and associations were searched. Only the publications from 2000 to 2017 were selected. Likewise, in order to perform an ordered review based on the correlation between the salivary proteins/nervous system proteins and physiological and pathologic conditions of neuropsychiatric conditions, the following search filters were also applied: "Humans" and "Clinical trial", in order to include, exclusively, articles of an experimental nature concerning the species *Homo sapiens*. Based on the extensive analysis of the 79 filtered and compiled articles, a survey of a representative sample of neuronal and salivary proteins identified in subjects with (i) neuropsychiatric conditions and (ii) healthy mental functioning was carried out. With the results obtained from this analysis, it was possible to create a neuronal proteome database, named Neuro.OralCard. The full database has not yet been deposited in the oral proteome databank of the OralCard (Arrais, 2013). This specific analysis enables the updating of the OralCard, which is allusive to the human Oralia in different pathologic states. Meanwhile, the full neuroproteome (UniprotKB) was annotated in a non-restricted access server, in the LIMMIT Lab, Faculty of Medicine, University of Lisbon, and accessible via: https://www.limmit.org/uploads/2/6/8/4/26841837/neuro.oralcard.xlsx (retrieved August 01, 2020).

4.4.2. Manual Annotation Of The Proteomics Data Of Neuropsychiatric Conditions And Database Creation

The detailed analysis of all the selected experimental studies (both enlarged and directed; *in vivo* and *in vitro*) made it possible to deliver the manual annotation of the data related to the neuronal produced proteins identified in the brain, blood, saliva and sperm, in patients with neuropsychiatric diseases or with a healthy mental condition (Schafer et al., 2013). The manually annotated information is essential for the functional interpretation of the collected proteomics data. In order to obtain a serial organization of the information collected, variables were constructed (**Figure 21**) integrating the diverse characteristics of the proteins, such as:

1. the identification data of the proteins (label in light blue);
2. the sample location and provenience (label in blue, dark blue and light gray);
3. the health/disease state relation (label in gray, dark gray and darker gray);
4. the characterization of the sample donor (label in light green and green);
5. the characterization of the sample collection and analysis methods (label in dark green); and
6. whether or not they have already been proposed by the author as biomarkers (label in dark green) and their presence in the saliva proteome (light red).

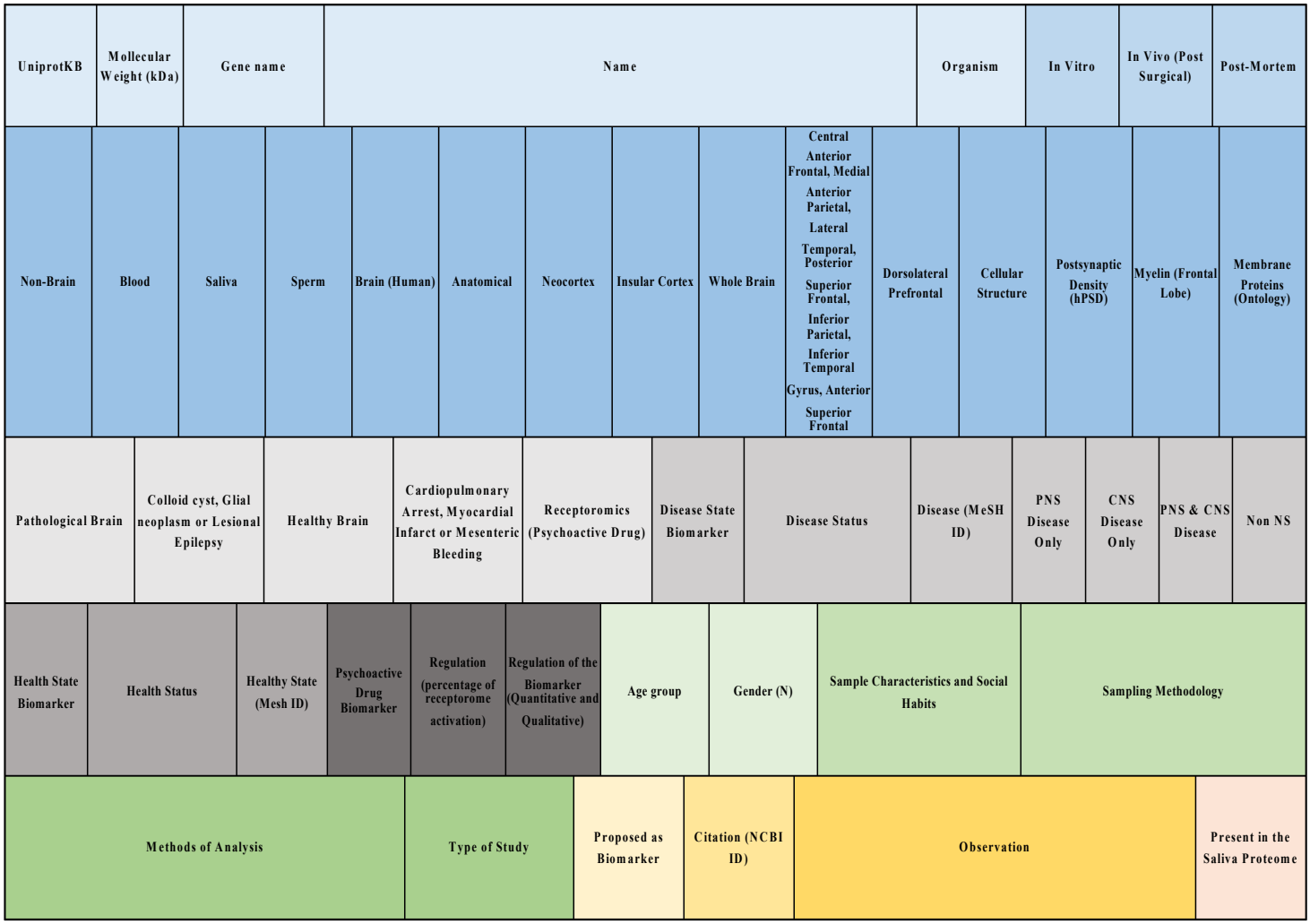


Figure 20. Headings Neuro.OralCard
The 51 variables used in the Neuro.OralCard to synthesize the Neuronal-Salivary proteome are represented in 6 separate categories.

Finally, the referred embedded type database was generated and is accessible online in: https://www.limmit.org/uploads/2/6/8/4/26841837/neuro.oralcard.xlsx (retrieved August 01, 2020; *in* Annex K: External Support).

4.4.2.1. Identification Of The Different Proteins Present In ICD-10 Chapters V (Mental And Behavioral Disorders) And VI (Diseases Of The Nervous System)

This section comprehends the items in light blue (UniprotKB; Molecular Weight (kDa); Gene name; Name and Organism). As regards to the identification of neuronal proteins, 5 fields were used to record the relative information of the proteins.

With the support of the research and selection in the online database UniProtKB (Tognolli et al., UniProt Consortium 1916) (https://www.uniprot.org/, retrieved August 03, 2020), a universal identification code (UniProtKB) was granted to each protein (UniProt Consortium, 2014).

Based on the use of the UniProt website (Boutet et al., 2016) (**Figure 21**), essential information about the identification of each protein identified in the course of the bibliographic analysis was gathered. Equally important, the protein data acquired from the bibliographic research was complemented with UniProt tools, namely, the database entry code, the name of the associated gene and whose organism it belongs to (it was filtered for the human species).

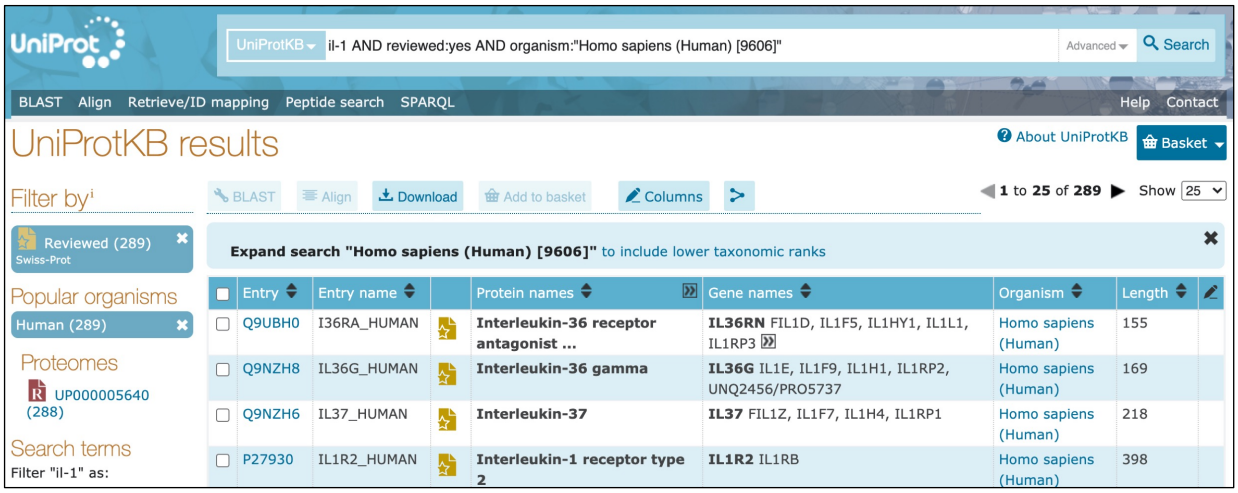


Figure 21. UniProt Website
As retrieved in August 03, 2020, an example (IL-1) of how each protein is consulted in the online knowledge database.

4.4.2.2. Origin And Location Of The Neuronal Samples Mapped

In accordance with the provenance of the annotated neuronal samples of the neuronal proteome under study, the proteins were associated to the following groups:

Firstly, they were clustered in the items in blue (*in vitro*; *in vivo* (Post-Surgical) and *post-mortem*);

Secondly, they were separated in the items in dark blue: a) Non-Brain: Blood, Saliva and Sperm; b) Brain (Human) (i) Anatomical: Neocortex, Insular Cortex, Whole Brain, Central Anterior Frontal, Medial Anterior Parietal, Lateral Temporal, Posterior Superior Frontal, Inferior Parietal, Inferior Temporal Gyrus, Anterior Superior Frontal, Dorsolateral Prefrontal; and c) Brain (Human) (ii) Cellular Structure: Postsynaptic Density, Myelin (Frontal Lobe) and Membrane Proteins (Ontology);

Thirdly, they were organized in the items in light gray: a) Pathological Brain: Colloid cyst, Glial neoplasm or Lesional Epilepsy; b) Healthy Brain: Cardiopulmonary

Arrest, Myocardial Infarct or Mesenteric Bleeding; and c) Receptoromics (Psychoactive Drug).

4.4.2.3. Correlation With Health And Neuropsychiatric States And Conditions

The annotation of this information allows not only to deduce if certain proteins are present in the state of health or disease, but also to understand if they are unique to a certain pathology (Schafer et al., 2014). In order to identify the diseases under study, the MeSH ID code and its elusive correlation to the ICD-10 (WHO, 2016) classification was assumed. Moreover, this annotation classifies the proteins according to their specific association and dysregulation with specific components of the nervous system.

Those variables comprehend the items in gray: a) Disease State Biomarker, Disease Status and Disease (MeSH ID); and b) PNS Disease Only, CNS Disease Only, PNS & CNS Disease, Non-NS. Likewise, they comprehend the items in dark gray: Health State Biomarker, Health Status and Healthy State (Mesh ID).

As far as the regulation of each protein, this refers to the variation of its expression in disease, compared to healthy control. The regulation of the salivary proteins identified in both mental and neurological diseases was based on the following formula:

Fold Change calculation = number of instances (gene or protein expression) in disease state / number of instances in health state = x.

If x (≥) 1: Predicts that the regulation is positive (+); and if $0 < x < 1$: Predicts that the regulation is negative (-). x is the coefficient of difference. Thus, whenever a protein presents a Fold Change calculation of 0,5, it indicates that it has decreased in disease 2 times as to its expression, compared to the healthy state. This analysis of the values enables a clearer identification of the variation in the amount of a given protein in a disease state compared to a healthy state.

Fold change calculation was not indicated for all the neuroproteomes, as only few and limited studies, in our specific neuronal-salivary literature research, published it. A larger research, with less restricted keywords, would gather more fold change quantifications.

This annotation comprehends the items in darker gray: Regulation of the Biomarker (Quantitative and Qualitative). Moreover, the variables (in darker gray): Psychoactive Drug Biomarker, Regulation (percentage of receptorome activation) were inserted. They were used to indicate the proteins that were identified in psychoactive drug trials, and their regulation, i.e., the percentage they bonded to their exact receptor.

4.4.2.4. Characterization Of The Donor Of The Specific Analyzed Sample

The characterization of the sampled donor includes age, gender, social habits and other sample characteristics, e.g., smoking or alcohol consumption, chronic use of medication. This information can be seen in the variables in light green: Age group and Gender (N); and in green: Sample Characteristics and Social Habits.

4.4.2.5. Sampling And Analysis Methods And Study Characterization

This section comprehends the items in light green: Sampling Methodology; in dark green: Methods of Analysis and Type of Study; and in medium yellow: Citation (NCBI ID). The methods used for the sample collection and subsequent analysis were written down for the purpose of characterizing the methodologies applied in the collection, identification and protein analysis. Resulting from the heterogeneity of sampling techniques, e.g., "PSD fractions of human postmortem brains" or " Frozen gray matter dlpfc blocks were obtained from the Stanley Foundation Brain Tissue Array

Collection", and proteomic analysis, e.g., " LC-MS/MS protein identifications from DT", this information becomes crucial in studies of this character, increasing the quality of interpretation of the results.

In order to characterize the study, its type (proteomics, transcriptomics, genomics, etc.) and the NCBI ID - related code of PubMed (https://www.ncbi.nlm.nih.gov/pubmed, retrieved August 04, 2020) was written down.

4.4.2.6. Detected As Biomarker And Present In Saliva

Information about the study was also recorded regarding whether or not the protein in study (1) was proposed as a biomarker of a specific health or neuropathological condition and (2) had already been identified in saliva. Moreover, a variable observation was added for additional information about the protein, e.g., "11 markers distinguished between early-onset MD and CT. Eighteen markers distinguished between MDD with or without comorbid anxiety".

This section comprehends the items in yellow: Proposed as a Biomarker (light) and Observation (dark); and the items in light red: Present in the Saliva Proteome.

4.5. *In Silico* Characterization And Bioinformatics Analysis

Different functional clustering methods were used for the functional characterization of the data collected in the Neuro.OralCard. In order to understand the strategy for using (i) the neuroproteome database and (ii) the different bioinformatics analysis, the reader will be led by procedural questions.

4.5.1. Clustering Analysis ("How To Identify The Proteome Composition?")

With the aid of a specialized tool for the calculation and design of Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/, retrieved July 15, 2020) (Oliveros, 2007), it was possible to complete an analysis of the protein origin data. This tool enabled the calculation of the intersections of two sets of elements: the neuronal proteome and the saliva proteome. This intersection permitted the identification of common or exclusive elements, or proteins, in the two sets of data (**Figure 22**).

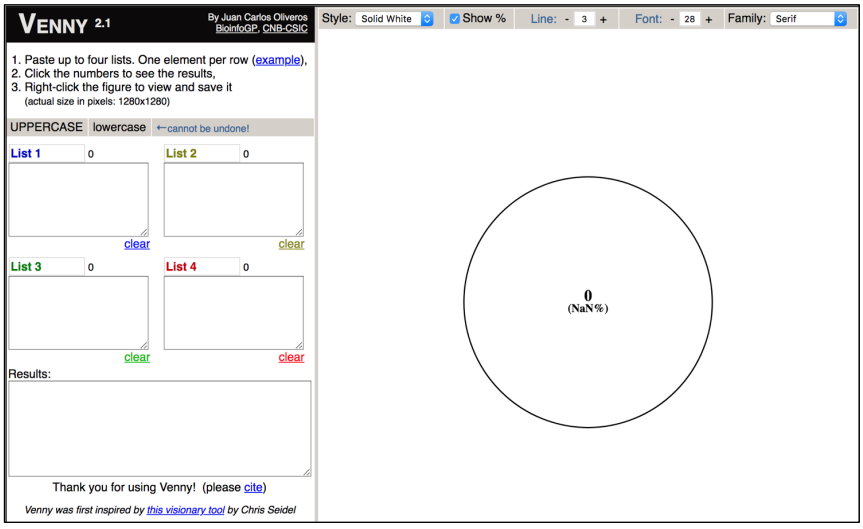


Figure 22. VENNY Website
As retrieved in July 15, 2020, it is shown the tool used to design Venn diagrams. The Venn diagram was used for the calculation of the intersections of two sets of elements: the neuronal proteome and the saliva proteome.

The saliva proteome database, published as the bioinformatics tool OralCard (Arrais et al., 2013), was obtained from SalivaTec, CIS, UCP (Rosa et al., 2012). This database, as a set of UniprotKB codes, was cross-mapped to the set of UniprotKB obtained by the Neuro.OralCard, the neuronal proteome database obtained through this work.

These two sets of data were represented by a Venn Diagram, obtained through the bioinformatics tool Venny 2.1. Venn set colors were plotted according to % of elements found.

4.5.2. PANTHER Functional Classification ("How To Classify The Proteome?")

With the support of PANTHER, a biological process and molecular function enrichment was performed.

Version 15.0 (released 2020-02-14) was used and contains 15,702 protein families, divided into 123,989 functionally distinct protein subfamilies.

After the collection and organization of all existing proteomics data regarding the pathologies under study, a functional analysis was performed according to their gene ontologies (**Figure 23**).

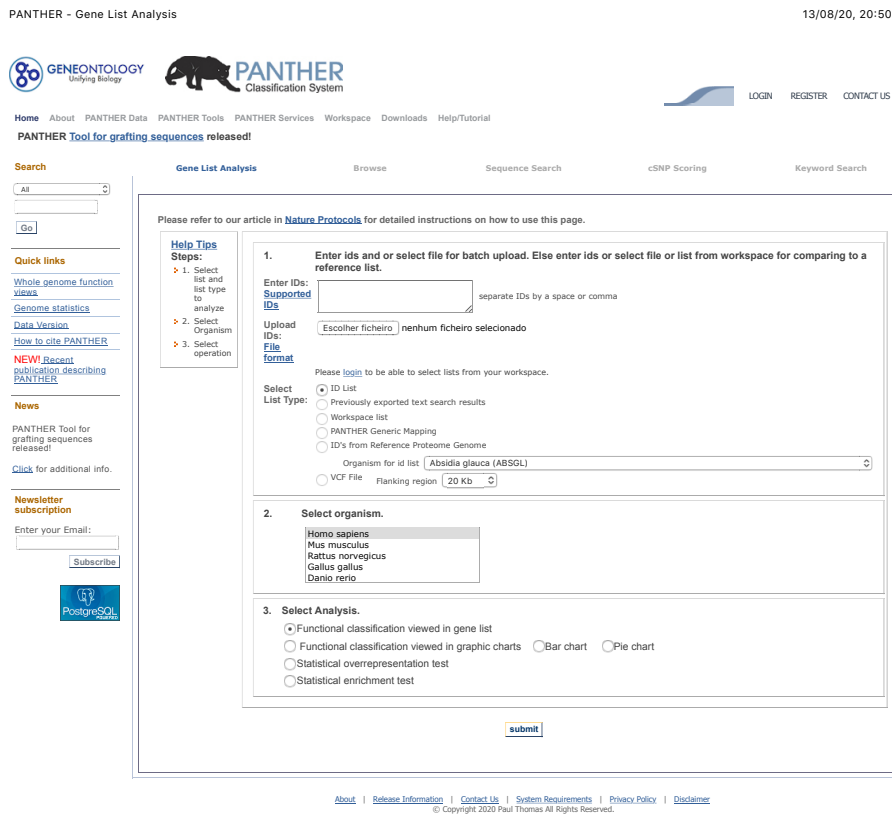


Figure 23. PANTHER Website As retrieved in August 13, 2020, it is shown the tool used for the biological process and molecular function enrichment.

The objective of this analysis was to produce not only an enrichment of the biological processes in which the proteins intervene, but the specific molecular functions involved, as well. This classification was performed using PANTHER (Protein ANalysis THrough Evolutionary Relationships) tool, which allows to classify genes and proteins according to their functions, and to obtain information about

biological processes, molecular functions, cellular components and signaling pathways (Mi et al., 2005; Mi et al., 2006; Mi et al., 2009).

In order to perform this characterization, the UniProtKB information collected in the neuronal database served as input in PANTHER. The respective UniProtKB of neuropsychiatric conditions are the codes of the proteins under study and collected in the database. After this insertion, the appropriate organism (*Homo sapiens*) was chosen, and also the type of analysis desired, e.g., "PANTHER Overrepresentation test"; the annotation version and the release date, e.g., "PANTHER version 13.1 Released"; the reference list, i.e., the proteome list to be compared to; the annotation data set, e.g., PANTHER GO-Slim Biological Processes; and the test type, e.g., Fisher's Exact with FDR multiple test correction. With this interface, it was, therefore, possible to choose the type of desired classification (**Figure 24**).

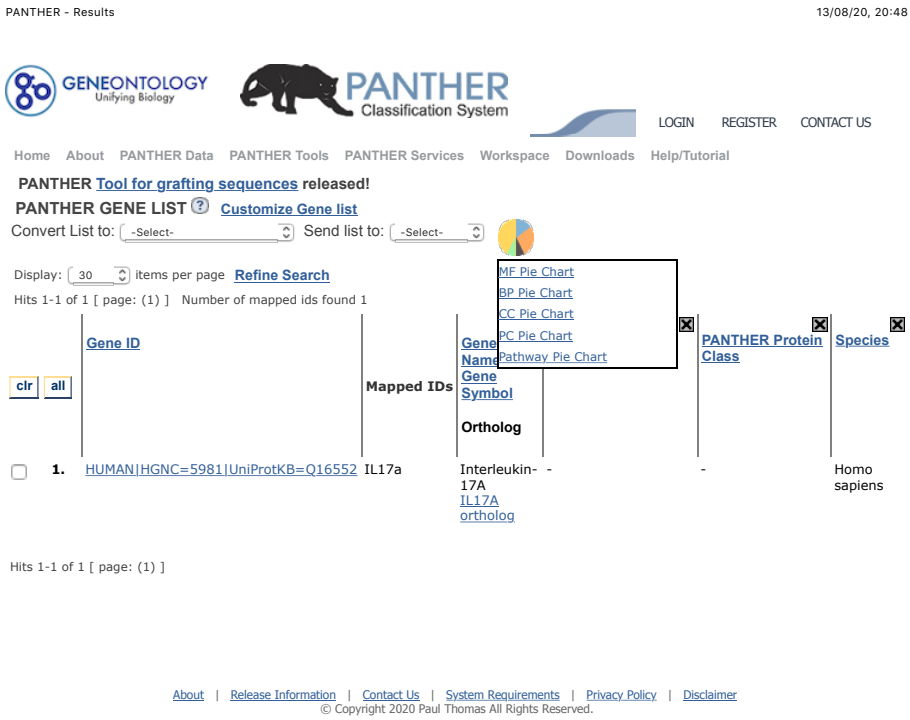


Figure 24. PANTHER Pie As retrieved in August 13, 2020, it is shown an example (IL-17A) of the procedure to choose the type classification.

MF Pie Chart allows the classification according to molecular function; BP Pie Chart classification, according to biological processes; CC Pie Chart, according to cellular components; PC Pie Chart, according to protein class; and Pathway Pie Chart, according to the signaling pathways.

A functional analysis of the data for the neuroproteome under study was compared against a reference list, the human proteome (Human - taxon 9606), reflecting all human proteins identified, published and annotated, on 11.07.2018.

The purpose of this comparison was to statistically [obtained values (neuronal proteome) vs. expected values (human proteome)] determine the difference between (i) the expression of proteins present in neuropsychiatric ontologies and in healthy neuropsychophysiology versus (ii) the human proteome.

In order to quantify the difference between the observed values and the expected values of each item, the fractional difference was calculated through the application of a Fisher's Exact with FDR multiple test correction.

4.5.3. Cytoscape Functional Clustering ("How To Detect The Proteome Networks?")

A network functional enrichment was performed (**Figure 25**) with Cytoscape v. 3.6.0.

Firstly, the analysis mode was set to ClueGO: Functions (v. 2.5.0) and the visual style to groups.

Secondly, the load marker was set to *Homo sapiens* [9606] and the following ontologies were updated (Ontology; Type; Updated on; Pathways/Genes).

Ontology REACTOME, type Pathways, updated on 17.02.2018 has 2155 pathways expressed by 10,609 genes (Croft et al., 2010). It is a database of reactions, pathways and biological processes. It visually represents biological pathways in full mechanistic detail, while making the source data available in a computationally accessible format.

Ontology GO, type Biological Process, updated on 16.02.2018 has 15,785 pathways expressed by 17,540 genes (Botstein et al., 2000). It is the best known and most used database of biological processes.

Ontology KEGG, type a.) COMPOUND and b.) KEGG, updated on 16.02.2018 has respectively a.) 267 pathways expressed by 3657 genes and b.) 316 pathways expressed by 7325 genes. It is an extensive collection of small molecules, like peptides, that are relevant for biological systems (Hattori et al., 2003).

Ontology GO, type a.) Immune System, b.) Molecular Function and c.) Cellular Component, updated on 16.02.2018 have respectively a.) 1170 pathways expressed by 3152 genes, b.) 4612 pathways expressed by 17,556 genes, and c.) 1924 pathways expressed by 18,620 genes. It is the GO collection of immunity compounds, molecular functions and cellular components (Diehl et al., 2007; Hill et al., 2008).

Thirdly, GO levels were set to specific, i.e., GO levels set to 7-15 and ClueGO predefined to 200 gene associations. Likewise, the pathways were prearranged to make GO term fusions, increasing the specificity of the GO hierarchical tree, i.e., a fusion of GO parent-child terms was made and based on similar associated genes.

Fourthly, the layout of the data visualization was set to edge: weighted spring embedded layout. This layout offered the best exploration tools for our dataset of proteins.

As we wanted a high pathway specificity between a considerable high number of proteins, this layout permitted the sufficient data separation for an adequate visualization of the protein networks.

Finally, we defined the tool to only and exclusively show *kappa* score and ontology relations. With this selection, we avoided plotting unnecessary information in our pathways.

This 5-step strategy is important because Cytoscape tends to plot an extensive and easily incomprehensible amount of information, which cannot be directly or indirectly digested. Therefore, the right choice of restricted options and parameters is essential for the adequate exploration of the data.

Methodology: Cytoscape analysis v. 3.6.0

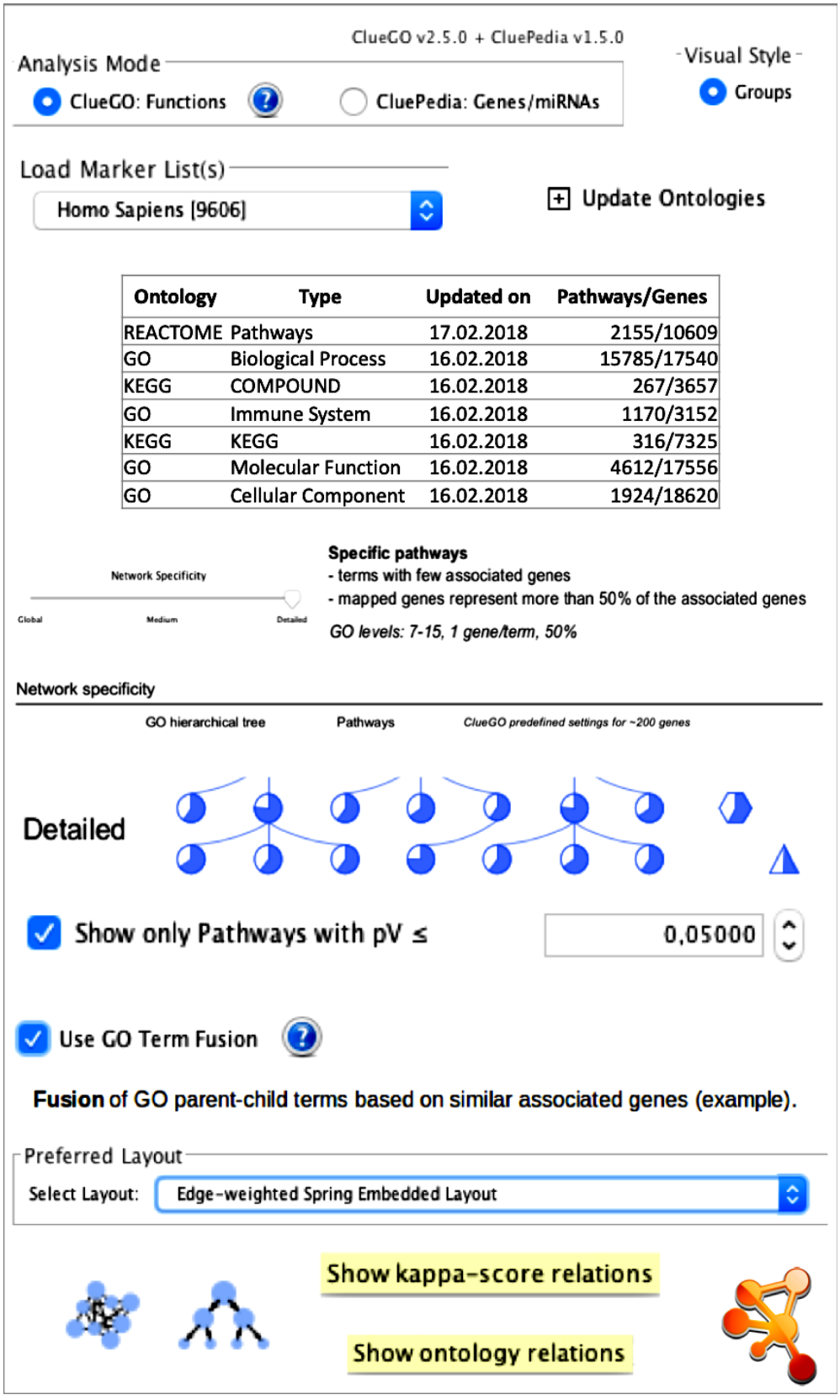


Figure 25. Cytoscape Analysis
The methodology for the Cytoscape analysis (v.3.6.0) is synthesized in this figure, reviewing the 5-step strategy.

4.5.3.1. ClueGO, Cytoscape Plug-In

ClueGO (Bindea et al., 2009) is a Cytoscape plug-in that visualizes the non-redundant biological terms for large clusters of genes in a functionally grouped network. ClueGO performs single cluster analysis and comparison of several clusters (lists of genes).

The ClueGO network is created with kappa statistics and reflects the relationships between the terms based on the similarity of their associated genes. ClueGO uses different data sources like Gene Ontology, KEGG, WikiPathways or Reactome. ClueGO also visualizes terms and pathways resulted from other enrichment analyses as functionally grouped networks.

4.5.4. *In Silico* Data Analysis By Web-Based Application

Regarding the *in silico* data analysis (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017) by web-based application and to better organize methodology used in chapters 4 and 6, a detailed explanation is given to the bioinformatics tools. This explanation comprises not only the underlying methodology for each analysis used in chapters 4 and 6, but also the software and knowledgebases used.

The two main software chosen (PANTHER v.15.0 and Cytoscape v.3.6.0) use different methodologies and show major differences on their interfaces. As outcome PANTHER was chosen for functional classification, while Cytoscape for functional clustering.

As an example, PANTHER, for the functional enrichment, offered a better and systematic classification of one single knowledgebase. While Cytoscape permitted not only a better graphic visualization but also the capacity to cluster the neuroproteome in a combined system of knowledgebases. However, in some specific analysis they both used similar knowledgebases, as an example in the functional enrichment by gene ontology (PANTHER GO Slim Molecular Functions / ClueGO Molecular Function Genes and PANTHER GO Slim Cellular Components / ClueGO Cellular Component Genes).

To fully explain the details of the software, the knowledgebases and protein annotation systems as well as the subjacent technical reasons for their application in a specific analysis, a suitable explanation is given.

4.5.4.1. PANTHER Functional Classification Vs. Cytoscape Functional Clustering

(A) In this thesis, PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was used as large curated biological database of protein families for high-throughput functionally analysis (Thomas et al., 2003).

Neuronal proteins are thus classified according to families and subfamilies, pathways, biological processes, molecular functions and cellular components. In this *in silico* characterization and bioinformatics analysis, the application and subjacent technical reasons for the specific selection of this software was to accurately infer the function of the neuronal proteome of the neurotypical young adults (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017).

PANTHER classification system was based on the evolutionary relationships of neuronal genes with the known functions, i.e., neuronal proteins (Mi, Muruganujan and Thomas, 2012).

By combining gene function, ontology, pathways and statistical analysis tools, PANTHER enabled to analyze the large-scale proteomic data obtained from the

neuronal-salivary proteome database. Using the Neuro.OralCard data and the tools on the PANTHER it was possible to discover, classify and analyze the neuronal proteome.

In PANTHER a pythogenetic tree ("subfamily membership", "gene function", "protein class") was found for each of the neuronal protein families. Swiss-Prot protein names were used to name subfamilies (membership), Gene Ontology (GO) terms were used for gene function, and PANTHER/X ontology terms were used for protein class.

In this study, the process for data generation comprised:

(i) the creation of phylogenetic trees;

(ii) the use of a PANTHER component named PANTHER/X (PANTEHR index);

(iii) the use of the application PANTHER pathways.

For the phylogenetic trees (i), PANTHER used GIGA algorithm, i.e., on each iteration it attempted to reconcile tree in event form of speciation and gene.

PANTHER/X (PANTEHR index) (ii) assisted in summarizing, navigating the molecular and biological functions of the neuronal proteome. Although PANTHER/X ontology has a hierarchical organization, it was used as a directed acyclic graph and so mapped to the GO and arranged in different ways to facilitate large-scale analysis of the Neuro.OralCard.

The application PANTHER pathways (iii) included 176 pathways using CellDesigner tool.

In the PANTHER Functional Classification, the analysis of the neuronal proteome was done by the following procedure:

Firstly, protein IDs from UniProt were inputted and PANTHER family and subfamily of the neuronal protein set was identified and classified. Secondly, an analysis of the GO molecular function ("what are the functions"), of the GO biological process ("what are the processes"), and of the GO cellular component ("where in the cell") is conducted. Thirdly, an analysis of the PANTHER protein class classified the IDs of PANTHER protein class for each of the neuronal proteins. Fourthly, an analysis of the pathways classified the neuronal pathways of the set of proteins.

(B) In this thesis, Cytoscape clustering system was used as a platform for visualizing molecular interaction networks and integrating neuronal protein expression profiles and other state data (the subphenomes/subgroups of this study) (Shannon et al., 2003). ClueGO plugin was used for network and molecular profiling analyses.

Cytoscape was thus chosen to visualize and analyze network graphs of the Neuro.OralCard. In this *in silico* characterization and bioinformatics analysis (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017), the application and subjacent technical reasons for the specific selection of this software was to (i) input, (ii) visualize and (iii) analyze functional clusters of neuronal proteins (Bell et al., 2006).

In the Cytoscape Functional Clustering, the analysis of the neuronal proteome was done by the following procedure:

Firstly (i), Neuro.OralCard was inputted, and molecular interaction networks from protein-protein interaction pairs were constructed. The complete set of annotation terms for all the proteins was updated, and a set of confidence values for your protein–protein interactions were defined. Automatically, it was revised the gene functional annotations from the Gene Ontology (GO) and KEGG databases.

Secondly (ii), for the visualization, a customize network data is displayed. A superposition of protein expression ratios and p-values on the neuronal network is shown. In this study spring-embedded layout's algorithms were used for the visualization.

Thirdly (iii), an analysis of the neuronal network and molecular profile was conducted. The network was filtered to select subsets of interactions based on the selected knowledgebases. The threshold number of nodes involved in interactions sharing and combining the same GO annotation, viz. GO Immune System Process, GO Biological Process Genes, GO Molecular Function Genes, and GO Cellular Component Genes, the same KEGG and the same REACTOME PATHWAYS was defined.

Then, nodes whose protein expression levels change significantly in one or more conditions according to the p-values previously loaded with the gene expression data were selected. Active subnetworks/pathway were thus selected. The neuronal network was screened against protein expression data to identify connected sets of interactions, i.e., interaction subnetworks, whose neuronal proteins showed particularly high levels of differential expression. The interactions contained in each subnetwork provided hypotheses for the regulatory and signaling interactions in control of the observed expression changes.

Finally, clusters (highly interconnected regions) were found in the neuronal network. Clusters in the protein–protein interaction network are protein complexes and parts of pathways. Clusters in a protein similarity network represented protein families.

4.5.4.2. Knowledgebases: Gene Ontology (GO), KEGG and REACTOME

The Gene Ontology (GO) is a knowledgebase created by a bioinformatics initiative to unify the representation of gene and gene products, e.g., proteins (Gene Ontology Consortium, 2015). More specifically, the GO consortium aimed to annotate genes and gene products, assimilate and disseminate annotation data and to enable functional interpretation of experimental data, for example via enrichment analysis (Thomas, 2017). In this study, GO focused on the function of the genes in the neuroproteome.

Ontology is a representation of things that are detectable or directly observable, and the relationships between those things. In this thesis, GO covered three main domains:

(i) cellular component, the parts of a neuronal cell or its extracellular environment;

(ii) molecular function, the elemental activities of a neuronal protein at the molecular level, such as binding or catalysis;

(iii) biological process, operations or sets of molecular events with a defined beginning and end, pertinent to the functioning of the brain. Genome annotation in brain research thus encompasses the practice of capturing data about a gene product, like a neuronal protein; and GO annotations accordingly use terms from the GO ontology to do so.

Gene Ontology was the main knowledgebase used in this thesis. In PANTHER, four separate GO PANTHER applications were used for a biological process and molecular function enrichment, viz. PANTHER GO Pathways, PANTHER GO Slim Cellular Components, PANTHER GO Slim Molecular Functions and PANTHER GO Protein Class.

In Cytoscape, and more specifically in the ClueGO, a Cytoscape Plug-In, four different ClueGO applications were used for network functional enrichment and as a network visualization tool, viz. ClueGO Immune System Process, ClueGO Biological Process Genes, ClueGO Molecular Function Genes, and ClueGO Cellular Component Genes.

Note that although the applications (PANTHER GO Slim Molecular Functions / ClueGO Molecular Function Genes and PANTHER GO Slim Cellular Components /

ClueGO Cellular Component Genes) in PANTHER (i) and Cytoscape (ii) used the same GO annotations, the enrichment approach and outcomes were entirely different. (i) was used for biological processes and molecular function enrichment and (ii) was used for network functional enrichment.

KEGG (Kyoto Encyclopedia of Genes and Genomes) (Kanehisa and Goto, 2000) is a knowledgebase dealing with genomes, biological pathways and diseases. KEGG was used for data analysis of the neuroproteome.

In this study, KEGG PATHWAY database was used for mapping representing experimental knowledge on neuronal metabolism. Each pathway map contained a network of neuronal molecular interactions and reactions and was designed to link genes in the genome to neuronal proteins in the pathway. It integrates therefore the building blocks and wiring diagrams of the neuronal system — more specifically, genetic building blocks of genes and proteins, chemical building blocks of small molecules and reactions, and wiring diagrams of molecular interaction and reaction networks.

The KEGG PATHWAY database includes not only the normal states but also the perturbed states of the neuronal systems. This knowledgebase was used mainly in Cytoscape, and more specifically in the ClueGO, Cytoscape Plug-In, for network functional enrichment and as a network visualization tool.

REACTOME is a knowledgebase of biological pathways (Croft et al., 2010). The content is cross-referenced to many bioinformatics databases and the rationale behind REACTOME is to visually represent biological pathways in full mechanistic detail.

The core unit of the REACTOME data model is thus the reaction. In this study neuronal proteins participating in reactions formed a network of biological interactions and were grouped into pathways. The neurobiological pathways in REACTOME included signaling, innate and acquired immune function, transcriptional regulation, translation, apoptosis and classical intermediary metabolism.

In REACTOME, the neurotypical adult biological processes were annotated by breaking them down into a series of molecular events, and these included the classical chemical interconversions of intermediary metabolism that direct molecules between cellular compartments.

The neuronal proteins were cross-referenced with UniProt and as localization of molecules to subcellular compartments is a key feature of the regulation of human neurological processes, proteins were associated with specific locations. Over-representation results were presented as a list of statistically overrepresented pathways, and, in this thesis, used mainly in Cytoscape, and more specifically in the ClueGO, Cytoscape Plug-In, for network functional enrichment and as a network visualization tool.

In chapter 6 of this thesis, a functional analysis of the immune response in neurotypical young adults is performed. For that purpose, in PANTHER and for a functional classification of the Human Th17 Cytokine Assay, the application PANTHER Slim Biological Processes will be used.

Complementarily, in the Cytoscape, and more specifically in the ClueGO, two different applications will be used for functional clustering of the immune interaction networks and as a network visualization tool, viz. ClueGO Immune System Process and KEGG PATHWAY.

Results And Discussion

This set of results was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[71]: Martins, J. E.[a,b,c,d], Rosa, N[c], Correia, M. J.[c], Simões, M.[a,b], Barros, M.[c].

The results, and their discussion, comprehend the data acquired in:

(4.A.) the neuronal-salivary proteome database;

(4.B.) in the *in silico* characterization and bioinformatics analysis.

The variables analyzed are both quantitative/continuous and qualitative/nominal, depending on the type of analysis that was performed.

4.A. Neuronal-Salivary Proteome Database

4.A.1. Human Neuronal Proteome Of Neuropsychiatric Conditions

As a result of the compilation of central and peripheral nervous system protein production, the functional characterization of the human neuronal proteome becomes fundamental to understand its molecular mechanisms.

Those molecular mechanisms enable the posterior identification of potential biomarkers of susceptibility for neuropsychiatric diseases.

4.A.1.1. Composition

How many neocortical proteins are biomarkers of a healthy brain?
How many postsynaptic density proteins are associated with central nervous system disease?

The identification of the neuronal proteins was achieved by analyzing the results of experimental proteomics studies.

These studies comprehend:

(1) Enlarged studies characterized by the high-throughput proteomics acquisition of data;

(2) Directed studies characterized by the detection and quantification of specific analytes.

These studies, therefore, comprehend both bottom-up and top-down proteomic approaches. In total, 79 articles were analyzed, published between the years 2000 to 2017, as shown in , for the respective keyword correlations. The 79 articles referenced for the creation of the Neuro.OralCard may be consulted online in: https://tinyurl.com/Neuro-OralCardReferences (retrieved July 16, 2020; ; *in* Annex K: External Support).

From this literature revision, an extensive omics annotation (manual and automatic) was made for the full range of neurologic diseases, and also for schizophrenia, disorders of adult personality, mood/affective disorders, and anxiety disorders.

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Type of Study	Keywords	N.º of Publications
Specific	"Saliva*"	4
Enlarged	"Proteomics"; "Genomics"; "Transcriptomics"; "Epigenomics"; "Metabolomics"; "Proteome"; "Neuroproteome"	13
	"Molecular AND Biomarker AND Happiness"	1
	"Proteome Mental"	7
	"Proteome Yoga"	1
Directed	"Neuronal Physiology"; "Biomarker"; "Protein"; "Mental Health"; "Meditation"	15
	"Genome"	1
	"Recept* Mental Neuro"	1
	"Inflammation AND Depression"	4
	"Protein AND Biomarker AND Happiness*"	4
	"Protein AND Biomarker AND Wellbeing*"	4
	"Consciousness Biomarker Protein*"	4
	"Consciousness Molecular Biomarker"	2
	"Proteo* Consciousness*"	2
	"Prote* Wellbeing Biomarker*"	1
	"Happiness Biomarker*"	8
	"Wellbeing Biomarker*"	7

Figure 26. Composition Of Literature Review
Specific, enlarged and directed studies were used for the construction of the neuronal-salivary proteome database. A total sum of 79 articles were used: 4 (specific), 22 (enlarged) and 53(directed).

Those included, for instance, genomics and proteomics studies from overall brain *post-mortem* and *in vivo/in vitro* structures, like the postsynaptic density, membrane or myelin proteomics of specific brain regions.

Moreover, protein data were complemented with receptorome studies of psychopharmacology and from online available databases ventures, e.g., Genes2cognition (http://www.genes2cognition.org/science/proteomics/synapse.html, retrieved August 05, 2020).

The final neuronal database, named Neuro.OralCard, revised not only neuronal produced but also peripheral proteins. These neuronal proteins ultimately aim to represent not only epigenome, transcriptome, proteome, and metabolome analysis, but also the main findings of functional cellular assays.

The results of this neurobiological approach imply that alterations in neurotransmission, in hormonal regulation, in metabolism, in the cell cycle, and in the immune system may be partially responsible for the neuronal and mental pathophysiology. The Neuro.OralCard database is constituted by 2416 proteins with regard to ICD-10 neuropsychiatric conditions and mental health functioning.

To our understanding, this is somehow an innovative database that gathers the information about the proteins present and altered in healthy vs. pathological states of the nervous system. This update/creation is essential not only for the constant publication of new experimental studies of neuronal proteomics, but also for other omics that intend to reflect neuronal condition, e.g., salivary proteomics studies of the nervous system. It covers data on proteins with altered expression profiles, as of yet unidentified, or confirmed experimental results, obtained by other authors with similar experimental studies (Zhang et al., 2013).

The information that characterizes the pathologies and conditions under study is compiled in https://www.limmit.org/uploads/2/6/8/4/26841837/neuro.oralcard.xlsx (retrieved August 01, 2020), allowing its users to analyze it in a simplified and systematic way. Salivary sampling and analysis have only recently been developed for the study of the neuronal proteome. Currently, few embedded type databases review the recent advances in molecular neurobiology. Likewise, there are few susceptible molecules already identified as salivary biomarkers of neuronal and mental conditions.

The Neuro.OralCard database aims to be used as a molecular data reference and support for biomarker discovering in neuropsychiatric illnesses, as well as non-pathologic conditions of the nervous system. **Figure 27** and **Figure 28** indicate the neuronal proteins identified, resulting from the experimental studies reviewed and analyzed throughout the course of this work.

				N. of Proteins	%
Type of Sample	In Vitro			47	1,94%
	In Vivo (Post Surgical)			627	25,90%
	Post-Mortem			1866	77,08%
	Other			393	16,23%
Tissue and Structure	Non-Brain	Blood		273	11,28%
		Saliva		24	0,99%
		Sperm		1	0,04%
	Brain (Human)	All Brain		2190	90,46%
		Anatomical	Neocortex	804	33,21%
			Insular Cortex	1307	53,99%
			Whole Brain	646	26,68%
			Central Anterior Frontal, Medial Anterior Parietal, Lateral Temporal, Posterior Superior Frontal, Inferior Parietal, Inferior Temporal Gyrus, Anterior Superior Frontal	736	30,40%
			Dorsolateral Prefrontal	165	6,82%
		Cellular Structure	Postsynaptic Density (hPSD)	1158	47,83%
			Myelin (Frontal Lobe)	61	2,52%
			Membrane Proteins (Ontology)	479	19,79%
Brain Type	Pathological Brain	All		888	36,68%
		Colloid cyst, Glial neoplasm or Lesional Epilepsy		627	25,90%
	Healthy Brain	All		1815	74,97%
		Cardiopulmonary Arrest, Myocardial Infarct or Mesenteric Bleeding		61	2,52%
		Receptoromics (Psychoactive Drug)		41	1,69%
Biomarker	Disease State Biomarker	All		2059	85,05%
		PNS Disease Only		9	0,37%
		CNS Disease Only		361	14,91%
		PNS & CNS Disease		9	0,37%
		Non NS		43	1,78%
	Health State Biomarker			802	33,13%
	Proposed as Biomarker			312	12,89%
Detected	Present in the Saliva Proteome			764	31,56%
	Present in the Oral Proteome			970	40,07%
All Proteins				**2421**	**100,00%**

Figure 27. Descriptive Analysis Of The Neuronal Proteome
The number of proteins and relative percentage (total neuronal proteome) are represented for the variables or categories: Type of Sample, Tissue and Structure, Brain Type, Biomarker and Detected.

Both figures show the distribution of the proteins identified and collected in different categories. **Figure 27** also offers a descriptive analysis with the percentage of proteins in a specific category. This descriptive analysis is important when questioning the statistical weight of the proteome which has been identified in a specific variable, i.e., Type of Sample, Tissue and Structure, Brain Type, Biomarker or Detected.

Figure 28 offers a better visualization of that distribution and refers to the number of samples (or proteins) identified in omics studies, techniques or methodologies already revised by the authors. In the Type of Sample, most of the samples came from *post-mortem* brain sampling (e.g., A3KMH1 von Willebrand factor A domain-containing protein 8) and a few from *in vitro* collections (e.g., O43169 Cytochrome b5 type B). *In vivo* (post-surgical) also offered a good quantity of proteins such as O43236 septin 4. In Tissue and Structure, the majority of the non-brain samples came from blood (e.g., O75444 Transcription factor Maf) and saliva (e.g., P01834 Ig kappa chain C region). However, most of the samples (90%) were originated from the Human Brain.

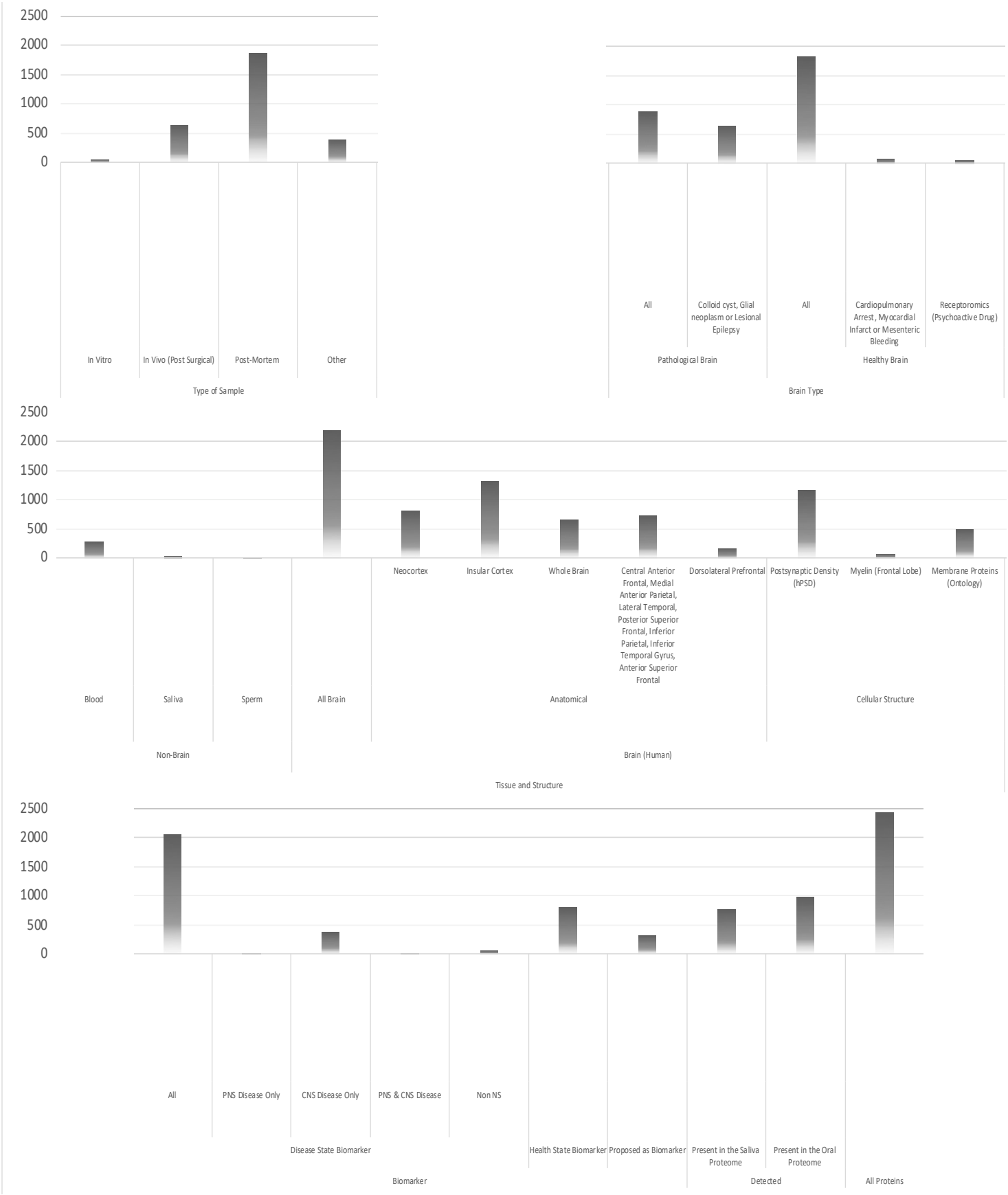


Figure 28. Distribution Of The Neuronal Proteome By Categories (X-Label; Y-Number Of Proteins)
The number of proteins is plotted for each of the 30 subvariables in the categories:
Type of Sample, Tissue and Structure, Brain Type, Biomarker and Detected.

From these, 50% came from anatomical cortical structures such as:

(i) the neocortex[72] (e.g., P02042 Hemoglobin subunit delta);

(ii) the insular cortex (e.g., P02768 serum albumin);

(iii) the dorsolateral prefrontal cortex (e.g., P17600 Synapsin-1);

(iv) other cortical regions (e.g., P02794 ferritin, heavy polypeptide 1), as the central anterior frontal, medial anterior parietal, lateral temporal, posterior superior frontal, inferior parietal, inferior temporal gyrus, anterior superior frontal.

The other 50% came from cellular structures such as the postsynaptic density (e.g., P13637 ATPase, Na+/K+ transporting, alpha 3 polypeptide), myelin (e.g., P62158 calmodulin (CaM)) and membrane proteins (e.g., P51148 Ras-related protein Rab-5C).

24% of the brain samples came from a pathological brain (e.g., P49841 glycogen synthase kinase-3 beta), i.e., colloid cyst, glial neoplasm or lesional epilepsy. Whereas, 74% came from a healthy brain (e.g., P49815 Tuberin (Tuberous sclerosis 2 protein)). 2% of the proteins were discovered in receptoromics studies of psychoactive drugs (e.g., P50406 5-hydroxytryptamine receptor 6).

From the total 2416 neuronal proteins, 85% were considered as a possible disease state biomarker, while 33% were considered as a possible biomarker of a mental health state or of neurotypical subjects. From those, only 13% were in fact identified and proposed as biomarkers. Those 13% are mostly biomarkers of central nervous system conditions, such as the P60903 Protein S100-A10 (p10 protein) identified in Post-Traumatic Stress Disorder (MESH ID 68,003,865) and Major Depressive Disorder (MESH ID and 68,013,313); or the P42263 glutamate receptor, ionotropic, AMPA 3 in Mental Retardation, X-Linked (MESH ID 68,038,901); or the P22314 ubiquitin-like modifier activating enzyme 1 in Spinal Muscular Atrophy, Distal, X-Linked 3 (MESH ID 67,564,506).

However, when compared to disease state biomarkers, only a very limited and minor quantity has been identified as mental health biomarkers or biomarkers of neurotypical subjects. The PMC4383272 (NCBI ID) proteomics study proposed two salivary sampled neuronal proteins by liquid chromatography-tandem mass spectrometry. Those were P0CG05 Ig lambda-2 chain C regions and Q9UGM3 Deleted in malignant brain tumors 1 protein (Glycoprotein 340). Both were considered present in the happiness state (MESH ID 68,006,240) induced by self (MESH ID 68,012,649) awareness (MESH ID 68,001,364).

In addition to the 2416 neuronal proteins identified in this study, the neuronal proteome was cross-referenced with the OralCard pool of salivary proteins (N=3030 proteins). This cross-referencing showed that 1/3 of the 2416 neuronal proteins are also detected in salivary samples and present in the salivary proteome. This 1/3 usually corresponds, in our database, to small peptides or proteins with a small molecular weight, e.g., the P01766 Ig heavy chain V-III region BRO (13 kDa).

This cross-analysis of proteins found both in the neuronal and salivary proteomes is further explained in the clustering analysis. This is a critical phase for conducting an experimental study design, which uses saliva as a sample for characterizing neuronal conditions (Arrais et al., 2013; Zhang et al., 2013).

The (i) neuronal proteome and (ii) the neuronal & salivary proteome are completely described in the following database, which is accessible online: https://tinyurl.com/DatabaseProteomeUniprotKB (retrieved July 17, 2020; *in* Annex K:

[72] microdomain-associated proteins in the dorsolateral prefrontal cortex, frontal superior gyrus of Brodmann Area 9, inferior frontal gyrus of Brodmann Area 45 (Behan et al., 2009); presynaptic proteins (SNAREs) and lipid rafts microdomains in the frontal cortex (Salaün, James and Chamberlain, 2004)

External Support). This database comprehends the Revised Human Neuronal Proteome and the Revised Human Neuronal & Salivary Proteome, on 12.07.2018. These revised proteomes include the variables: Entry; Entry name; Protein names; Gene names; Involvement in disease; Pharmaceutical use; Gene ontology (molecular function); Gene ontology (biological process); GO; PubMed ID; Mass and Pathway.

This proteome characterization was possible due to the information gathered in the UniProt database consortium (UniProt Consortium, 2016, Boutet et al., 2016). An example of a protein that is found in the neuronal & salivary proteome is P37837, TALDO_HUMAN, Transaldolase (EC 2.2.1.2), TALDO1 TAL or TALDO TALDOR. P37837 is a protein implicated in the Transaldolase deficiency (TALDOD) [MIM:606003], which is an inborn error of the pentose phosphate pathway resulting in early-onset multisystem disease.

This protein has the molecular function of monosaccharide binding (GO:0048029) and sedoheptulose-7-phosphate:D-glyceraldehyde-3-phosphate activity (GO:0004801). It has the biological function of energy reserve metabolic process (GO:0006112) and has the pathway of carbohydrate degradation and pentose phosphate pathway. The protein has been identified in the PubMed ID studies: 8,300,619; 9,339,383; 9,524,206; 1,070,229 and has the molecular mass of 37.54 kDa.

4.A.1.2. Origin Of The Catalogued Neuronal Samples

Is it possible to understand the P01584:Interleukin-1 beta role in schizophrenia?
How was P04792: Heat shock protein 1 identified and annotated?

The neuronal samples analyzed in the proteomics studies can be derived from different types of samples, different brain tissues, regions and structures and different brain types. Those proteins were also classified as biomarkers for a specific condition, or not, and could, or not, be detected in saliva. For better understanding and data visualization, the following hierarchical representation is presented (**Figure 29**).

For instance, P01584: IL1B IL1F2, Interleukin-1 beta (IL-1 beta) (Catabolin), present in *Homo sapiens*, is normally found on blood samples but can be identified in pathological brains.IL-1 beta was identified as a disease biomarker of schizophrenia (MESH ID 68,012,559), as a protein affecting only the Central Nervous System. A protective allele of IL-1 beta reduces its expression regardless of diagnosis. IL-1 beta was identified both in male and female subjects. The sampling was in whole blood and the analysis method was real-time RT-PCR. This gene expression type of study (NCBI ID: 22,804,923) proposed IL-1 beta as a biomarker. IL-1 beta has already been identified in the salivary proteome.

Likewise, P04792: HSPB1, the Heat shock 27 kDa protein 1, present in *Homo sapiens*, can be found on neocortical samples in the postsynaptic density (hPSD). It is considered a biomarker of the Charcot-Marie-Tooth disease, Type 2E (MESH ID 67,535,413), as a protein affecting only the Peripheral Nervous System. HSPB1 was identified both in male and female subjects. Human postsynaptic density's samples were isolated from neocortical samples of nine adult individuals undergoing neurosurgical procedures. The protein preparations were allocated into three pools, each showing a similar pattern of staining on SDS-PAGE and the expected enrichment of 12 hPSD marker proteins including neurotransmitter receptors, scaffold and signaling proteins relative to cortical synaptosomes. Proteomic profiling using LC-MS/MS provided the comprehensive protein identification. This proteomics study type (NCBI ID: 3,040,565) proposed HSPB1 as a biomarker. Likewise, HSPB1 has already been identified in the salivary proteome.



Figure 29. Neuronal Proteome

Hierarchical representation of the neuronal proteome (N=2421 proteins). A circular plot presents the relative percentage of the total number of proteins for each of the 30 subvariables in the categories: Type of Sample, Tissue and Structure, Brain Type, Biomarker and Detected.

4.A.1.3. Characterization Of The Sample's Donor

In order to more accurately characterize the individuals in the sample, data were collected regarding their age, gender and relevant social habits. Regarding the analysis of the age group, the range of variation of all individuals is [0;81] years old. This large age variability may lead to genomic alterations, repercussions on biological processes and modifications due to aging in the neuronal system. All these factors may, consequently, lead to changes in the expression of some proteins (Fleissig et al., 2010).

In what concerns gender analysis, the experimental studies analyzed included both male and female subjects. Different social habits were present in the donors, such as smoking, which may lead to an increase in systemic inflammation (Fleissig et al., 2010).

4.A.1.4. Analytical And Sampling Methods

In this work, as mentioned before, large-scale proteomics studies were analyzed, which resulted in the identification of high numbers of proteins but also targeted studies, which allowed the identification of a smaller number and specific proteins.

The studies aimed to analyze proteins in which there is previous evidence of their possible implication in disease and health, that is, identify and quantify proteins that may play an important role in a specific physiology. The annotation of the methods of sampling and analysis is relevant, when analyzing the data, because their heterogeneity can influence the detection of proteins. This heterogeneity makes difficult the direct interpretation of the data obtained in the proteomics studies.

4.B. *In Silico* Characterization And Bioinformatics Analysis

A functional characterization of the neuronal proteome was executed to understand the biological processes of neuronal proteins present in the Neuro.OralCard database. This characterization was performed according to the protocol defined in methods with the same denomination.

4.B.1. Clustering Analysis

How many proteins are present in the saliva?
How many neuron-produced proteins are present in saliva?
How many proteins are present in the brain?

The clustering analysis of the Neuro.OralCard database is shown in **Figure 30** and fully detailed in the online database: https://tinyurl.com/VENNYDatabaseNeuro-OralCard (retrieved July 18, 2020; *in* Annex K: External Support).

VENNY 2.1 software was used for that purpose, as a support tool for constructing Venn diagrams with separate lists of data. In this database, the neuronal proteins identified are cross-referenced with the salivary proteome in OralCard (Rosa et al., 2012; Arrais, 2013). The 2 proteomes compared comprehend 2416 unique neuronal proteins and 3030 unique salivary proteins.

From those:

1651 elements are included exclusively in the Neuronal Proteome;

Two thousand two hundred and sixty-five elements are included exclusively in the Salivary Proteome;

Seven hundred and sixty-five common elements are included both in Neuronal & Salivary.

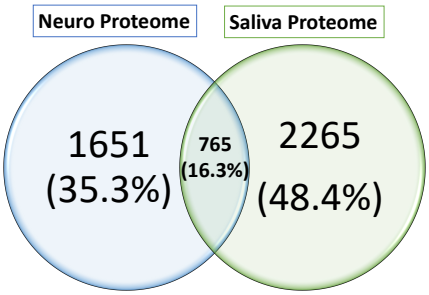


Figure 30. Clustering Analysis
A Venn diagram is used to describe the composition of the neuronal and salivary proteomes. The neuronal proteome (exclusively) is composed by 1651 (35.3%) proteins, the salivary proteome (exclusively) is composed by 2265 (48.4%) proteins and the neuronal-salivary proteome (exclusively) is composed by 765 (16.3%) proteins.

Therefore, of the total neuronal proteins identified (2416), 765 (1/3) are identified in samples of total saliva. Likewise, of the total salivary proteins identified (3030), 765 (1/4) are neuronal produced proteins. Compared to blood, plasma and cerebrospinal fluid proteomics (Loo, Yan, Ramachandran and Wong, 2010; Wormwood et al., 2015; Castagnola et al., 2017), saliva offers sufficient diagnostic consistency. Hence, this alternative type of sample, when probing into the nervous system, may be a significant and relevant option.

This analysis makes it possible to verify the existence of an increasingly relevant number of proteins identified in salivary samples from neuronal origin. Also important is that the total number of the salivary proteins identified also depends on the interest shown by investigators, and salivary proteomics is only an emerging field. Furthermore, the sampling from total saliva is relatively easy compared to neuronal samples, eventually justifying the recent increase of the number of proteins identified in the studies analyzed (Lamster and Ahlo, 2007; Nunes et al., 2015).

Henceforth, the method of collecting saliva, requiring less time and less technical skills from the professional/clinician/researcher, is interesting. Thus, the identification of the salivary proteomes identified in neuronal conditions, through the analysis of salivary samples, seems to reinforce the hypothesis that saliva may be used as diagnostic fluid for neuropsychiatric diseases.

However, it is important to standardize saliva collection methods, so that they are used as routine analysis requirements in clinical settings (Castagnola et al., 2011).

4.B.2. PANTHER Functional Classification

How different is the neuroproteome expression from the whole Homo sapiens proteome?
What are the biological processes overexpressed in the neuronal proteome?
What are the most significant and overexpressed pathways in the brain?
What are the most relevant and overexpressed molecular functions in the neuronal proteome?
What is the significance of the expressed neuroimmune proteins in the brain?
How many protein classes are present in the neuroproteome and how many genes code them?

The Neuro.OralCard identified 2416 unique neuronal produced proteins as shown in **Figure 31** (UniprotKB Entries). A PANTHER functional classification was then executed on those identified proteins. The functional clustering allowed the cataloging of the proteins according to the "Biological Process", "Molecular Functions", "Cellular Components" and "Signaling Pathways" ontology, using the PANTHER tool (Mi et al., 2005; Mi et al., 2006; Mi et al., 2009).

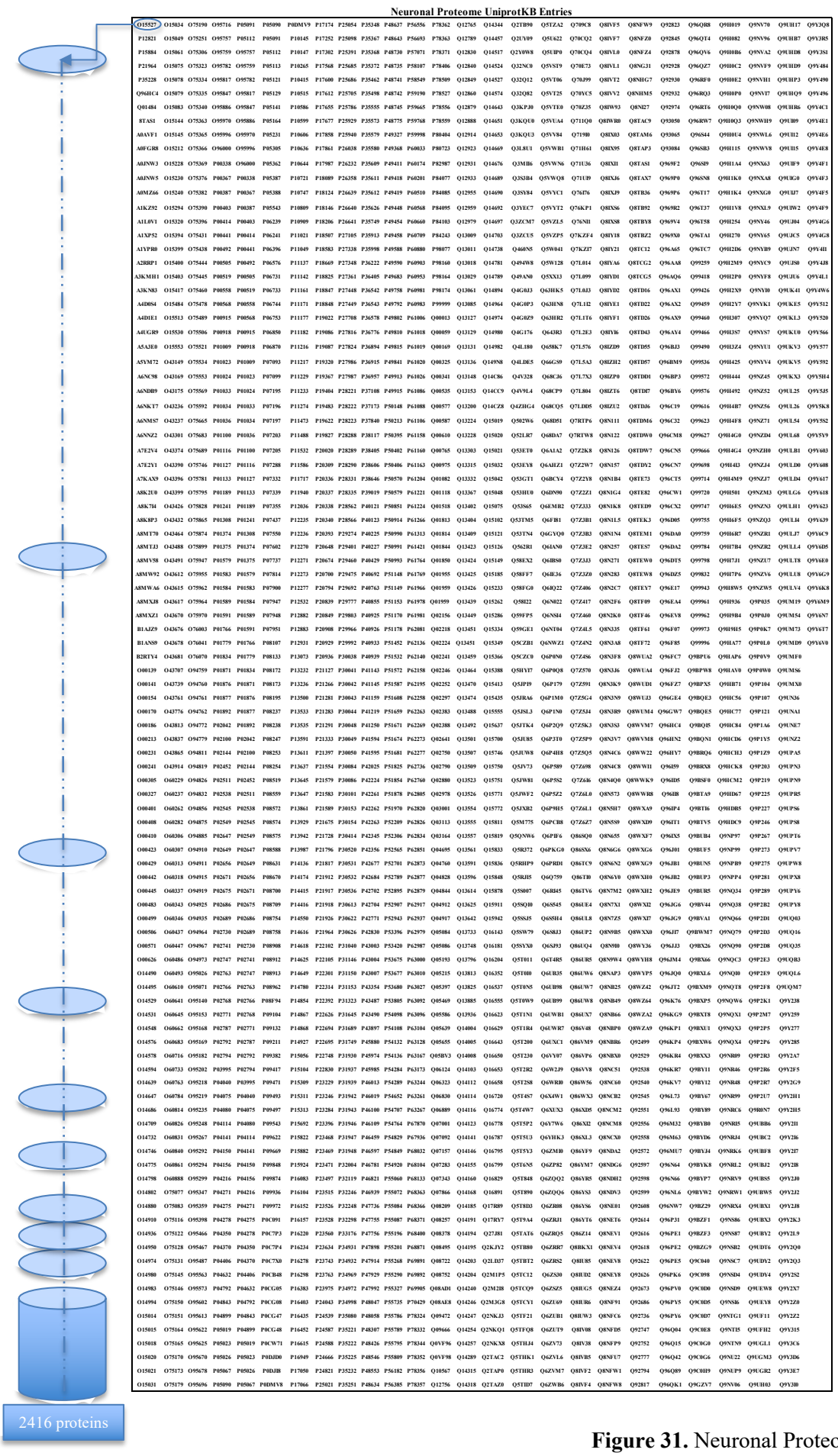


Figure 31. Neuronal Proteome UniprotKB Entries
The identified 2416 unique neuronal produced proteins are displayed by UniprotKB code (e.g.,Q15031).

PANTHER overrepresentation test (released 05.12.2017) analysis type was executed with annotation version 13.1. The neuronal proteome was analyzed against the reference *Homo sapiens* proteome (with a N=21042). Different plots were acquired (x: annotation data set; y: log of fractional difference [neuronal proteome vs. *Homo sapiens* full proteome]).

The proteins which are significantly increased (p-value of <0.05) are considered the most important by the plotted regulation (greater number of proteins), when compared to the human proteome. In a first analysis, the x variable was set to GO (Gene Ontology) - Slim Biological Process. In this analysis (with a p-value set to <0.05), it is shown which biological processes are overexpressed or underexpressed in the neuronal proteome vs. the full human proteome. As shown in **Figure 32**, there is an underexpression (GO) of proteins related to: gamete generation; RNA metabolic processes; reproduction and transmembrane receptor protein kinase activity.

Contrastingly, there is an overexpression (GO) of: anatomical structure development; asymmetric protein localization; behavior; cellular glucose homeostasis; endocytosis; exocytosis; gluconeogenesis; glycolysis; nervous system development; neurological system processes; neuron-neuron synaptic transmission; neurotransmitter secretion; oxidative phosphorylation; protein folding; receptor-mediated endocytosis; response to endogenous stimulus; sensory perception of sound; synaptic transmission; synaptic vesicle exocytosis; system process; and tRNA aminoacylation for protein translation.

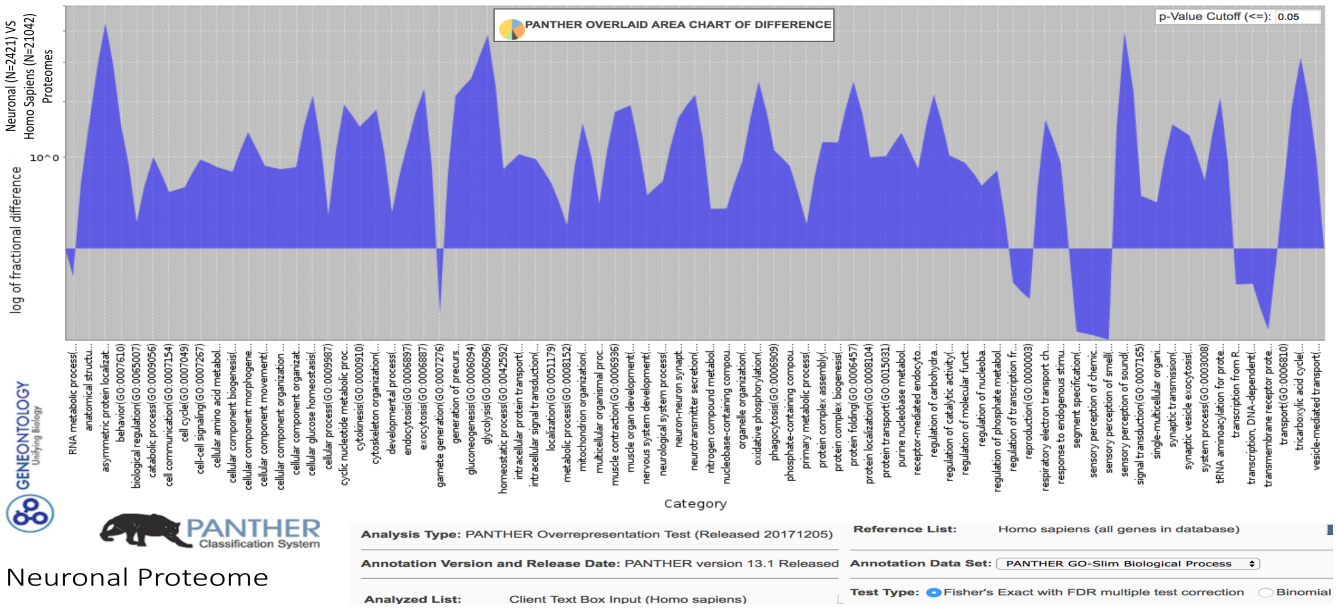


Figure 32. PANTHER Functional Classification: Overrepresentation Single
The proteins significantly increased (p-value of <0.05) are plotted by regulation (greater number of proteins), when compared to the human proteome. X variable was set to GO (Gene Ontology) - Slim Biological Process. The biological processes shown are overexpressed or underexpressed in the neuronal proteome.

In a second analysis (**Figure 33**), the x variable was set to multiple GO (Gene Ontology): (a) PANTHER pathways (graph on the top left); (b) PANTHER Slim cellular components (graph on the top right); (c) PANTHER Slim molecular functions (graph on the bottom left); and (d) PANTHER protein class (graph on the bottom right).

In (a), the most significant and relevant overexpressed pathways are: 5HT1, 5HT2, 5HT4 type receptor mediated signaling pathways; adrenaline and noradrenaline

biosynthesis; axon guidance mediated by semaphorins; Beta1, Beta2, Beta3 adrenergic receptor signaling pathways; corticotropin-releasing factor; cytoskeletal regulation by Rho GTPase; D1/D5 dopamine receptor mediated signaling pathway; endogenous cannabinoid signaling; enkephalin release; GABA-B receptor II signaling; glycolysis; heterotrimeric G-protein signaling pathway; Histamine H1 and H2 receptor mediated signaling pathways; inflammation mediated by chemokine and cytokine signaling pathway; ionotropic glutamate receptor pathway; metabotropic glutamate receptor group I, II and III pathways; muscarinic acetylcholine receptor 1 and 3 signaling pathways; nicotinic acetylcholine receptor signaling pathway; opioid prodynorphin pathway; opioid pro-enkephalin pathway; opioid proopiomelanocortin pathway; oxytocin receptor mediated signaling pathway; Parkinson disease pathway; pentose phosphate pathway; and pyrimidine metabolism.

In (b), the most significant and relevant overexpressed proteins are constitutive of the following cellular components: dendrite; endosome; intracellular; microtubule; mitochondrion; neuromuscular; neuro-projection; plastid; postsynaptic membrane and synapse.

In (c), the most significant and relevant overexpressed molecular functions are: ATPase activity; heterotrimeric G-protein signaling pathway; intermediate filament binding; microtubule binding; microtubule motor activity; motor activity; proton-transporting ATP synthase complex and structure molecule activity.

In (d), the most significant and relevant overexpressed protein classes are: ATP synthase; G protein; aminoacyl-tRNA synthetase; anion channel; calcium channel; chaperone; chaperonin; cytokine; ion channel; kinase; membrane trafficking regulatory protein; transporter; tubulin; and voltage-gated calcium channel and sodium channels; whereas the underexpressed protein classes are: C2H2 zinc finger transcription factor; KRAB box transcription factor.

This analysis allowed to catalog different GO of the full range neuronal proteins identified in the Neuro.OralCard. In total, the analysis shows that the neuronal proteins are highly involved, with a p-value of <0.05 when compared to the full human proteome, in: 79 specific biological processes, 60 precise pathways, 32 particular cellular components, and 41 distinctive molecular functions. The full proteome is integrated into 55 main protein classes. Those GOs are therefore more characteristic of the nervous system.

To summarize, **Figure 34** and **Figure 35** present a distribution of the expressed genes (with a p-value cutoff <0.05) of the described GO. The neuronal proteome is maintained by 2317 genes. 1819 genes assure 117 categorically different neuronal pathways. For instance, the inflammation mediated by chemokine and cytokine signaling pathway (P00031) is widely expressed and has 76 gene hits. Contrastingly, the 2-arachidonoylglycerol biosynthesis (P05726) is not so extensively expressed and has solely 2 gene hits. Likewise, the most significant neuronal molecular functions are expressed by 1926 genes and comprehend 9 unique categories. Binding (GO:0005488) and catalytic activity (GO:0003824) are the most expressed functions with 750 and 680 gene hits, respectively.

The neuronal cellular components are maintained by 2268 genes and organized in 8 main categories. Exclusive synaptic (GO:0045202) and membrane (GO:0016020) components comprehend 50 and 350 gene hits, correspondingly.

Four thousand one hundred and thirty-one genes ensure 14 main and separate biological processes. Metabolic (GO:0008152) and immune system (GO:0002376) processes have a widespread expression, but one over 780 and the other 80 gene hits, respectively.

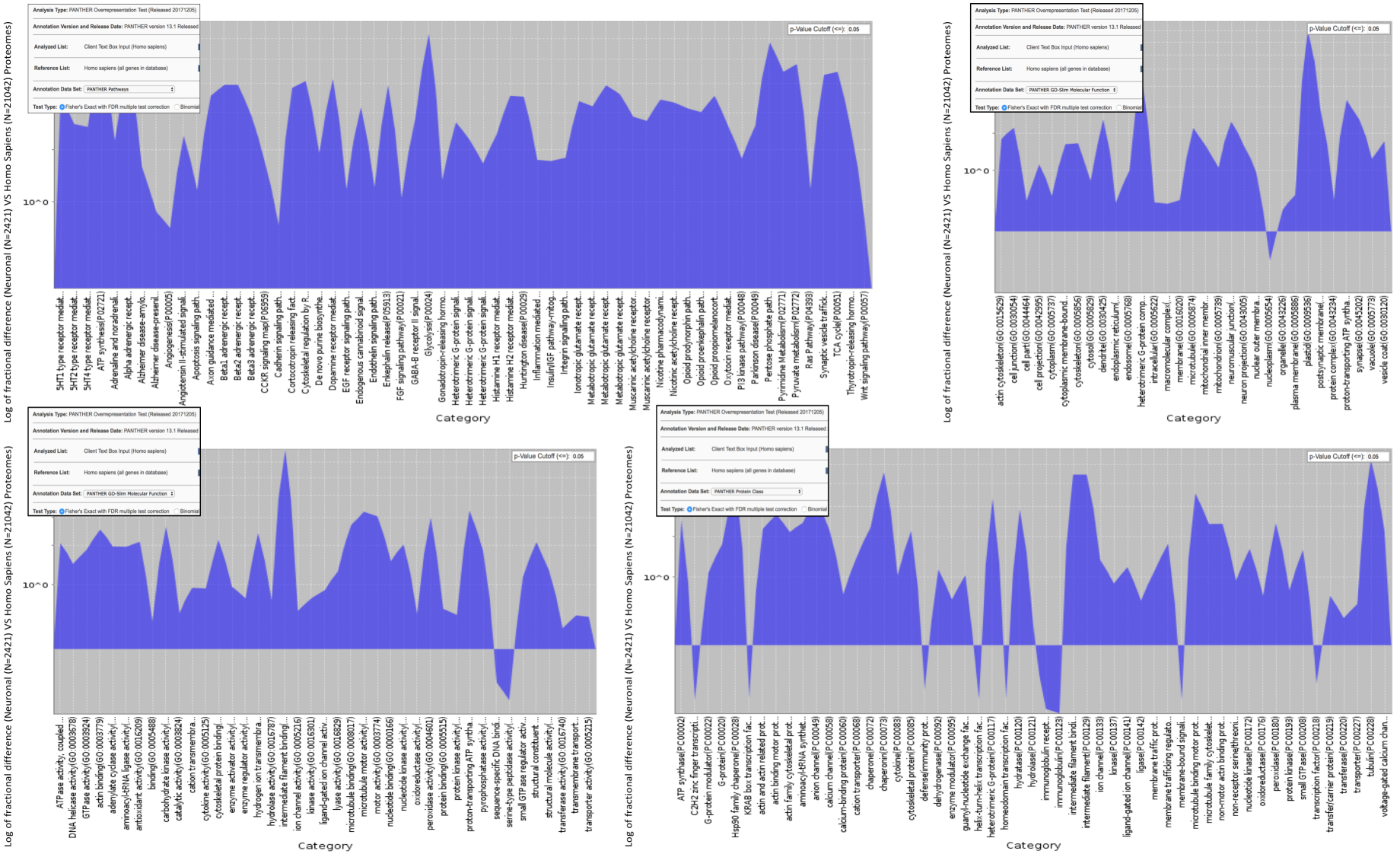


Figure 33. PANTHER Functional Classification: Overrepresentation Multiple The proteins significantly increased (p-value of <0.05) are plotted by regulation (greater number of proteins), when compared to the human proteome. X variable was set to multiple GO (Gene Ontology): (a) PANTHER pathways (graph on the top left); (b) PANTHER Slim cellular components (graph on the top right); (c) PANTHER Slim molecular functions (graph on the bottom left); and (d) PANTHER protein class (graph on the bottom right). The biological processes shown are overexpressed or underexpressed in the neuroproteome.

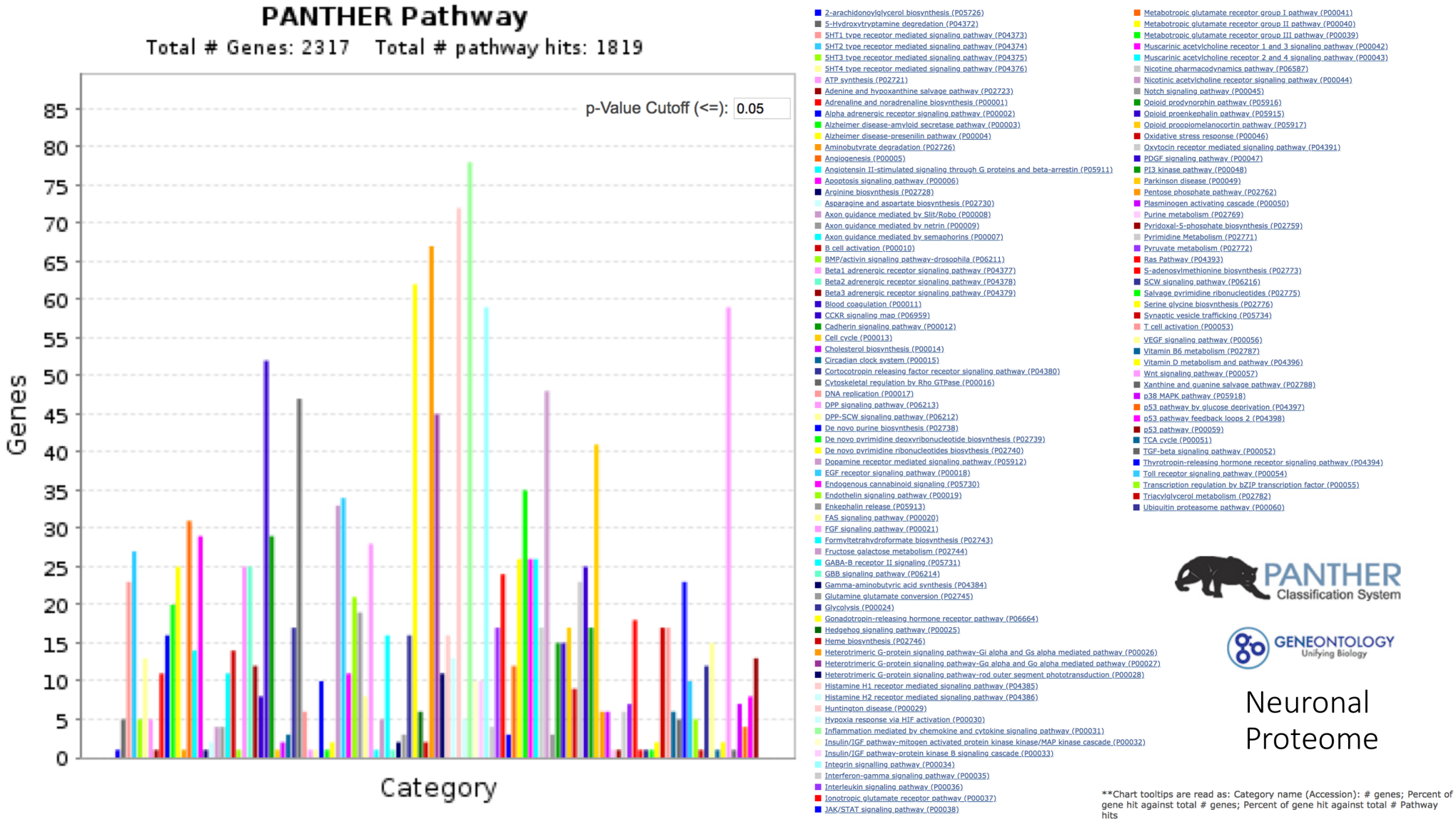


Figure 34. PANTHER Functional Classification: Gene Ontology Single

The neuronal proteins are implicated, with a p-value of <0,05, when compared to the full human proteome in 117 distinctive neuronal pathways. Those GOs are more characteristic of the nervous system. This plot summarizes the distribution of the expressed genes (with a p-value cutoff <0.05) of the specific GO.

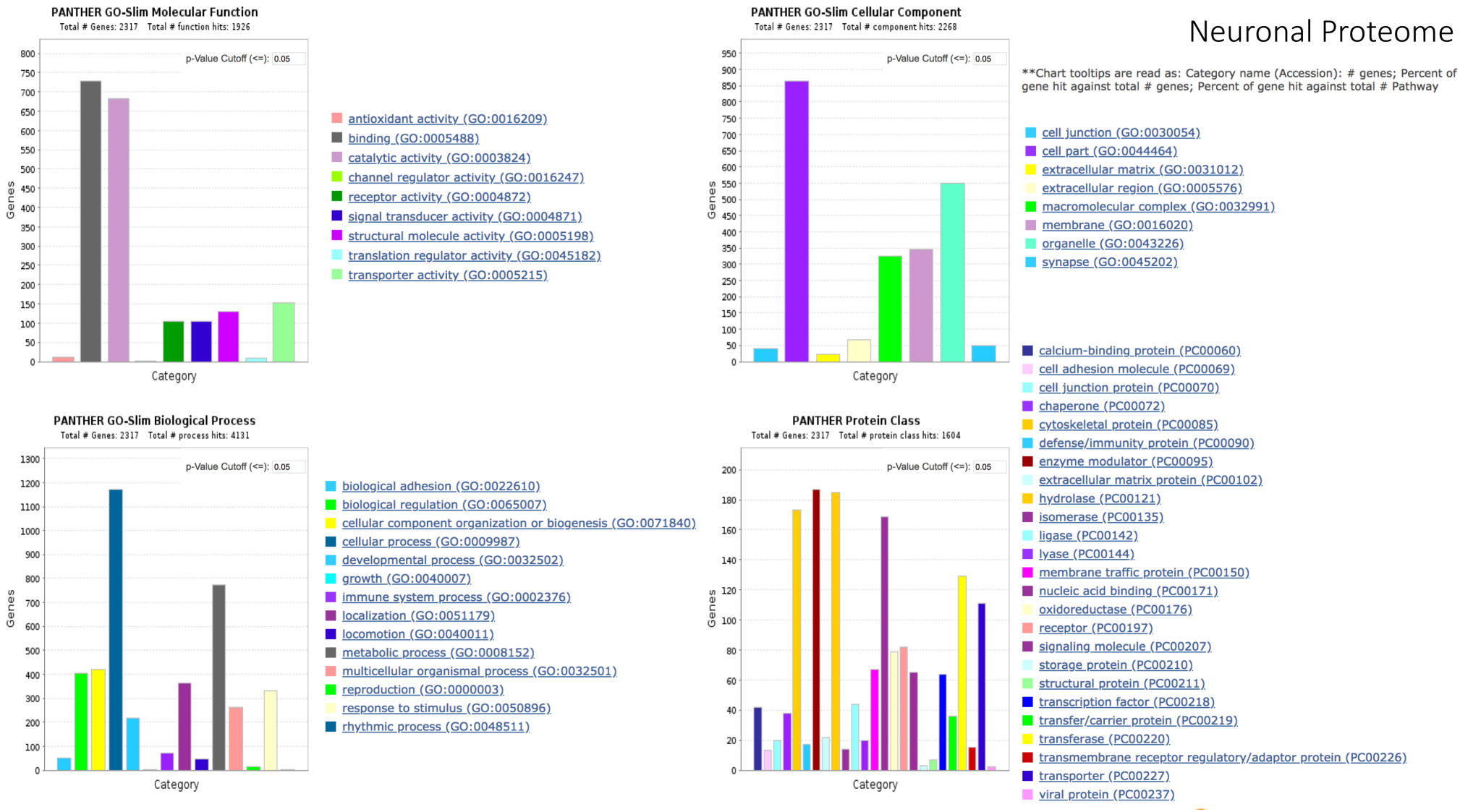


Figure 35. PANTHER Functional Classification: Gene Ontology Multiple

The neuronal proteins are implicated, with a p-value of <0,05, when compared to the full human proteome in (a) 9 distinctive molecular functions (graph on the top left), in (b) 8 particular cellular components (graph on the top right), in (c) 14 specific biological processes (graph on the bottom left), and in (d) 25 precise protein classes (graph on the bottom right). Those GOs are more characteristic of the nervous system. This plot summarizes the distribution of the expressed genes (with a p-value cutoff <0.05) of the specific GO's.

Lastly, 25 main protein classes are kept by 1604 genes. Enzyme modulators (PC00095) and signaling molecules (PC00207) are principal classes with over 190 and 70 gene hits, distinctly.

4.B.3. Cytoscape Functional Clustering

How is it composed the neuroproteome network of neurotypical subjects?
What immune system processes are clustered in neocortical proteome network?
Is it possible to identify the neuropeptide network present in the different brain structures?
Is it possible to characterize the neuroproteome network present in saliva?
Is it possible to extract a network obtained from a set of neuronal proteins quantified in saliva?

A first identification of the neuronal proteins, present in different and specific conditions and structures, was an essential element for the evaluation of potential functional biomarkers. This step was acquired in the previous functional clustering.

However, it is imperative to understand (i) how these molecules interact with each other, (ii) in which molecular networks they participate, and (iii) which individual and network functions they acquire. This type of molecular biology analysis is referenced as a functional network analysis and Cytoscape have been a good bioinformatics tool to make this enrichment (Bindea et al., 2009).

All the variables used in Neuro.OralCard and described in **Figure 27**, **Figure 28** and **Figure 29** were exported to a Cytoscape database. Moreover, all the UniProtKB was fed into each array. Then, the different Cytoscape algorithms, described in Methods, made the clustering of proteins and functional networks, for each of the arrays. The Cytoscape database with the neuronal proteome, via UniProtKB coding, is accessible online in: https://tinyurl.com/CytoscapeDBNeuroOralCard (retrieved July 19, 2020; *in* Annex K: External Support).

Afterwards, this database was used to perform the Cytoscape functional clustering. The functional clustering was made according to the guidelines described in Methods. The ontologies described have all been used to extract the 28 variables/arrays in our Cytoscape database. Those 28 variables/arrays originated 28 molecular maps.

A molecular map is composed of a heading and GO-term analysis parameters (on the top) (**Figure 36**) and the respective functional network (on the bottom) (**Figure 37**).

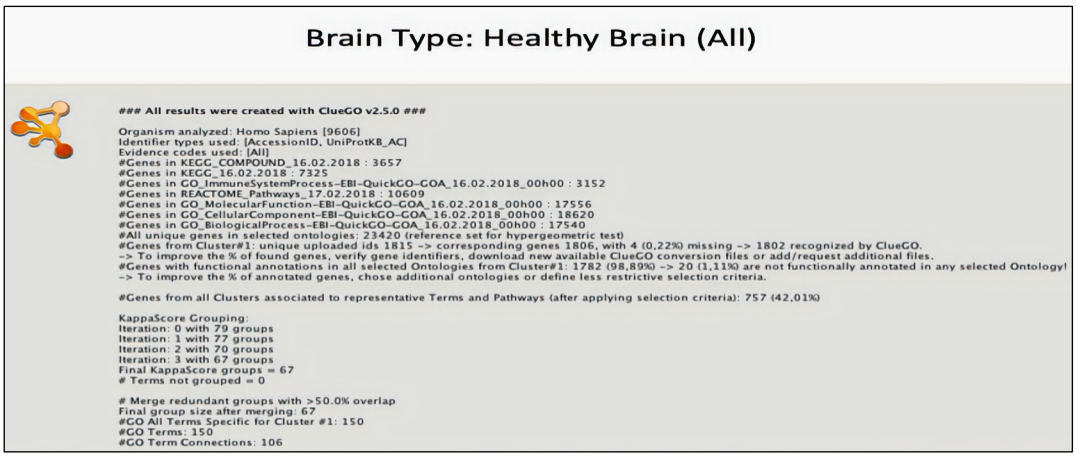


Figure 36. Cytoscape Functional Clustering: Heading And Extraction Parameters
Molecular map composed of a heading (Brain Type: Healthy Brain (All)) and GO-term analysis parameters. The analysis offered a minimum kappa score of 67. The final functional clustering offered 150 unique GO terms (or functions), establishing a network of 106 molecular function connections.

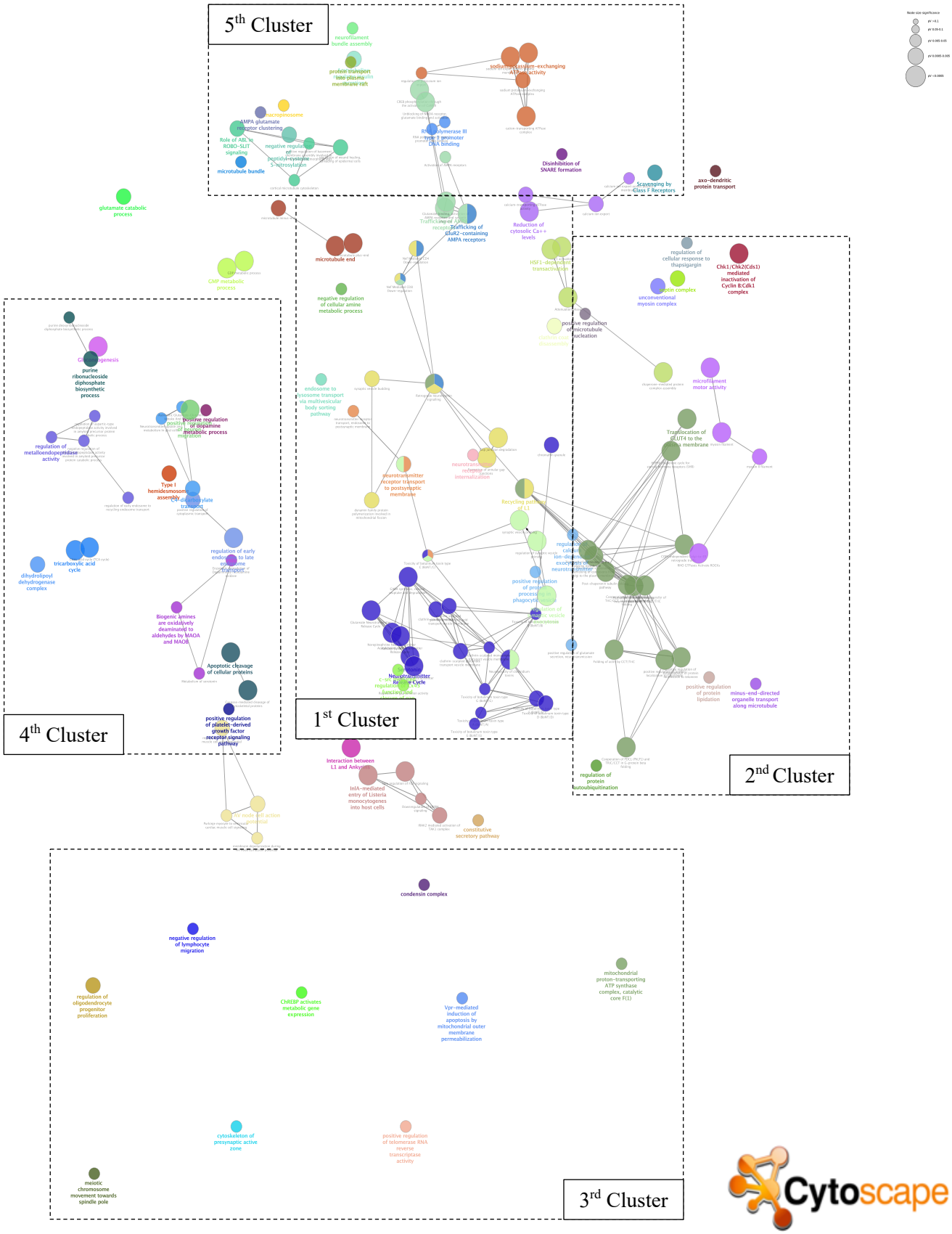


Figure 37. Cytoscape Functional Clustering: Functional Network Healthy Brain Molecular map showing the functional network of the neurotypical brain of healthy individuals. The different molecular functions that are overexpressed in the healthy brain are shown, when compared to the Homo sapiens proteome. The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

In the example of the molecular network map, in **Figure 36** and **Figure 37**, a functional network of all the samples corresponding to a brain without any pathological condition was plotted; hence a neurotypical brain of healthy individuals.

In **Figure 36**, it is shown that the functional clustering was made for *Homo sapiens* [9606], with ClueGO v. 2.5.0.

The type of identifier used was UniProtKB_AC, which was mapped with KEGG (7325 KEGG genes and 3657 COMPOUND genes), GO (3152 Immune System Process genes), REACTOME (10,609 Pathway genes), GO (17,556 Molecular Function genes), GO (18,620 Cellular Component genes), GO (17,540 Biological Process genes). From those GOs, the unique genes found in all ontologies were 23,420.

Those unique genes, with the specific and restricted guidelines defined for the Cytoscape functional clustering in the Methods section, found 757 functionality clusters (42%). Those clusters associated the terms and the pathways of the correlation between UniProtKB of the Healthy Brain proteome and the ontology databases. This correlation is given by the *kappa* score.

As shown, the analysis offered a minimum *kappa* score of 67 and all terms were grouped. Then, all redundant groups with >50% iteration overlap were merged. The final functional clustering offered 150 unique GO terms (or functions), establishing a network of 106 molecular function connections.

In **Figure 37**, the functional network of the healthy brain proteome is therefore shown. All the proteins of all the neurotypical samples were plotted, showing the different molecular functions that are overexpressed in the healthy brain, when compared to the *Homo sapiens* proteome. Therefore, a visual exploration of data is presented for the brain functions that are overexpressed vs. the complete set of human biomolecular functions (annotated in the *Homo sapiens* proteome, via UniprotKB coding).

The biological processes and molecular functions were organized in pathways divided by colors. An interception circle, or a node, is a function that is shared by different biological processes. Therefore, a single function can be involved in several biological processes and one biological process can affect numerous molecular functions.

The size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

Hence, in **Figure 37**, a main molecular network (in the center, in blue, yellow and light green) of the healthy brain was maintained by adequate correlation, suitable connection and fine association of the principal functions, which modulate the serotonin neurotransmitter release cycle, the c-src mediated regulation of Cx43 function and closure of gap junctions, the regulation of synaptic vesicle exocytosis, the positive regulation of protein processing in phagocytic vesicle, the regulation of calcium ion-dependent exocytosis of neurotransmitters, the neurotransmitter receptor internalization, the neurotransmitter receptor transport to postsynaptic membrane, the trafficking of GluR2-containing AMPA receptor, the trafficking of AMPA receptors, the RNA polymerase III type 1 promoter DNA binding and the recycling of L1.

This 1st cluster, in **Figure 37 (Amplified A)**, characterized almost by synaptic functions, was also maintained by isolated functions associated with it, but without a specific term connection. Those are: sodium-potassium-exchanging ATPase activity, disinhibition of SNARE formation, interaction between L1 and ankyrins, and endosome to lysosome transport via multivesicular body sorting pathway.

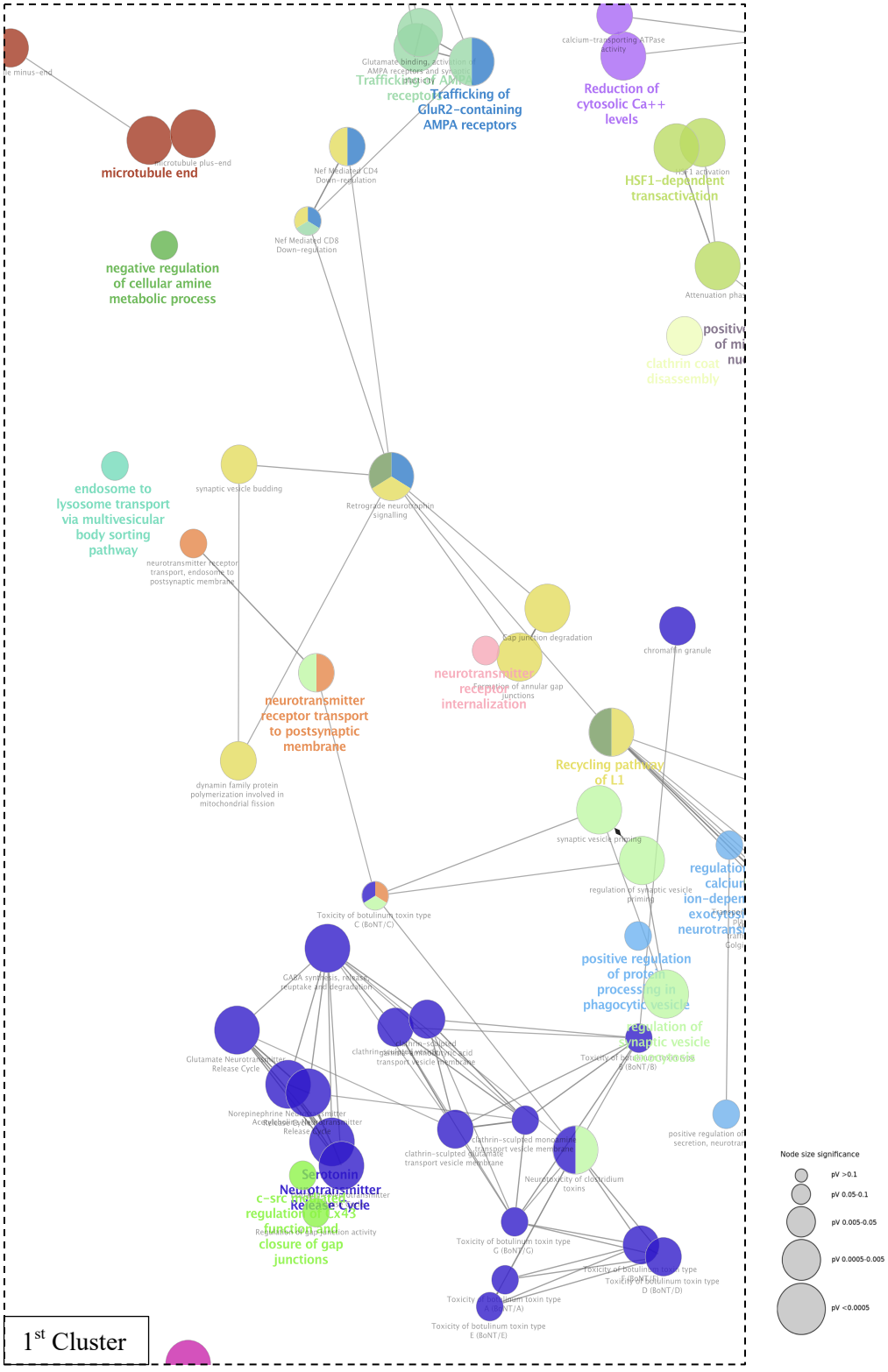


Figure 37 (Amplified A). Cytoscape Functional Clustering: Functional Network Healthy Brain, 1st Cluster
Figure 37 is amplified by clusters. Only the 1st cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

A 2nd cluster (on the right, in dark green, purple and orange), in **Figure 37 (Amplified B)**, characterized by intracellular neuron components and structures, was

visualized and coupled with the recycling of L1 of the 1st cluster. The cluster is composed of the functions: translocation of GLUT4 to the plasma membrane, positive regulation of microtubule nucleation, microfilament motor activity, HSF1-dependent transactivation, axo-dendritic protein transport, scavenging by Class F receptors, reduction of cytosolic Ca++ levels, unconventional myosin complex, septin complex, regulation of cellular response to thapsigargin, Chk1/Chk2(Cds1) mediated inactivation of Cyclin B: Cdk1 complex, minus-end-directed organelle transport along microtubule, positive regulation of protein lipidation, and regulation of protein autoubiquitination.



Figure 37 (Amplified B). Cytoscape Functional Clustering: Functional Network Healthy Brain, 2nd Cluster
Figure 37 is amplified by clusters. Only the 2nd cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

A 3rd cluster (on the bottom), in **Figure 37 (Amplified C)**, characterized by cell proliferation and differentiation, was formed by the isolated functions of: Vpr-mediated induction of apoptosis by mitochondrial outer membrane permeabilization, condensin complex, ChREBP activated metabolic gene expression, negative regulation of lymphocyte migration, regulation of oligodendrocyte progenitor proliferation, cytoskeleton of presynaptic active zone, meiotic chromosome movement towards spindle pole, positive regulation of telomerase RNA reverse transcriptase activity, and mitochondrial proton-transporting ATP synthase complex, and catalytic core F(1).



Figure 37 (Amplified C). Cytoscape Functional Clustering: Functional Network Healthy Brain, 3rd Cluster
Figure 37 is amplified by clusters. Only the 3rd cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

A 4th isolated cluster (on the left, in darker green, light blue and dark purple), in **Figure 37 (Amplified D)**, characterized by cell metabolism, was maintained by: AV node cell action potential, positive regulation of platelet-derived growth factor receptor signaling pathway, apoptotic cleavage of cellular proteins, biogenic amines which are oxidatively deaminated to aldehydes by MAOA and MAOB, regulation of early endosome to late endosome transport, C4-dicarboxilase transport, type 1 hemidesmosome assembly, positive regulation of dopamine metabolic process, positive regulation of neuron migration, tricarboxylic acid cycle, dihydrolipoyl dehydrogenase, regulation of metalloendopeptidase activity, purine ribonucleoside diphosphate biosynthetic process, and gluconeogenesis.

A 5th isolated cluster (on the top left, in lighter green, green, and magenta), in **Figure 37 (Amplified E)**, characterized by protein metabolism, was sustained by: the glutamate catabolic process, microtubule bundle, the role of ABL in ROBO-SLIT signaling, AMPA glutamate receptor clustering, macropinosome, negative regulation of

peptidyl-cysteine S-nitrosylation, microtubule end, GMP metabolic processes, negative regulation of cellular amine metabolic processes, neurofilament bundle assembly, protein transport into plasma membrane raft, and acetylcholine which regulates insulin secretion.

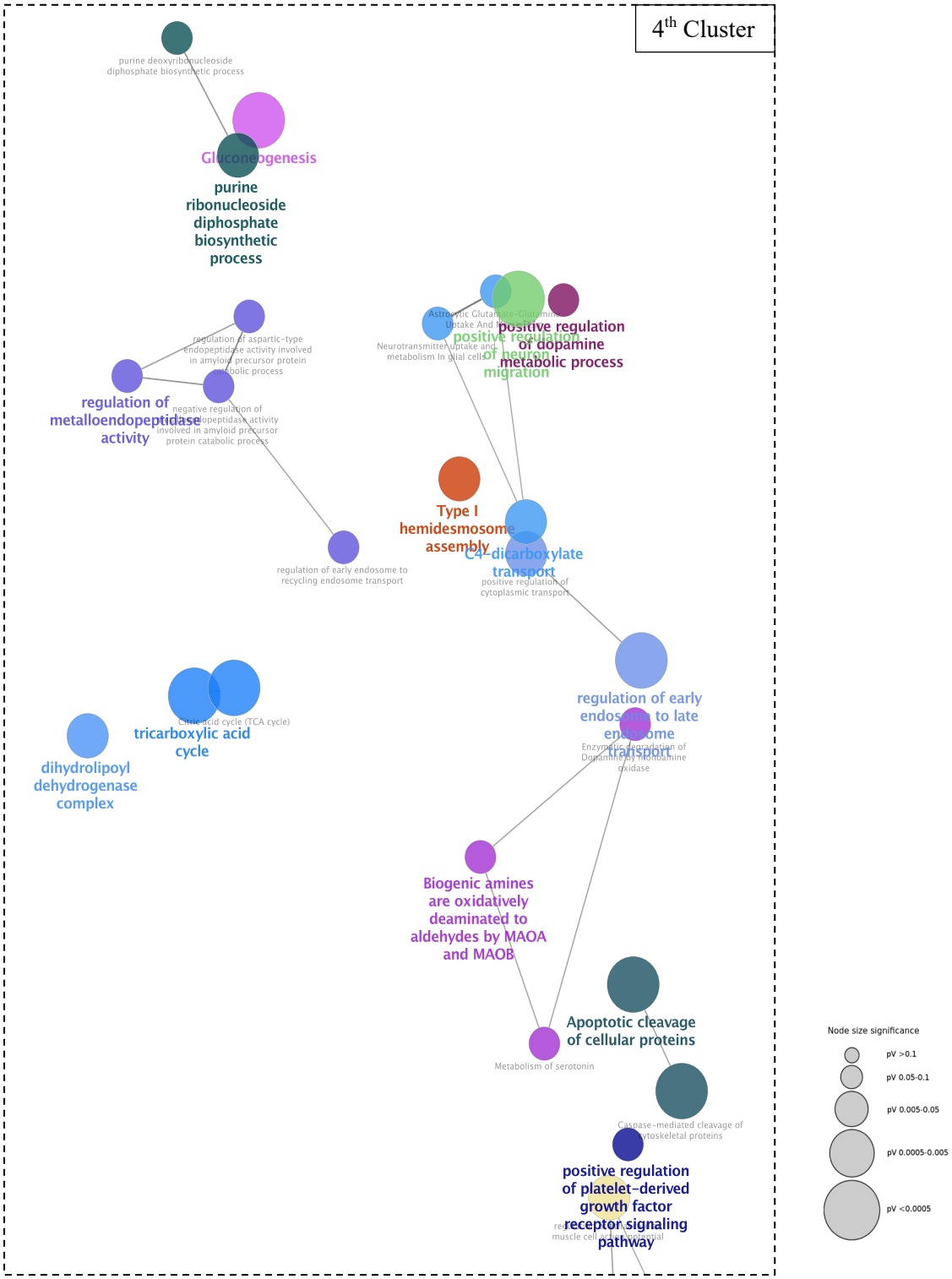


Figure 37 (Amplified D). Cytoscape Functional Clustering: Functional Network Healthy Brain, 4th Cluster
Figure 37 is amplified by clusters. Only the 4th cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).



Figure 37 (Amplified E). Cytoscape Functional Clustering: Functional Network Healthy Brain, 5th Cluster
Figure 37 is amplified by clusters. Only the 5th cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

The same analysis can be made about another molecular network. **Figure 38** and **Figure 38 (Amplified)** is another example, showing a map of all the proteins detected in all neocortical samples of all subjects. This map plots the biological processes that are overexpressed in this brain tissue and structure. Proteins associated both with a neurotypical/non-pathological development and neuropsychiatric conditions are gathered. The objective of this analysis was only to understand what the molecular functions that are most likely to be specific of neocortical samples are.

As described previously in **Figure 36**, a similar functional clustering was made for *Homo sapiens* [9606], with ClueGO v. 2.5.0. The same type of identifier was used and mapped with the same GOs. In this neocortical functional clustering, 377 functionality clusters (54.96%) were found. Those clusters associated the terms and pathways of the correlation between UniProtKB of the Tissue and Structure: Neocortex proteome (**Figure 27**, **Figure 28** and **Figure 29**) and the ontology databases.

As shown, the analysis offered a minimum *kappa* score of 39 and all terms were grouped. The final functional clustering offered 87 unique GO terms (or functions) establishing a network of 74 molecular functional connections.

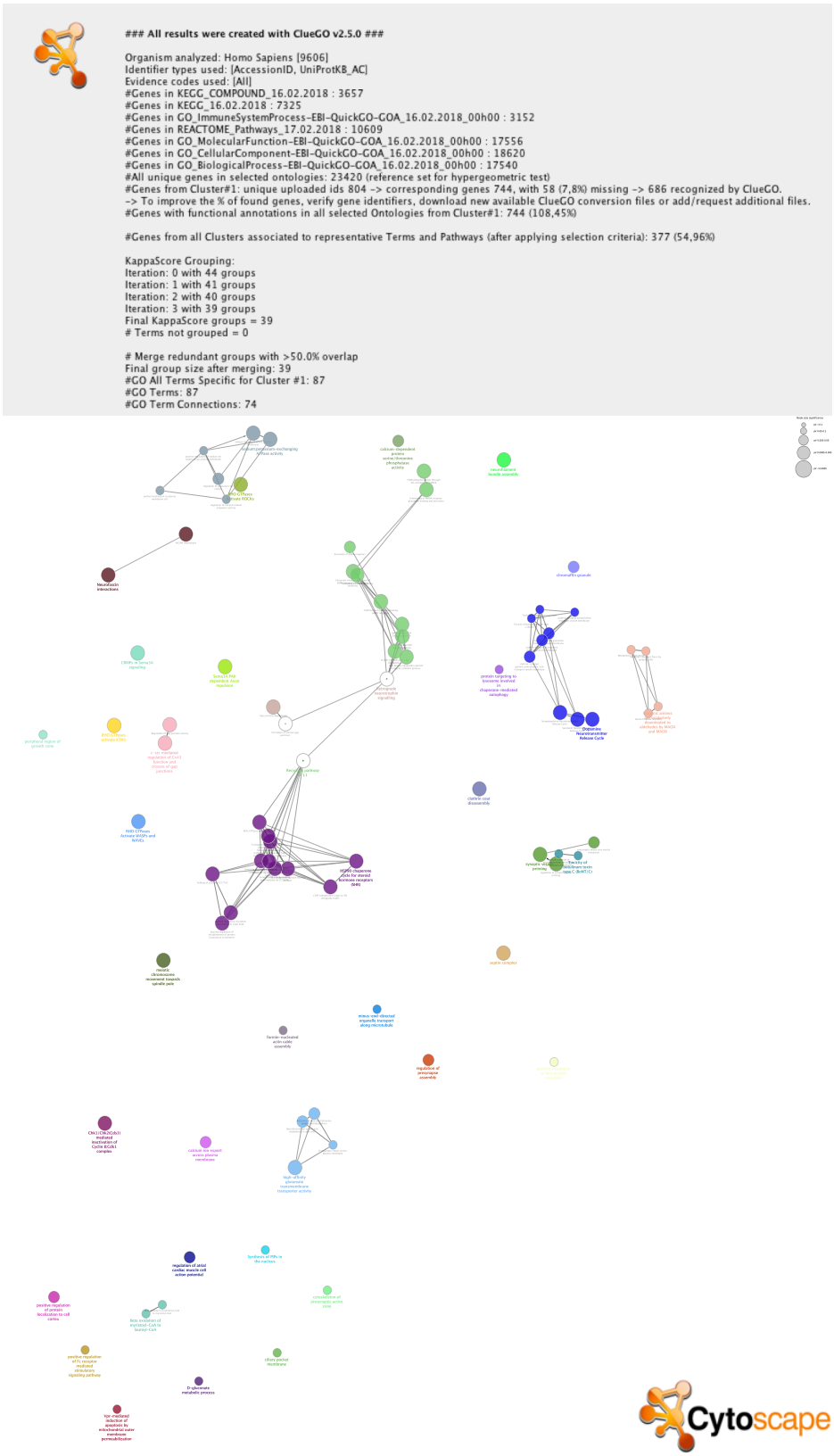


Figure 38. Cytoscape Functional Clustering: Functional Network Neocortex
Molecular map showing the functional network of the proteins detected in all neocortical samples of all subjects. 377 functionality clusters (54.96%) were found with a minimum kappa score of 39. The final functional clustering offered 87 unique GO terms (or functions) establishing a network of 74 molecular functional connections.

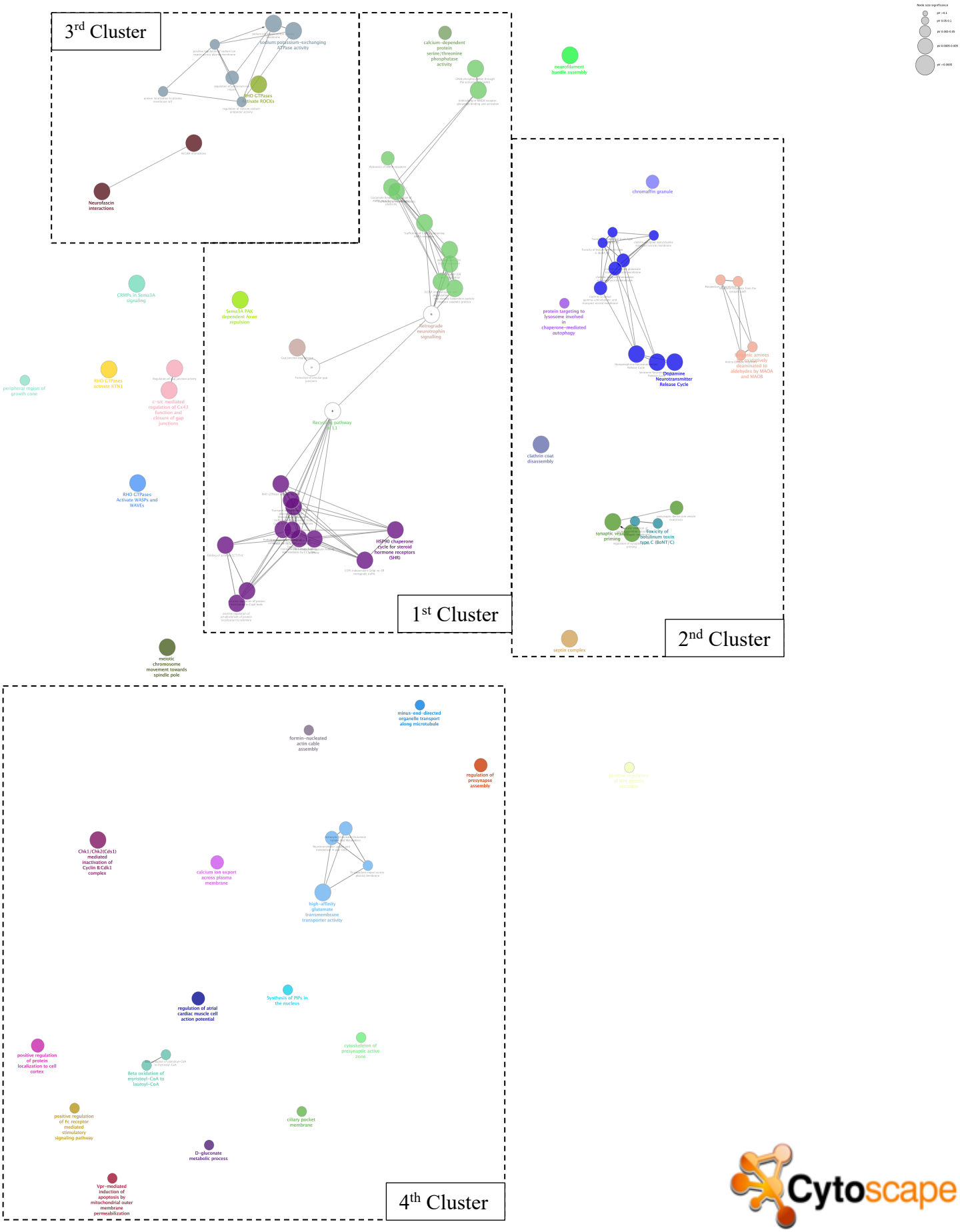


Figure 38 (Amplified). Cytoscape Functional Clustering: Functional Network Neocortex Amplified Total

Hence, in **Figure 38**, a main molecular network (in the center, in purple and light green), in **Figure 38 (Amplified A)**, of the neocortex[73] was maintained by adequate correlation, suitable connection and fine association of the principal functions regulating the: recycling pathway of L1, HSP90 chaperone cycle for steroid hormone receptors (SHR), retrograde neurotrophin signaling, calcium-dependent protein serine/threonine phosphatase activity, neurofilament bundle assembly, Sema3A PAK dependent axon repulsion, CRMPs in Sema3A signaling, c-src mediated regulation of Cx43 function and closure of gap junctions, RHO GTPases activate KTN1, RHO GTPases activate WASPs and WAVEs, meiotic chromosome movement towards spindle pole, and the peripheral region of the growth cone.

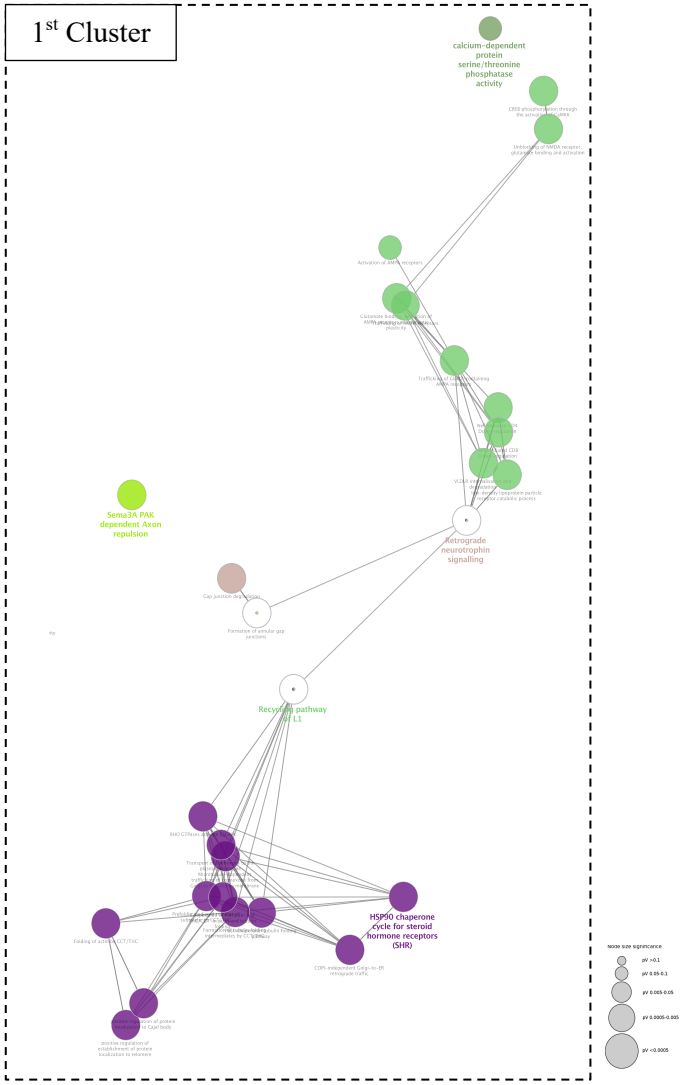


Figure 38 (Amplified A). Cytoscape Functional Clustering: Functional Network Neocortex, 1st Cluster
Figure 38 is amplified by clusters. Only the 1st cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

[73] microdomain-associated proteins in the dorsolateral prefrontal cortex, frontal superior gyrus of Brodmann Area 9, inferior frontal gyrus of Brodmann Area 45 (Behan et al., 2009); presynaptic proteins (SNAREs) and lipid rafts microdomains in the frontal cortex (Salaün, James and Chamberlain, 2004)

A 2nd isolated cluster (on the right, in dark purple, dark green and light orange), in **Figure 38 (Amplified B)**, characterized by intracellular neuron regulation, is composed of the following functions: dopamine neurotransmitter release cycle, chromaffin granule, protein targeting to lysosome involved in chaperone-mediated autophagy, clathrin coat disassembly, synaptic vesicle priming, septin complex, and biogenic amines oxidatively deaminated to aldehydes by MAOA and MAOB.

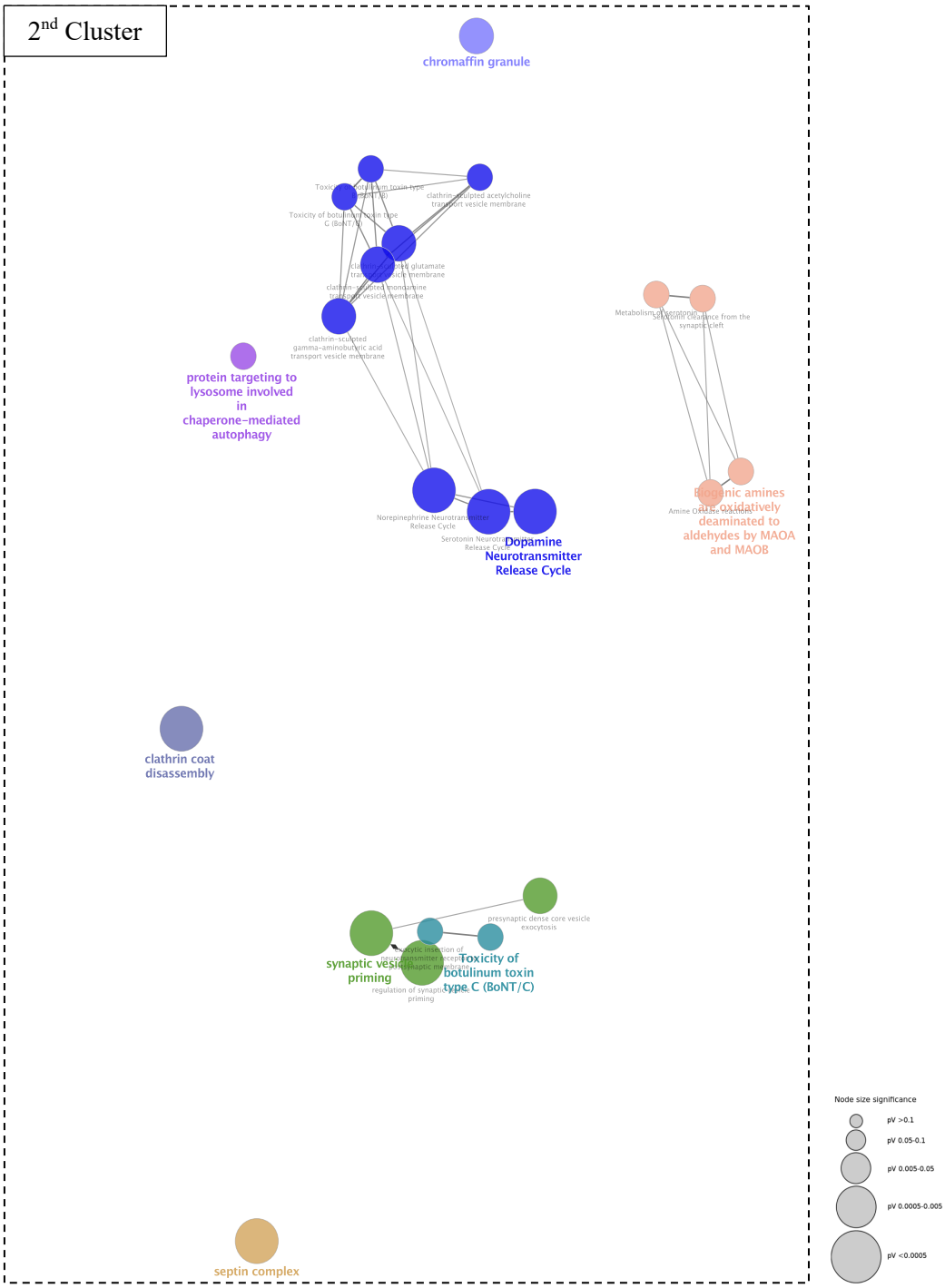


Figure 38 (Amplified B). Cytoscape Functional Clustering: Functional Network Neocortex, 2nd Cluster
Figure 38 is amplified by clusters. Only the 2nd cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

A 3rd isolated cluster (on the top, in gray, magenta and dark green), in **Figure 38 (Amplified C)**, characterized by neuron metabolism, is maintained by: sodium-potassium-exchanging ATPase activity, RHO GTPases activate ROCKs and neurofascin interactions.



Figure 38 (Amplified C). Cytoscape Functional Clustering: Functional Network Neocortex, 3rd Cluster
Figure 38 is amplified by clusters. Only the 3rd cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

A 4th cluster (on the bottom), in **Figure 38 (Amplified D)**, characterized by membrane signaling and transmembrane transport, was formed by the isolated functions of: Chk1/Chk2(Cds1) mediated inactivation of Cyclin B- Cdk1 complex, calcium ion export across plasma membrane, high-affinity glutamate transmembrane transporter activity, positive regulation of protein location to the cell cortex, positive regulation of Fc receptor mediated stimulatory signaling pathway, beta oxidation of myristoyl-CoA to lauroyl-CoA, Vpr-mediated induction of apoptosis by mitochondrial outer membrane permeabilization, D-gluconate metabolic processes, ciliary pocket membrane, regulation of atrial cardiac muscle cells action potential, synthesis of PIPs in the nucleus, cytoskeleton of the presynaptic active zone, formin-nucleated actin cable assembly, minus-end-directed organelle transport along microtubule, regulation of the presynapse assembly, and positive regulation of Wnt protein secretion.

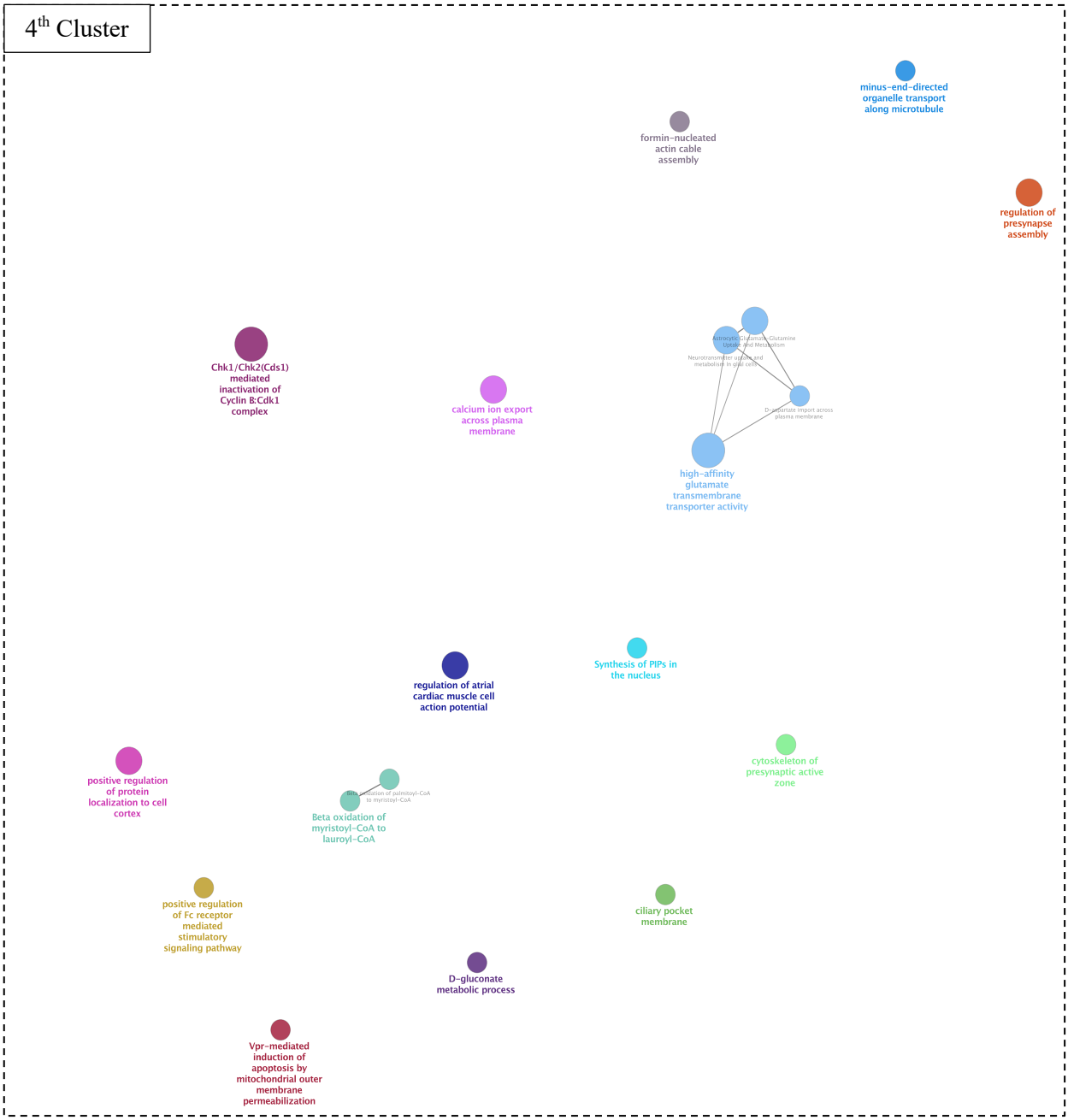


Figure 38 (Amplified D). Cytoscape Functional Clustering: Functional Network Neocortex, 4th Cluster
Figure 38 is amplified by clusters. Only the 4th cluster is presented.
The molecular functions are organized in pathways divided by colors and the size of the circles is related to the significance of the node, i.e., pV<0.0005 (the biggest circle), pV=0,005-0,05, pV=0,05-0,1, pV>0.1 (on the top right).

This Cytoscape functional clustering and analysis may be performed for all the arrays/variables of the Neuro.OralCard. Indeed, all the database was extracted, and 28 molecular networks were obtained.Henceforth, the 28 molecular network maps were compiled in a ".swf" file, Shockwave Flash language, Macromedia platform (Adobe Flash). The ".swf" file assembled the different maps with interactive tools, in order to be used as a user interface atlas. Subsequently, those compiled molecular maps were published online as an open access atlas (Carroll, 2011), in the LIMMIT Lab, Faculty of Medicine, University of Lisbon website. Lastly, a molecular biology atlas of the neuronal proteome, named MolecularCog(G) Atlas, was completed and is accessible in: https://www.limmit.org/molecularcogatlas.html (retrieved August 07, 2020; *in* Annex K: External Support).

The 28 molecular network maps were divided in 4 sections, as following.

Section A is made of: 1. Type of Sample: *In Vitro*; 2. Type of Sample: *In Vivo* (Post-Surgical); 3. Type of Sample: *Post-Mortem*; and 4. Type of Sample: Other.

Section A extracted the molecular functions according to the type of sample. An independent molecular map was given to all proteins obtained separately by *in vitro*, *in vivo* or *post-mortem* brain sampling.

Section B is made of: 5. Tissue and Structure: Blood; 6. Tissue and Structure: Saliva; 7. Tissue and Structure: All Brain; 8. Tissue and Structure: Neocortex; 9. Tissue and Structure: Insular Cortex; 10. Tissue and Structure: Whole Brain; 11. Tissue and Structure: Central Anterior Frontal, Medial Anterior Parietal, Lateral Temporal, Posterior Superior Frontal, Inferior Parietal, Inferior Temporal Gyrus, Anterior Superior Frontal; 12. Tissue and Structure: Dorsolateral Prefrontal; 13. Tissue and Structure: Postsynaptic Density (hPSD); 14. Tissue and Structure: Myelin (Frontal Lobe); and 15. Tissue and Structure: Membrane Proteins (Ontology).

Section B extracted the molecular functions according to the type of tissue or structures from which the sample was collected. An independent molecular map was given to all the neuronal proteins obtained separately in saliva or blood, or in all the brain samples collectively. Moreover, individual maps were given for samples coming from specific brain regions: neocortex[74], insular cortex, etc. Likewise, specific molecular maps were obtained for brain specific components, i.e., postsynaptic density, myelin and membrane proteins.

Section C is made of the: 16. Brain Type: Pathological Brain (All); 17. Brain Type: Pathological Brain (Colloid cyst, Glial neoplasm or Lesional Epilepsy); 18. Brain Type: Healthy Brain (All); 19. Brain Type: Healthy Brain (Cardiopulmonary Arrest, Myocardial Infarct or Mesenteric Bleeding); and 20. Brain Type: Healthy Brain (Receptoromics (Psychoactive Drug)).

Section C extracted the molecular functions according to the type of brain/subject the sample was collected from. An independent molecular map was given to all the neuronal proteins obtained separately in pathological and healthy brains. Moreover, individual maps were given for proteomes sampled from brains with a colloid cyst, glial neoplasm or lesional epilepsy. Likewise, specific molecular maps were obtained from the healthy brains of subjects whose cause of death was cardiopulmonary arrest, myocardial infarct or mesenteric bleeding. Lastly, a molecular map was obtained from receptoromics studies, i.e., receptor omics *in vitro* studies with a psychoactive drug.

Section D is made of: 21. Biomarker: Disease State (All); 22. Biomarker: Disease State (CNS Disease Only); 23. Biomarker: Disease State (PNS Disease Only); 24. Biomarker: Disease State (PNS & CNS Disease); 25. Biomarker: Disease State (Non-NS); 26. Biomarker: Health State Biomarker; and 27. Biomarker: Proposed as Biomarker.

Section D extracted the molecular functions according to the type of biomarker that was attributed to the neuroproteome. This section compiles molecules that were initially proposed as biomarkers of neuropsychiatric diseases or of neurotypical subjects. ICD-10 chapters: 'Diseases of the Nervous System' and "Mental and Behavior Disorders". An independent molecular map was given to all the neuronal proteins thought as biomarkers of central nervous system disease only, peripheral nervous system disease only, central and peripheral nervous system disease, non-nervous system disease, or of all combined neuropsychiatric conditions together. Moreover, an individual map was also

[74] microdomain-associated proteins in the dorsolateral prefrontal cortex, frontal superior gyrus of Brodmann Area 9, inferior frontal gyrus of Brodmann Area 45 (Behan et al., 2009); presynaptic proteins (SNAREs) and lipid rafts microdomains in the frontal cortex (Salaün, James and Chamberlain, 2004)

given for the all the proteins considered biomarkers of neurological and psychiatric health. Lastly, a molecular map was obtained from the neuronal proteins that have completely passed the guidelines of biomarker research and were identified, quantified and proposed as biomarkers of susceptibility.

Section E is made of: 28. Detected: Present in the Saliva Proteome. Section E extracted the molecular functions of all the neuronal proteomes that have already been identified in saliva. This is the most important molecular map in our study, as it reflects the actual neuronal biological processes that can be collected from salivary samples.

A full analysis of the MolecularCog(G) Atlas was not the objective of this chapter. A forthcoming work will be published, comprising the complete analysis of the 28 molecular maps obtained by this Cytoscape functional clustering.

4.C. Limitations On The Identification Of Neuronal-Salivary Proteome

The human body is composed of different fluids such as blood, urine, saliva, tears, and sweat, and all contain a wide variety of proteins. These fluids circulate in the body, fill an organ or body cavity, are secreted or excreted from the body, and play vital roles in the overall health and well-being of an individual. Body fluids provide a unique window into the biological processes and functioning of the human body. Some fluids, such as blood, urine, and cerebrospinal fluid (CSF), have found widespread clinical applications for the monitoring of human health and the diagnosis of diseases.

Blood products are by far the most popular and well-accepted choice for clinical testing, because blood is the circulating fluid that surrounds all tissue and organs and potentially collects by-products from diseased areas. Changes in the concentrations of specific plasma proteins have been associated with disease processes, leading to well-accepted clinical applications. Mining human body fluids to discover disease biomarkers is challenging, but it is hoped that changes in the proteome may be observed even before clinical symptoms become obvious, for instance in a sample of neurotypical young adults.

Whole saliva is a plasma ultra-filtrate composed of water, peptides and proteins (including enzymes), hormones, sugars, lipids, electrolytes, and several other components, that bathes the oral cavity. Whole saliva is made up of several salivary and non-salivary components. It contains secretions from the salivary glands (parotid, submandibular, and sublingual glands, and minor salivary glands) and non-salivary components such as serum and blood derivatives and a serum-like transudate that emanates from the gingival sulcus (Kaufman and Lamster, 2002).

The salivary glands are composed of two types of epithelial cells: acinar and ductal. Saliva is generated in the acinar cells and stored in granules that are released upon secretory stimulation. The ductal cells line the salivary ducts and direct secreted saliva into the mouth. The unique contribution of gingival fluid to whole saliva provides saliva with markers that are derived from the circulation. This gingival contribution makes it feasible to exploit whole saliva diagnostically to monitor disease biomarkers typically present in serum, albeit at different concentrations.

While the biological and mechanistic rationale for non-oral systemic diseases to be reflected diagnostically in saliva is currently unclear, efforts are in progress to elucidate the systemic connectivity. Using animal models, it is shown, upon development of systemic diseases (e.g., melanoma or lung cancer), that there are disease-specific biomarker changes in saliva (Gao et al., 2009). Moreover, nearly 40% of the proteins that have been suggested to be candidate markers for systemic diseases such as cancer, cardiovascular disease, and stroke can be found in whole saliva.

One of the possible hypotheses for a mechanistic rationale is that systemic diseases are known to ectopically produce hormones, lymphokines, and cytokines that can exert systemic effects. These disease-secreted mediators, upon reaching the salivary gland, can alter gene expression and translation of ectopic proteins that can serve as surrogate biomarkers in saliva. Another possible hypothesis for a mechanistic rationale is that proteins in whole saliva originate from not only the secretion of salivary glands (i.e., submandibular, sublingual, parotid, and minor glands) but also from leakage of plasma.

Henceforth, several sources can contribute to the overlap of protein identifications of saliva and plasma: (i) plasma and saliva may share essential proteins needed to maintain their physiological functions as body fluids; (ii) leakage of plasma into saliva through intracellular or extracellular routes, including outflow of gingival crevicular fluid; (iii) proteins derived from cell debris may be in close contact with either fluid.

The usefulness of saliva for the diagnosis of systemic diseases needs to be explored further and in greater depth. To date, the application of salivary proteins for the monitoring of brain diseases has not been widespread. The question remains whether saliva can be harnessed for biomarker discovery for neuropsychiatric diseases as well.

On the one hand, biomarkers in saliva may be able to provide insight into the health of the brain and the central nervous system. On the other hand, the identification of salivary proteins also present in brain samples needs to be taken with extreme caution (Walton, 2018).

4.C.1. Salivary Proteome Vs. Plasma Proteome

Saliva can be seen in many cases as a reflection of the physiological function of the body (Lima et al., 2010). However there have been concerns about the use of saliva for diagnostic purposes due to its low concentration of analytes in comparison to blood (Miller, 1994). Nevertheless, with the advent of highly sensitive molecular methods and nanotechnology, as the case in this thesis, this has become a less important limitation.

Loo and colleagues (2010) compared the salivary and plasma proteomes based on their gene ontology (GO) in terms of cellular component, molecular process, and biological function. In general, the salivary and plasma proteomes showed similar distributions in all 3 major GO categories. As expected for body fluids, the GO study of cellular components displayed that both saliva and plasma are overrepresented with extracellular proteins when compared with the overall human proteome.

As it will be shown in chapter 5, the salivary proteome contains a larger proportion (14.5%) of low-molecular-weight proteins (< 20 kDa). The highest fraction of proteins found in whole saliva range in size between 20 and 40 kDa (26%). In total, 65% of the saliva proteins have a molecular weight less than 60 kDa. Hence, with regard to the proteins found in common between saliva and plasma, the molecular weight distributions are similar to the distributions of the salivary proteome with a tendency toward the low-molecular-weight end.

Other differences in protein composition and function can be viewed from the comparison of the salivary and plasma proteomes. Human plasma/serum is dominated by immunoglobulins and albumins that make up 60-80% of the total weight (Bjorhall et al., 2005). The most abundant 22 proteins in plasma represent 99% of the total protein content of plasma. These abundant proteins saturate gels and columns for protein separation and display, and make the identification of lower-abundance proteins challenging.

Because of the wide dynamic range presented by plasma biofluids [greater than 10^{10} (Issaaq et al., 2007)], depletion of abundant plasma proteins is critical for improving the prospects of the identification of lower-abundance plasma proteins (Whiteaker et al., 2007). However, for whole saliva, the top 20 most abundant proteins represent only

approximately 40% of the saliva protein content. This situation makes it a far easier task to use salivary fluids for potential biomarker discovery (Loo et al., 2010).

Many of the abundant proteins in human saliva from healthy individuals have been individually studied and characterized previously. These include amylases, proline-rich proteins, statherin, histatin, mucin, and cystatins (Oppenheim et al., 2007). Fang and colleagues (2007) have identified low-abundance salivary proteins by utilizing capillary isotachophoresis/capillary zone electrophoresis (CITP/CZE), viz. a similar technique to Experion™ Automated Electrophoresis System (Biorad®), and reported a total of 1479 proteins in whole saliva.

Loo and colleagues (2010) demonstrated that saliva contains more than 2000 proteins, which was also demonstrated by this thesis. It should be noted that 27% of the salivary proteome overlapped with the plasma proteome. These 27% overlapped proteins actually enter saliva directly from blood by ultrafiltration, selective transport and active transport (Jusko and Milsap, 1993), which was also demonstrated by this thesis. The flow of plasma components into the saliva may involve several processes (Nunes and Macedo, 2013): (a) ultrafiltration through gap junctions between secretory cells. In this case, only molecules with a molecular weight below 1.9 kDa can be transported (ions, water, and some hormones); (b) selective transport through passive diffusion of lipophilic molecules (steroid hormones); (c) active transport through ion channels; and (d) active pumping of Na^+ ions with concomitant entrance of H_2O. Moreover, the saliva also contains compounds that are synthesized primarily in acinar cells and, to a lesser extent, in duct cells (e). That is to say that some saliva components are not related to plasma concentrations, but they are related to the local glandular answer. Furthermore, the concentration of numerous electrolytes changes due to active ion transport (f).

The same low overlap is seen between the salivary and neuronal proteome. However, despite the seemingly low overlap of proteins identified in the whole saliva and neuronal, their gene ontology distributions are remarkably similar such as molecular function, biological processes, and cellular components, as also shown in chapters 4 and 6 of this thesis. While this suggests that circulatory biomarkers may be detectable in saliva (Loo, Ramachandran and Wong, 2010), there is a greater implication in reference to the clinical role of salivary fluids for brain health. Thus, saliva is functionally equivalent to serum in terms of reflecting the health status of the human body (Walton, 2018).

4.C.2. Identification Of Salivary Proteins Also Present In Brain Samples

Farah and colleagues (2018) reviewed a multitude of studies revealing that saliva may be a valuable source of biomarkers to facilitate early diagnosis of neuropsychiatric conditions, neurodegenerative diseases and neurodevelopmental disorders. The two main physiological fluids that are used for the detection of such markers are CSF and blood.

Saliva is secreted in the mouth by the principal salivary glands: the sublingual, submandibular and the parotid, which are under direct parasympathetic innervation of the cranial nerves VII (glossopharyngeal) and IX (facial). The facial nerve innervates both the sublingual and submandibular glands through the submandibular ganglion, while the parotid gland is under glossopharyngeal innervation via the otic ganglion (Moore and Dalley, 2018). Therefore, this close relation between the salivary glands and the nervous system could render these glands' secretions as a useful pool of biomarkers that represent effectively neurophysiology (Shi et al., 2011), viz. in this study the normal physiology of the nervous system of neurotypical young adults without neuropsychiatric conditions.

The source of these biomarkers remains to be properly identified. One probable source is that they are transported directly from the CNS via the axons of the facial and glossopharyngeal nerves to the salivary glands to ultimately be vacated along with saliva

secretion (Farah et al., 2018). Another probable source is that the constituents of saliva enter saliva through blood via passive diffusion, active transport or extracellular ultrafiltration (Pfaffe et al., 2011). Contrastingly, the neuronal constituents present in plasma enter blood through the blood-brain barrier. This is a barrier system in which the capillary endothelial cells in the brain are closely connected to each other while interacting with surrounding pericytes and astrocytes. It precisely controls the exchange of substances between blood and brain tissue, which is essential for maintaining the stability of the microenvironment in the brain. Studies show that the cells that make up the blood-brain barrier regulate the development and function of the blood-brain barrier by expressing tight and adherent connexins, transporters, and related signaling molecules. In addition, neurons and microglia are also involved in the regulation of the blood-brain barrier under physiological conditions (Abbott et al., 2010; Serlin et al., 2015).

Different factors affect the molecular transport in the blood-brain barrier (Banks, 2009): charge, tertiary structure and degree of protein binding. Chief among these factors, however, is molecular weight. The best approximation of the influence of size on blood-brain barrier penetration is that it is inversely related to the square route of molecular weight. Small peptides and proteins with molecular weights in excess of 600 Da are known to cross the blood-brain barrier. However, bigger substances are documented to cross the blood-brain barrier by the mechanism of transmembrane diffusion, or active transport, for instance: cytokine-induced neutrophil chemoattractant-1 (CINC-1) with 7800 Da (Pan and Kastin, 2001), or BDNF with 27 kDa (Pezet and Malcangio, 2004).

4.C.2.1. Neuronal-Salivary Proteome Of Neuropsychiatric Conditions

Schizophrenia and bipolar disorder fall under the title of neuropsychiatric disorders, which present with multiple cognitive and behavioral symptoms and other DSM-V criteria (American Psychiatric Association, 2013). Thus, the exploration of physiological fluids for easily accessible and accurate biomarkers seems essential in efforts to provide innovative and better diagnosis and monitoring.

Immune system deregulations have been put into evidence for bipolar disorder and schizophrenia (Kim et al., 2007). From this standpoint, Iavarone and colleagues (2014) conducted a salivary proteome analysis and found eight immune system-related proteins to be elevated in the saliva of schizophrenic and bipolar patients when compared to healthy controls. The identified proteins were α-defensins 1–4, S100A12, cystatin A and the S-derivatives of cystatin B (proteins also identified in this study, and presented in chapter 5: pipeline oral proteome study). All of the mentioned proteins are involved in innate immunity and therefore indicate an immunologic imbalance in schizophrenic and bipolar patients (Pietzsch and Hoppmann, 2009).

4.C.2.2. Neuronal-Salivary Proteome Of Neurodegenerative Diseases

Contrastingly, neurodegenerative diseases like Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis have been widely studied in salivary biomarker research. When examining the concentration of α-syn in saliva, one study found that the proportion of the toxic oligomeric form of the protein was higher in patients with Parkinson's disease than in healthy controls (Vivacqua et al., 2016). With the objective of establish α-syn as a prominent salivary biomarker of Parkinson's disease diagnosis, Devic and colleagues (2011) used western blotting and spectrometry to establish the presence of α-syn in human saliva. These findings were replicated by Al-Nimer and colleagues (2014) and validated by ELISA assay. They detected a significant increase in α-synolig and α-syn olig/α-syntotal ratio in the saliva of Parkinson's disease patients as compared to healthy controls. These results observed in saliva are mirrored in CSF

(Parnetti et al., 2013). Similar to what is seen in Parkinson's disease, huntingtin protein (Htt) is capable of being detected in saliva. Knowing that testing CSF and blood for Htt protein for diagnostic confirmation of HD can be performed, it is still an impractical measure due to the invasiveness of the testing procedure and because of low Htt concentrations in the acquired Blood and CSF samples (Wild et al., 2015). In a study conducted by Bloom and colleagues (2016), it is shown that Htt protein was successfully detected, using ELISA, in saliva. There was a significant increase in total Htt protein concentration in saliva samples obtained from Huntington's disease patients when compared to controls. Likewise, and in the study performed by Obayashi and colleagues (2008) using a YK070 chromogranin A EIA kit, salivary CgA levels were found to be only significantly elevated in patients with terminal amyotrophic lateral sclerosis in comparison with healthy controls.

4.C.2.3. Neuronal-Salivary Proteome Of Neurodevelopmental Disorder

Similarly, neurodevelopmental disorders, like and attention deficit hyperactivity disorder and autism spectrum disorder, have been recently studied in salivary biomarker research (Estes and McAllister, 2015; Hipólito and Martins, 2018).

Ngounou Wetie and colleagues (2015) conducted a pilot study in which they examined discrepancies in salivary protein levels and signatures (Stoop, 2012) between autism spectrum disorder patients and healthy controls. By employing Mass Spectrometry-based proteomics, they were able to identify a variability in salivary proteomic levels. A further analysis of protein–protein interactions revealed protein complex formation might be compromised in autism. In the same context, Castagnola and colleagues (2008) conducted a proteomic study aimed at identifying changes in the post-translational modifications of salivary proteins and they were able to detect salivary protein hypophosphorylation in neuroatypical subjects. These findings support that hypophosphorylation serves more as an explanation for failed protein mechanisms rather than a biomarker and therefore could explain the essence of protein–protein interaction deficits.

4.C.3. Protein Degradation, Protein Quality And Protein Stability

The major challenges with preserving whole saliva relate to the complexity of its composition and its inherently high proteolytic activity (Helmerhorst and Oppenheim, 2007). Proper handling and storage are required to preserve the integrity of potential salivary biomarkers and prevent salivary autoproteolytic degradation, because rapid protein degradation occurs within 30 minutes after sample collection and degradation start already during collection (Esser et al., 2008). Short-term storage of freshly collected samples on ice is the more effective method without interfering with the chemistry of the proteome (Thomadaki et al., 2011).

Before saliva can be used as a diagnostic fluid for protein markers in the clinic, its stability should be determined. Studies on protein stability in saliva samples (Morris, 2002; Ng, 2003; Schipper et al., 2007) have been conducted and because different fundamental and prominent factors may affect sample quality, saliva collection methods and processing must be refined. Specialized protocols have been proposed for proper saliva collection (Henson and Wong, 2010). There are at least six proteases present in saliva which are probably involved in salivary proteolysis. However, also 13 protease inhibitor proteins were identified which may counteract protease activity. Nevertheless, the overall balance is clearly in favor of degradation.

Cocktails of inhibitors (aprotinin, pancreatic trypsin inhibitor, leupeptin, and antipain) are known to be effective against salivary proteases (Thomadaki et al., 2011),

although experimental results were disappointing. Likewise, as published by Esser and colleagues (2008) three degradation products with masses of 2937 Da, 3370 Da and 4132 Da were discovered, which can be used to monitor the degradation process and to determine the quality of a saliva sample before protein analyses. These types of approaches were not used in this thesis.

More promising (Schipper et al., 2007) were the non-specific approaches of protease inhibition, i.e., cooling on ice of whole saliva samples ensures protein stability for up to 1 hr. He confirmed that overall protein stability of saliva samples is maintained if successively stored at −80 °C. Both procedures applied in this thesis.

Subsequently, whole saliva centrifugation is encouraged, by most of the studies (Esser et al., 2008; Sun et al., 2009), to separate the cells from the protein-containing supernatant. Protein degradation in whole saliva is 7.5-fold faster than in whole saliva supernatant (Sun et al., 2009). This was the reason why in this study all the 256 salivary samples (T0 and T1) were rapidly transferred to Biobanco-iMM in [15m;1h45m] and centrifuged at 10,000 × g for 10 minutes. Rapid centrifugation after collection thus will prolong the stability of soluble whole saliva proteins. The resulting cell sediments of the salivary samples of the neurotypical subjects were frozen. In the future and if a research hypothesis is driven that way, other protein extraction procedures for identification of biomarkers associated with this fraction may be conducted.

Caution should be exercised when biomarker assays are conducted at ambient temperature or at 37 °C, since salivary enzyme activities will be reactivated, and this may cause loss of biomarkers during analysis (Xie et al., 2008), viz. the effect of sample handling. In this thesis, all wet-lab analysis was conducted with a dry ice support for sample transportation and handling between experiments. Dry ice is the solid form of carbon dioxide and is useful for preserving samples in dry ice sublimates at −78.5 °C. Then as defended by the majority of the studies (Schipper et al., 2007) in salivary biomarkers research, for long-term storage, the method of choice for maintaining the integrity of saliva samples consisted of freezing at −80 °C. Hence, the saliva aliquots were preserved at -80ºC in the Biobanco-iMM biobank (Afonso, Protereira and Dias, 2014).

As shown, this study used already a refined SOP and adequate processing (Rosa et al., 2016). In the study conducted by Lee and colleagues (2017) stabilizing salivary levels of Aβ42 peptides by adding thioflavin S to prevent aggregation and using sodium azide to inhibit bacterial growth, enhanced Aβ42 detection in the sample. In this study, following Rosa and colleagues (2016) standards, it was added a sample buffer with β-mercaptoethanol. Then, the saliva samples and the ladder were subjected to the same denaturing conditions (95. for 10 min). The migration times and the concentration of each protein in the sample wells were normalized to the ladder using internal markers. Finally, the variability within each band was calculated by the variation coefficient of the Total Protein values relatively to all the proteins in each group of molecular weights (MW).

This raises the question that if proper extraction, handling and treatment of the saliva sample with agents and compounds known to stabilize the measured molecule will result in better sample quality and therefore better biomarker detection. Consequently, the measurements of neuronal-salivary proteome were normalized (in chapter 5 and chapter 6) against total salivary proteins to account for shifts in concentrations of proteins brought about by irregular salivation. In salivary composition and secretion, it is established that there exist circadian rhythms that normally govern saliva production, composition and flow rate (Dawes, 1972). This variable was adequately controlled in all the experimental studies in this thesis, i.e., all the 256 salivary samples from participants and controls were always collected during the same time frame in order to avoid normal shifts in saliva protein concentrations from confounding the evidence.

Aside from physiological changes, qualitative and quantitative salivary alterations in secretion or composition, induced by either systemic or oral conditions, can cause functional deficiency of the saliva. Oral cancers are associated with significant changes of the salivary proteins.

Similarly, changes in the salivary protein composition have been observed in systemic diseases. For instance, Sjogren's syndrome, an autoimmune disease, causes reduction in saliva volume. Alterations in glycosylation of salivary mucins have been associated with cystic fibrosis. Increased levels of amylase and IgA are observed in diabetic patients. A number of salivary components, including cortisol, amylase, and lysozyme, are altered under stress conditions.

In this thesis, a rigorous effort was made to control the participants of this study to brain and systemic pathologies. Thus, the sample of 128 neurotypical young adults were considered healthy and showed no signs or symptoms of neurological and psychiatric pathologies, or uncontrolled disorders of the kidney, liver, lungs, heart, and musculoskeletal, rheumatologic, metabolic, or severe chronic or terminal diseases.

A key initial step for saliva to be of practical use for health monitoring is the cataloging of its protein components. However, because of its complexity, variation in protein abundance and post-translational modifications [75], a comprehensive characterization of the protein composition of salivary fluid could not be achieved through traditional biochemical approaches until the introduction of MS-based, high-throughput proteomics technologies. These physiologic and pathologic alterations in salivary secretion suggest that analysis of saliva, should take into account also protein degradation and carbohydrate post-translational modifications.

Glycosylation of salivary proteins is believed to play a role in salivary protective functions. Characterizations of the glycosylated proteins in saliva and plasma have reported over 70 proteins in saliva (Ramachandran et al., 2006, 2008; Larsen et al., 2007; Sondej et al., 2009) and over 300 in plasma (Liu et al., 2005) as glycosylated proteins. The result of the annotation information extracted from the UniProt knowledge base indicates that potentially more glycosylated proteins exist in saliva and plasma. Indeed, the glycosylation profiles generated from lectin blots indicated that the majority of salivary proteins are potentially glycosylated (Sondej et al., 2009).

However, stronger analytical evidence on protein stability is needed to ensure the validity of salivary biomarker study outcomes, and, indeed, what works in the lab still has several barriers to face before it can be implemented in the clinic. A good diagnostic test must have high sensitivity and specificity, but also be high throughput, low cost and portable (Walton, 2018).

As applied in chapter 5 of this thesis, Guo (2006) could identify 1381 proteins employing a capillary electrophoresis approach. However, because of the complexity of whole saliva, each proteomic strategy leads to partial overlapping subsets of saliva proteins; for instance, with 2D gel (Huang, 2004; Wilmarth, 2004; Vitorino, 2004; Hu, 2005; Walz, 2006) or free flow electrophoresis (Xie, 2005). Therefore, different proteomics strategies contribute to a comprehensive view of the whole saliva proteome.

[75] protein synthesis occurs during a process called 'translation'. Posttranslational modification of proteins, being one of the later stages in protein biosynthesis, refers to the reversible or irreversible chemical changes proteins may undergo after translation. Viz., they are chemical modifications of a polypeptide chain that occur after DNA has been transcribed into RNA and translated into protein. These chemical alterations range from the enzymatic cleavage of peptide bonds to the covalent additions of particular chemical groups, lipids, carbohydrates, or even entire proteins to amino acid side chains. These chemical modifications of a polypeptide chain after its biosynthesis extend the range of amino acid structures and properties, and consequently, diversifies structures and functions of proteins (Brenner and Miller, 2014).

4.C.4. Neuro.OralCard, Neuropeptide And Neuroimmune Response

In Neuro.OralCard, immunoglobulins and neuropeptides contribute from 5 to 15% of the total number of salivary proteins, suggesting that these molecules could result from leakage from plasma. The precise mechanism of leakage of plasma proteins and whether specific plasma proteins are selectively transported into the salivary system are unknown. However, leakage of plasma into saliva through intracellular or extracellular routes, including outflow of gingival crevicular fluid, is likely (Yan et al., 2009)

This observation is further explored in chapter 5 and tested in chapter 6 of this thesis, and can have relevant implications in neurophysiology of healthy subjects.

However, as shown by Yan and colleagues (2009), compared with the total human proteome, the salivary proteome is overrepresented in the extracellular component and in the cytoplasmic and cytoskeleton components, an indication of secretion. In contrast, intracellular components are underrepresented in saliva. With regard to biological processes, compared with the human proteome, the salivary proteome is overrepresented in the categories of response-to-stimulus, response-to-stress, and cell organization and biogenesis, but are underrepresented in cell communication and other primarily metabolic processes. The over-representation of proteins involved in response-to-stimulus and response-to-stress processes presumably reflects the functions of saliva in the body's defense system.

That being said, scientists cannot depend on one biomarker to ultimately achieve an accurate monitoring of neurologic and mental health (Pacheco, Martins and Simões, 2019). Instead, a definitive biomarker profile, by studying the neuronal-salivary proteome, must be shaped to be used in neurophysiology. The research made in this chapter remains inefficient in discerning the reliability of salivary biomarkers in brain research, but explores its possibility. Proper saliva collection and processing protocols must be better standardized in order to decrease biases and allow an accurate identification.

The excerpt of the research outline (**Figure ε**) systemizes the Section II: Molecular Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 4 in the overall experiment.

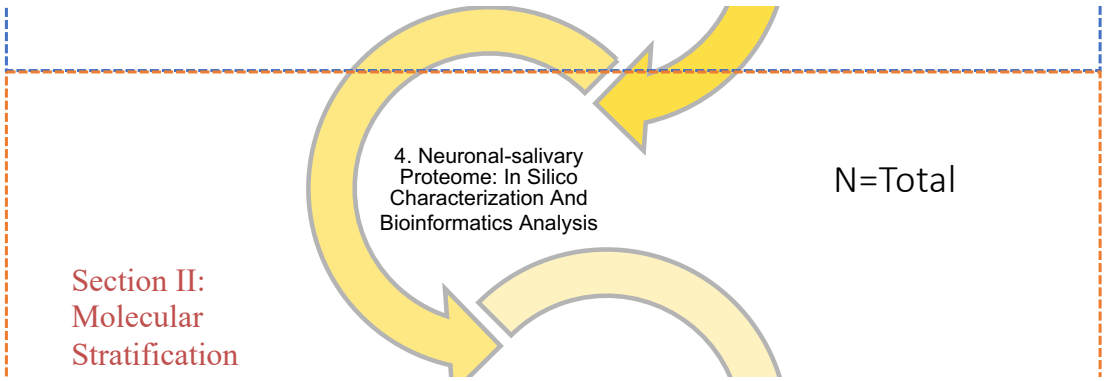


Figure ε. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section II: Molecular Stratification
The chapter 4 (Neuronal-Salivary Proteome: *In silico* Characterization and Bioinformatics Analysis) is displayed, which comprehended the methodology that led to the construction neuroproteome database and annotation of the neuronal proteins present in salivary samples as reviewed from the literature at 15.08.2020.

Chapter 5. Protein Profiling of Cognition and GUI Toolbox Development in Neurotypical Young Adults

Preamble ("How to scan the total protein profile of neurotypical young adults?")
Neuronal protein networks identifiable in the salivary protein profile may be useful to characterize neurotypical mental health states and traits. A pipeline oral protein profile study, which consisted of a total protein profiling, had explored an innovative probing of saliva. Subsequently, a full approach on 92 neurotypical young adults showed specific expected protein profiles present in saliva. Those molecular profiles were significantly correlated with the previously identified mental health strata (Hipólito and Martins, 2017). Afterwards, a GUI toolbox, MODeLING.Vis, was developed for the data mining of the acquired neuroproteomic data. The developed toolbox permitted data exploration and hypothesis testing of the biomolecular data. From a common scientific interest perspective, the outcome of this analysis was quantifying the [9.1;30]kDa molecular weight range. This range is characteristic of the small neuroimmune molecules and neuropeptides and, thus, offers a solution for probing into the neurocognitive response.

Materials And Methods

Chapter 5 comprises the methodology that led to the establishment of the Neuro.SalivaPrint (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017). This chapter comprised two methodologies used in: (i) the acquisition and characterization of the total protein profile; and (ii) the creation of Graphical User Interface (GUI) toolbox for the data visualizing and data mining of the total protein profile.

5.1. Intervention Protocol

An Intervention Protocol was applied for pre-molecular sample screening. The experimental procedure that was used for mental health stratification was a cognitive behavioral intervention, which is fully described in chapters 2 and 3 of this thesis. Four specific mental health states and traits, i.e., clinical subtypes, were found. We then conducted subsequent and complementary molecular analysis on those 4 subphenomes.

This molecular analysis continued our sample stratification by total protein characterization.

5.2. Full Sample Screening

As published in chapters 2 and 3, the neurotypical young adults' sample was initially separated into independent groups:

(a) Experimental group (64 subjects);

(b) Control group (sham intervention) (64 subjects).

Afterwards, these groups were stratified into the top and bottom phenomes, resulting in 4 subgroups: (i) the top phenome of the Experimental group, (ii) the top phenome of the Control group, (iii) the bottom phenome of the Experimental group, and (iv) the bottom phenome of the Control group.

From the original total 128 subjects, that stratification assigned N=92 in 4 subgroups (subphenomes), which were considered comparable for a) age (median 21,495 y.o.), b) gender mean=50% males, and BMI (21,325 kg/m^2). Salivary samples were collected from each subject and stored in average 6 aliquots of 250 µl for post-processing.

5.3. Pipeline Oral Protein Profile Study

A preliminary exploratory study had already been completed and justified the *rationale* of this study. This biomedical analysis methodology (Martins et al., 2016a) was conducted, by SalivaTec, and generated preliminary data (presented in Results) with a sample size of 22 control subjects (T-1) and 5 preliminary subjects (T-1 [before and after the experimental procedure]), for which the total protein profiles were characterized by capillary electrophoresis. An overall limitation of exploratory studies is to understand if the variability measured is higher than the natural variability. Therefore, we made a prior sampling, defined as a timepoint prior to our intervention (T-1) in order to assess the variability of our data, independently of our study and our stratification.

We understand the limitation of proposing a (T-1) with only 22 control subjects and 5 preliminary subjects (T-1 [before and after the experimental procedure]). However, this limited sample had already offered some distinct Molecular Weight (MW) range intervals, as shown in Results.

We used this timepoint only to compare it with the MW intervals generated by the study (T0 and T1), and not to calculate the significative differences between Δ[T-1;T0] or Δ[T-1;T0]. T-1 offered us an expected protein profile of our population (neurotypical young adults), defined in this study as the population protein profile. This T-1 control group could only be used for testing the variability of the MW range intervals of our sample against our population. No statistical testing was thus applied.

5.4. Salivary Total Protein Profile

The collection and processing of saliva were performed adopting an easy, safe and cost-effective method, established in SalivaTec lab (Rosa et al., 2016). To avoid the intersubject circadian rhythm variability in saliva composition and food debris (Dawes, 1972; Cuevas-Córdoba and Santiago-Garcia, 2014), salivary collection was all executed at the same time interval [09:15;12:00] (a.m.) after a morning fasting.

Subjects were asked to rinse their mouths with water and discard it before sample collection. Briefly, two sublingual cotton rolls were placed for 2 minutes. To recover the saliva sample (1–2 ml), cotton rolls were collected in a falcon tube. During the collection process, the sample tubes were kept at 4°C. Then, to guarantee protein stability and avoid the whole-saliva proteolysis (Esser et al., 2008), all the salivary samples (T0 and T1) were rapidly transferred to Biobanco-iMM in [15m;1h45m]. Subsequently, they were centrifuged at 10,000 × g for 10 minutes. Afterwards, the saliva aliquots were stored and preserved at -80ºC in the Biobanco-iMM biobank (Afonso, Protereira and Dias, 2014) and subjects' information added to the Biobanco-iMM databank.

The salivary total protein profile was assessed by capillary electrophoresis using an Experion™ Automated Electrophoresis System (Biorad®) with standard protein chips (Experion™ Pro260 Analysis Kit) and samples were analyzed according to Biorad technical specifications. The protein profile and the quantification of the abundant protein bands were determined using Experion™ Software, version 3.20. In order to determine if there are characteristic variability patterns in the protein profiles obtained from healthy neurotypical subjects, to all saliva samples, sample buffer with β-mercaptoethanol was added. Then, the saliva samples and the ladder were subjected to the same denaturing conditions (95. for 10 min). The migration times and the concentration of each protein in the sample wells were normalized to the ladder using internal markers. Finally, the variability within each band was calculated by the variation coefficient of the Total

Protein values relatively to all the proteins in each group of molecular weights (MW).

5.5. Experion™ Automated Electrophoresis System (Biorad®)

Experion™ automated electrophoresis system (Biorad®) was used to identify and characterize the total protein profiles of the 92 subjects. We used the salivary samples, which were collected and stored in Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal. The collection was made according to the Biobanco-iMM and SalivaTec Standard Operation Protocol (SOP) (Rosa et al., 2016).

Later, those samples were safely and methodically transferred to SalivaTec, Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa. The samples were then processed and prepared for the capillary electrophoresis protocol, established by the Experion™ automated electrophoresis system (Biorad®) (Chang, 2005).

The quantification methods for protein profiling are validated by Biorad® (Wu and Strong, 2007). This system integrates whole protein quantitation in a single process in which protein separation, staining, band detection and quantitation are automatically executed (Kim et al., 2013).

This system accomplishes analysis in a single 30-minute automated step and includes the following components: automated electrophoresis station, priming station, system operation and data analysis tools and analysis kits, which include the chips and reagents for protein (Pro260 kit). This kit has a resolution and quantitation of 10–260kDa proteins while also separating and detecting 2.5–2000 ng/µl proteins.

Gel images were then processed and displayed on a computer monitor, and protein bands identified by migration relative to the MW marker.

The density of the protein bands was determined using Experion Imaging software (Biorad®, Hercules, CA), expressed in density units (Willoughby, Stout and Wilborn, 2007). All procedures followed the manufacturer's protocol.

5.5.1. Limitations Of The Experion™ And Capillary Electrophoresis

The Experion™ Automated Electrophoresis System (Biorad®) is an instrument that presents important concerns when analyzing protein profiles, showing limitations in extracting solid conclusions in protein analysis and in protein quantification. First, his resolution is low, and the electrophoretic bands that are seen in a single electrophoretic peak is a consequence of that low resolution (ng/µl). Henceforth, this system shows some inaccuracy and its application to exhaustive protein profiling are limited.

Moreover, the electrophoretic profile by Experion™ can be confounded by the presence of post-translational modification in salivary proteins (Bank et al., 1991; Hu, Jiang and Wong, 2010), more specifically glycosylation[76].

One other concern about conducting capillary electrophoresis for exhaustive protein profiling is the fact that the existence of a high concentration of mono- and polymeric sugars in saliva would also alter the obtained results. Sondej and colleagues (2009) showed that by understanding global changes in the salivary glycosylation profile, we will gain important insights on the glycoforms present in saliva and in the use of electrophoresis, e.g., two-dimensional gel electrophoresis, to preliminary screen salivary biomarkers.

[76] reaction in which a carbohydrate, i.e., a glycosyl donor, is attached to a hydroxyl or other functional group of another molecule (a glycosyl acceptor). These covalent and generally enzymatic modification of a protein following protein biosynthesis alters the predicted molecular weight of the same protein (Sarni-Manchado et al., 2008).

Preliminary electrophoretic screening should thus be followed by better analytical and quantification methods like MS/MS analysis or biometric immunoassays (the case in this thesis).

However, striking a balance between greater purity and faster protein purification is a current theme. Faster methods and higher purity are desired, but also important is to retain the functionality and structure of a protein.

Efficient protein purification requires accurate detection, size analysis and quantification at multiple stages in the purification pathway. In addition to being dependent on the buffer system at each step, these intermediate detection and quantification steps often involve a large number of samples.

Otherwise, Experion™ Automated Electrophoresis System (Biorad®) uses Caliper's LabChip technology and makes those more advanced approaches to protein separations more accessible for the academic market, in a low-cost solution. Some of the latest innovations in this type of detection include the use of microfluidics, which involves the manipulation of liquid samples in miniaturized systems (chips). Being less expensive than traditional gel electrophoresis and increasing sample throughput, it still generates high-quality digital sizing and concentration data on a moderate sensitivity protein assay (Smith, 2005).

For that reason, in this study, Experion™ was firstly used to perform a rapid electrophoretic separation of the total salivary samples of all the participants in this study.

Secondly, as the salivary samples were assessed for protein quality, a consequent analysis for simple protein mixtures was performed.

Thirdly, the effort of the research, with this low-end method and low-cost technique, was to still find differences in the electrophoretic separation by Experion™ in the totality of the salivary samples.

For that matter, a rigorous data analysis was performed in both:

(i) the acquisition and characterization of the total protein profile;

(ii) the creation of Graphical User Interface (GUI) toolbox for the data visualizing and data mining of the total protein profile.

Capillary electrophoresis was rather used for a preliminary stratification of the totality of salivary sample, which made possible the preselection of subjects that advanced to a more accurate and precise method of protein detection and quantification, i.e., a multiplex-biometric immunoassay, by Bio-Plex® MAGPIX™ Multiplex Reader.

The Chapter 6 of this thesis is dedicated to that analysis and explores better the concerns and limitations offered by a low-end technique like capillary electrophoresis. To note that one of the main restrictions of this thesis was the budget that was allocated, and that one of the main efforts was to still run it and extract adequate molecular results and reasonable conclusions.

5.6. Statistical Analysis

An initial inferential analysis, in the 5 preliminary subjects (T-1 [before and after the experimental procedure]), was made for the preliminary results, i.e., the Pipeline Oral Protein Profile Study.

The main objective was to test the evidence that there is a significant change in the protein profile (T0) after a stratification protocol (T1). In order to do that, a potency (T-Student) of 80%, a significance level of 5%, and an effect size corresponding to 40% of the estimated value for the standard deviation of the variation of the MWs in the preliminary study was considered.

Taking this option in the account, which corresponds to a medium Cohen's effect size, an appropriate full sample size for each group (experimental and control) of 64 elements was found.

Afterwards, we performed overall data analysis: data acquisition and data processing. This analysis permitted data visualization and exploration of the protein profiles. Double entry charts (x: MW; y: fluorescence) and (x: $[MW]_{interval}$; y: frequency), 3 component PCA and 2 dimensional electrophoretograms were used as graphic and visual exploratory tools.

5.7. Data analysis

5.7.1. Data Acquisition (Experion™ Automated Electrophoresis System (Biorad®)

Once protein separation was complete, the software subtracted background noise, identified and integrated peaks, and assigned their sizes and concentrations. Experion™ software displays all three forms of data simultaneously: Virtual gel, Electrophoretogram and Results table.

SalivaTec had already validated this software for the salivary protein profiling (Esteves et al., 2017). Raw data were thus analyzed with Experion™ software, which had already been used in several profiling studies (Hirayama et al. 2009; Minami et al.2009; Saito et al. 2009).

The data analysis workflow, starting with the raw data, included noise-filtering, baseline correction, peak detection and integration of the peak area from sliced electropherograms. Such functions are commonly used by data processing software, such as MassHunter from Agilent Technologies, or XCMS (Smith et al. 2006).

The width of each electropherogram was defined to 0.02 m/z. On average, Experion™ detected 1000 peaks, rounded to a decimal of a kDa (MW), in each saliva sample.

The standard deviation of the relative peak areas of the protein-derived peaks was treated automatically by Experion™ software algorithms and defined to 0.5 kDa. This allowed the best peak acquisition for our data.

All peak areas were divided by the area of the internal standard (relative area) to normalize the signal intensities, and to avoid capillary electrophoresis detector sensitivity bias among multiple measurements.

5.7.2. Data Processing (MATLAB Toolbox)

Once data acquisition was completed, we processed it with MATLAB Toolbox. We used a GUI as a data processing and data exploration tool. The accurate m/z value for each peak detected within the time domain was calculated with Gaussian curve-fitting m/z domain peak.

The alignment of peaks in multiple measurements was done, using an algorithm in MATLAB, and is further explained in Results.

Other techniques and software have been used for the same purpose (Baran et al. 2006; Soga et al. 2006) with slight modifications. We used the expectation-maximization algorithm to pick up representative peaks and MW range intervals. Other authors have used, for instance, the Douglas-Peucker algorithm (Wallace et al. 2004).

From unit m/z electropherograms, our algorithm found corresponding peaks across multiple samples, and optimized the numerical parameters of the normalization function, as already proposed by Reijenga and colleagues (2002).

5.8. Software

The following pieces of software were used:

(i) Experion Imaging software (Biorad®, Hercules, CA) for proteomic data acquisition, quantification and treatment;

(ii) LabWare™ LIMS for laboratory information management;

(iii) MATLAB™ for data visualization and data exploration of the protein profiles. A specific GUI toolbox was created, in MATLAB™, for that purpose: MODeLING.Vis.

Results And Discussion

This set of results was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[77]: Martins, J. E.[a,b,c,e], Simões, J. F. [a,b,c], D'Alimonte, D. [d], Sousa, S[a,c]., Esteves, E.[a,c], Rosa, N[c], Hipólito, I.[a,e,g], Rodrigues, T.[f], Correia, M. J.[c], Simões, M.[a,b], Barros, M.[c].

The results, and their discussion, comprehend the data acquired in:

(5.A.) the pipeline oral protein profile study;

(5.B.) the expected protein profile;

(5.C.) MODeLING.Vis;

(5.D.) the neuroinflammatory and neuropeptide panel choice.

The variables analyzed are both quantitative/continuous and qualitative/nominal.

5.A. Pipeline Oral Protein Profile Study

A preliminary study was conducted in a convenience sample, of neurotypical young adults, comprising (a) 22 control subjects (in T-1), as a population protein profile control group, and (b) 5 preliminary subjects of (i) the top phenome of the Experimental group (in T-1 [before and after the experimental procedure]).

Both groups were recruited and screened using the inclusion criteria of this study. Those results, obtained by Rosa and colleagues (SalivaTec, Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS)), firstly addressed the research hypothesis of finding salivary total protein profiles for the molecular stratification of a mental health state.

Firstly (a), a sample of 22 control subjects (T-1) was analyzed in Experion™ Automated Electrophoresis System (Biorad®). The raw data is presented in **Figure 39,** showing an extract of how the raw electrophoretic profile was obtained. In Experion™, in instrument No. 4170, an electrophoretic run has 10 wells for 10 different samples.

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[77] [a]LIMMIT Lab, Faculty of Medicine, University of Lisbon, Lisbon, Portugal
[b]Mind-Brain College, University of Lisbon, Lisbon, Portugal
[c]Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa, Viseu, Portugal
[d]CIMA, Centre for Marine and Environmental Research, University of Algarve, Faro, Portugal
[e]Lisbon Mind and Cognition Group, Institute for Philosophy of Nova, New University of Lisbon, Lisbon, Portugal
[f]IMM, Faculty of Medicine, University of Lisbon, Lisbon, Portugal
[g]School of Humanities and Social Inquiry Faculty of Law, Humanities, and the Arts, University of Wollongong, Australia

A run name was defined, as well as the sample name, and the lower, higher and system peak were selected by the researcher. Each well (in this case, well No. 1) detected different number of peaks with a specific x value = MW peaks (kDa).

Each peak was determined by the migration time (seconds) of the molecule or cluster of molecules. Please note that the presence of clusters of proteins in a single peak is a consequence of the low resolution of the instrument, viz. Experion™ Automated Electrophoresis System (Biorad®). The extract of the run shown in **Figure 39** found 20 peaks. Those different MW peaks have an attributed y value = immunofluorescence.

Run Name	Instrument	Well	Sample Name	Peak Number	Mig. Time (s)	Mol. Wt. (kDa)	Corrected Area	Concentration (ng/µl)	% Total	Total Concentration (ng/µl)	Peak Height	Observation
1st prelim	4170	1	#1	1	11.79	0.00	86.42	0.00	0.00	1602.24	18.64	
1st prelim	4170	1	#1	2	14.42	0.00	61.37	0.00	0.00	1602.24	42.65	
1st prelim	4170	1	#1	3	15.93	0.00	61.24	0.00	0.00	1602.24	24.27	
1st prelim	4170	1	#1	4	17.10	1.20	3067.92	0.00	0.00	1602.24	1364.82	Lower Marker
1st prelim	4170	1	#1	5	21.53	7.02	684.83	0.00	0.00	1602.24	303.12	System Peak
1st prelim	4170	1	#1	6	21.97	7.60	884.71	0.00	0.00	1602.24	343.91	System Peak
1st prelim	4170	1	#1	7	23.53	9.65	17.86	7.27	0.45	1602.24	16.01	
1st prelim	4170	1	#1	8	24.80	13.12	5.68	2.31	0.14	1602.24	8.22	
1st prelim	4170	1	#1	9	25.73	16.02	173.99	70.85	4.42	1602.24	110.01	
1st prelim	4170	1	#1	10	27.58	22.41	6.91	2.82	0.18	1602.24	10.16	
1st prelim	4170	1	#1	11	28.99	28.79	145.56	59.27	3.70	1602.24	61.10	
1st prelim	4170	1	#1	12	30.70	36.98	36.23	14.75	0.92	1602.24	36.17	
1st prelim	4170	1	#1	13	32.50	46.75	683.78	278.43	17.38	1602.24	261.55	
1st prelim	4170	1	#1	14	34.84	61.44	1635.71	666.04	41.57	1602.24	1288.47	
1st prelim	4170	1	#1	15	36.15	70.09	1216.71	495.43	30.92	1602.24	521.43	
1st prelim	4170	1	#1	16	39.96	101.51	2.53	1.03	0.06	1602.24	5.87	
1st prelim	4170	1	#1	17	44.93	149.76	9.92	4.04	0.25	1602.24	6.64	
1st prelim	4170	1	#1	18	53.60	260.00	184.19	75.00	0.00	1602.24	130.84	Upper Marker
1st prelim	4170	1	#1	19	60.13	0.00	0.54	0.00	0.00	1602.24	2.07	
1st prelim	4170	1	#1	20	66.32	0.00	2.63	0.00	0.00	1602.24	13.43	



Figure 39. Extract Of The Raw Electrophoretic Profile And Variables
The analysis comprises the run name, the instrument, the well, the sample name, the peak number, the migration time (s), the molecular weight (kDa), the corrected area, the concentration (ng/µl), the % total, the total concentration (ng/µl), the peak height, and observation.

The immunofluorescence is equal to the area (corrected area) under the curve. This area is equal to the integral function of y variable:

$[\int y^n dx = (1/n+1)\, y^{n+1},\ n \neq -1]$, where y = immunofluorescence.

Finally, the total concentration (ng/µl) (the % of concentration attributed to a specific peak), and the peak height for improved data analysis are also presented.

Later, the full raw electrophoretogram obtained by Experion™ Imaging software was exported to a comma-separated values file, ".csv", and can be consulted in: https://tinyurl.com/RawProteinProfilePopulation (retrieved July 20, 2020; *in* Annex K: External Support).

This ".csv" file shows the raw electrophoretic pattern of our population, i.e., the population protein profile. Those 22 control subjects were sampled in order to obtain specific preliminary MW intervals for our population of neurotypical young adults. Subsequently, a preliminary strategy was implemented for the search of those intervals, as shown in **Figure 40.**

Initially, the x-values (electrophoretogram) were rounded to decimals because it is the minimum kDa physical quantity considered distinct and specific of a protein or peptide (x = 9.1; x = 9.2;… ; x = 235.7).

Afterwards, the MW intervals were clustered according to their absolute rounded values (x-values). This clustering permitted the setting (further defined as kernels) of 19

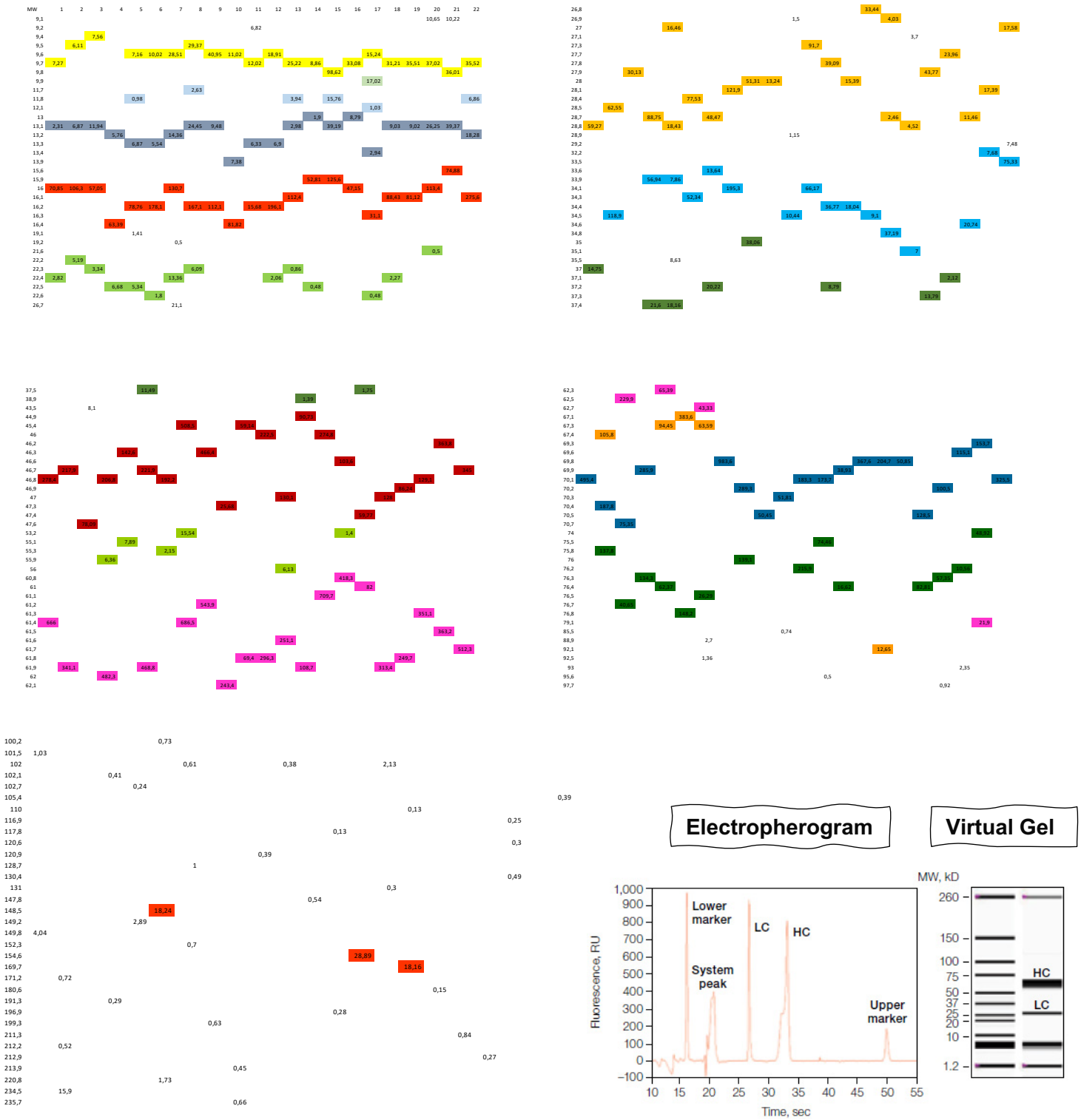


Figure 40. Strategy For MW Ranges Of The 22 Control Subjects (T-1)
Twenty-two control subjects sampled to show specific preliminary MW intervals for our population of neurotypical young adults. Preliminary strategy implemented for the search of those intervals and raw electrophoretic pattern of our population, i.e., the population protein profile.

A limitation of this clustering is that it does not permit a y-value (immunofluorescence or concentration (ng/µl)) consideration.

However, a y-value consideration was not the objective of this preliminary data visualization

Actually, for that purpose we later developed the data mining solution MODeLING.Vis.

Concluding, this strategy offered some distinct but simple MW range intervals, as shown in **Figure 41**.

We used them for data exploration and comparison with the intervals later generated by this study. Therefore, no adequate statistical testing was applied.

Interval	MW	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
9.1-9.2	9,2											6,82									10,7	10,2	
9.4-9.8	9,5	7,27	6,11	7,56		7,16	10	28,5	29,4	41	11	12	18,9	25,2	8,86	98,6	33,1	15,2	31,2	35,5	37	36	35,5
9,9	9,9																	17					
11.7-12.1	11,9					0,98			2,63					3,94		15,8		1,03					6,86
13-13.9	13,3	2,31	6,87	11,9	5,76	6,87	5,54	14,4	24,5	9,48	7,38	6,33	6,9	2,98	1,9	39,2	8,79	2,94	9,03	9,02	26,3	39,4	18,3
15.6-16.4	16,1	70,9	106	57,1	63,4	78,8	178	131	167	112	81,8	15,7	196	112	52,8	126	47,2	31,1	88,4	81,1	113	74,9	276
21.6-22.6	22,3	2,82	5,19	3,34	6,68	5,34	1,8	13,4	6,09				2,06	0,86	0,48			0,48	2,27		0,5		
26.8-28.8	27,8	59,3	62,6	30,1	88,8	34,9	77,5	48,5	122	51,3	13,2	1,5	91,7	39,1	15,4	33,4	6,49	4,52	43,8	24	11,5	17,4	17,6
32.2-34.8	34		119		56,9	7,86	52,3	13,6	195	38,1		10,4	66,2	36,8	18	9,1	37,2	7			20,7	7,68	75,3
37-38.9	37,5	14,8			21,6	18,2	11,5	20,2						8,79	1,39			1,75	13,8	2,12			
44.9-47.6	46,5	278	218	78,1	207	143	222	192	509	466	25,7	59,1	223	130	90,7	275	104	59,8	128	86,2	129	364	345
53.2-56	55,1				6,36	7,89		2,15	15,5					6,13			1,4						
60.8-62.7	61,7	666	341	230	482	65,4	469	43,3	687	544	243	69,4	296	251	109	710	418	82	313	250	351	363	512
67.1-67.4	67,3		106			94,5	384	63,6															
69.3-70.7	70,1	495	188	75,4	286				984	289	50,5	51,8	183	174	38,9	368	205	50,9	129	100	115	154	325
74-76.8	76		138	40,7	134	62,4	148	26,3		139			216	74,5	16,6				82,8	57,4	10,6	48,9	
79,1	79,1																					21,9	
92,1	92,1																12,7						
148.5-169.1	158						18,2								28,9		18,2						

5 main ranges of MW

Figure 41. Experion™ Electrophoresis System: Protein Profiling (Bands) Strategy offering preliminary and distinct MW range intervals, necessary for the data exploration and the comparison with the intervals, which will be later generated in this chapter.

Secondly (b), a sample of 5 healthy preliminary volunteers (T-1 [before and after the experimental procedure]) was analyzed in Experion™ Automated Electrophoresis System (Biorad®). In **Figure 42.A**, **Figure 42.B** and **Figure 42.C**, the total protein profile determined by capillary electrophoresis is shown, as described in the section Methods.

In this type of representation, each detected peak corresponds to a possible group of proteins with identical electrophoretic mobility and, therefore, in that migrate in same MW range. Please note that an error variable should be considered correspondent to the lack of accuracy offered by Experion™ analysis, and the clustering of proteins is not due to any biological process but also related to this inaccuracy.

A previous study by our research group had identified potential groups of proteins in these MW ranges, in saliva samples by LC-MS/MS (Rosa et al., 2016). These data, together with the data deposited in the OralCard (Rosa et al., 2012; Arrais et al., 2013), allowed the identification of saliva proteins by MW scale (**Figure 43** and **Figure 44**). **Figure 42.A** shows the electrophoretogram of subject 1 at 16 kDa (T-1[before and after experimental procedure]) screening a migration peak of a set of proteins. As an example of proteins migrating, the profilin-1, cystatin, prolactin and calmodulin are suitable candidates. Five protein peaks are exposed in **Figure 42** and will be further discussed in **Figure 43**. **Figure 42.B** is a preliminary electrophoretogram (T-1 [before and after the experimental procedure]) and shows that proteins present in saliva can be organized (in a visual exploratory analysis), in this preliminary sample, in those five main ranges of Molecular Weight (MW), specifically: 9.6 kDa, 16 kDa, 47 kDa, 62 kDa and 71 kDa, in (T-1 [before and after the experimental procedure]).

Each subject (**Figure 42.C**) shows different protein concentration (evaluated by the peak area (ng/µl)) post-experimental procedure, i.e., $\Delta[P]_{\text{after-before(experimental procedure)}}$).

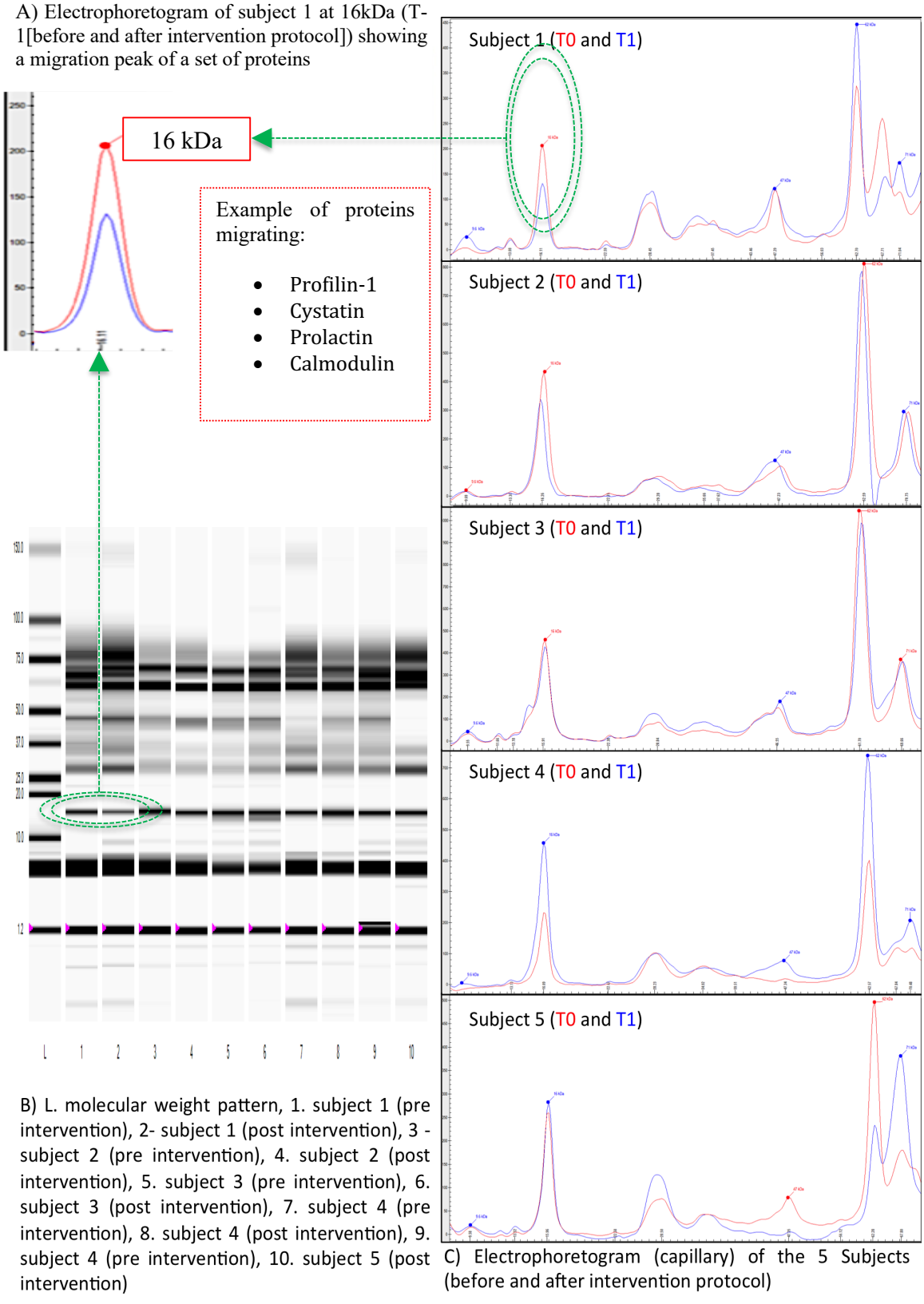


Figure 42. Pipeline Oral Protein Profile Study A) Electrophoretogram Of Subject 1 At 16 kDa (T-1[Before And After Experimental Procedure]) Showing A Migration Peak Of A Set Of Proteins; B) Electrophoretogram T-1[Before And After Experimental Procedure]). L. Mw Pattern; 1.2 - Subject 1; 3.4 - Subject 2; 5.6 - Subject 3; 7.8 - Subject 4; 9.10 - Subject 5; C) Saliva Total Protein Profile By Capillary Electrophoresis Of The 5 Subjects (T-1[Before(In Red) And After(In Blue) Experimental Procedure])

Figure 43 presents the proteins previously identified in saliva (Rosa et al., 2016), by 2D-LC-MS/MS, and listed in the OralCard (Arrais et al., 2013), which have MW range correspondent to our five main ranges.

Molecular Weight Migration Cluster by Experion (kDa)	Molecular Weight (kDa)	Uniprot Code	Protein Name	Biological Regulation	Cellular Component Organization or Biogenesis	Cellular Process	Developmental Process	Immune System Process	Localization	Metabolic Process
	8	P02814	Submaxillary gland androgen-regulated protein 3B (Proline-rich peptide P-B)*							
9.6	10	P07108	Acyl-CoA-binding protein (ACBP)	x					x	x
	11	P05109	Protein S100-A8 (Calgranulin-A)			x		x		x
	11	P01040	Cystatin-A (Cystatin-AS)	x						x
	15	P07737	Profilil-1	x						
	15	Q01469	Fatty acid-binding protein			x	x		x	x
	16	P01034	Cystatin-C (Cystatin-3)	x						x
	16	P01036	Cystatin-S (Cystatin-4)	x						x
16	16	P01037	Cystatin-SN (Cystatin-SA-1)	x						x
	16	P09228	Cystatin-SA (Cystatin-2)	x						x
	17	**P12273**	**Prolactin-inducible protein**					**x**		
	17	P12273	Prolactin-inducible protein					x		
	17	P27482	Calmodulin-like protein 3			x				
	17	P02810	Salivary acidic proline-rich phosphoprotein 1/2*							
47	47	P01009	Alpha-1-antitrypsin	x						x
62	58	P04745	Alpha-amylase 1							x
	58	P14618	Pyruvate kinase PKM							x
71	69	P02768	Serum albumin						x	
	69	P15311	Ezrin (Cytovillin)		x	x	x			

Figure 43. Proteins Identified In Saliva By Capillary Electrophoresis And Mass Spectrometry
Proteins previously identified in saliva, by 2D-LC-MS/MS, and listed in the OralCard, which have a MW range correspondent to the five main ranges (9.6 kDa, 16 kDa, 47 kDa, 62 kDa and 71 kDa).
Adapted from Arrais and colleagues (2013) and Rosa and colleagues (2016).

These could be interesting potential proteins for *in silico* correlation with the general oral protein profile (Rosa et al., 2012; Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017; Cruz et al., 2018). Note that each peak in **Figure 42.C** corresponds not to one but to several proteins that migrate with similar MW, and an error correspondent to the lack of accuracy offered by Experion™ analysis.

Additionally, **Figure 44** shows quantitative data of the expressed proteins, post intervention, for each of the five ranges of MWs:

A) Proteomic characterization and change/variability (ng/µl), on the five MWs of interests, before and after the experimental procedure;

B) the difference Δ[P] after-before (experimental procedure);

C) Protein concentration (ng/µl). A raw table of concentration (ng/µl) of each group of proteins in 5 specific MW regions of interest is shown: 9.6 kDa, 16 kDa, 47 kDa, 62 kDa and 71 kDa.

For instance, proteins from the 71 kDa range had a higher difference of expression post intervention, Δ[P] after-before (experimental procedure) (**Figure 44.B**).

The change/variability (ng/µl), shown in **Figure 44.B**, was the variable that helped calculating the sample size of the full sample study (Results ii. Expected Protein Profile), as described in the statistical method.

This analysis showed, with the significance level calculated in the statistical analysis, that there were increases and decreases after the experimental procedure:

a) 9.6 kDa: increases in all subjects;

b) 16 kDa: decreases in subjects 1 and 2 and increases in subjects 3, 4 and 5;

c) 47 kDa: increases in subjects 1, 2, 3 and 4 and decreases in subject 5;

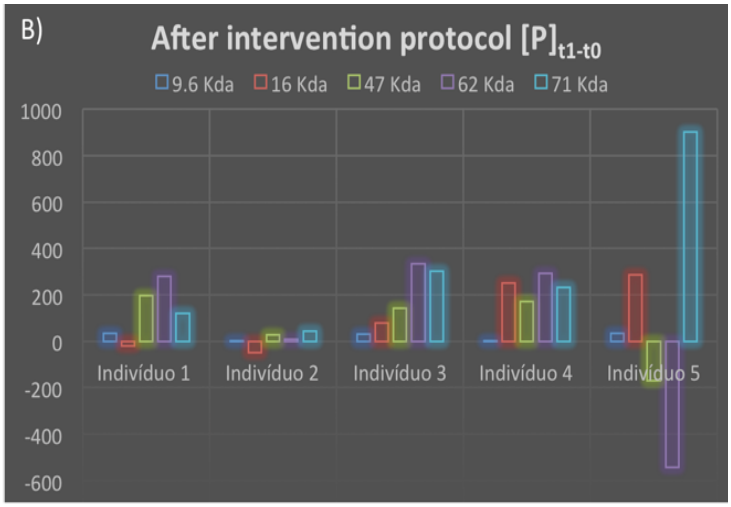
d) 62 kDa: increases in subjects 1, 2, 3 and 4 and decreases in subject 5;

e) 71 kDa: increases in subjects 1, 2 and 5 and decreases in subject 3.

Moreover, the protein concentration (ng/µl) shown in **Figure 44.C**, indicates that there is a difference in average of the 5 proteomic variables.

Subject	MW	$[P]_{t0}$	$[P]_{t1}$	$[P]_{t1-t0}$
	9.6 kDa	0	35,48	35,48
	16 kDa	184,48	164,16	-20,32
1	47 kDa	117,55	315,83	198,28
	62 kDa	241,38	519,77	278,39
	71 kDa	72,22	192,47	120,25
	9.6 kDa	12,85	15,39	2,54
	16 kDa	356,41	308,93	-47,48
2	47 kDa	176,32	204,36	28,04
	62 kDa	494,03	503,48	9,45
	71 kDa	265,07	307,98	42,91
	9.6 kDa	28,51	60,02	31,51
	16 kDa	573,26	651,11	77,85
3	47 kDa	120,6	262,24	141,64
	62 kDa	845,93	1178,57	332,64
	71 kDa	389,57	691,57	302
	9.6 kDa	0	0,81	0,81
	16 kDa	240,95	491,49	250,54
4	47 kDa	9,29	181,56	172,27
	62 kDa	304,68	596,83	292,15
	71 kDa	80,01	310,36	230,35
	9.6 kDa	20,5	54,97	34,47
	16 kDa	338,8	625,52	286,72
5	47 kDa	174,8	4,94	-169,86
	62 kDa	547,92	4,94	-542,98
	71 kDa	280,19	1182,87	902,68

A)



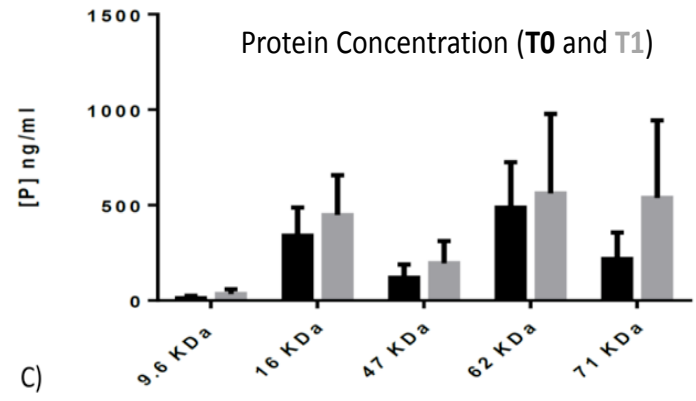


Figure 44. Quantitative Data Of The Pipeline Oral Protein Profile Study A) Proteomic Characterization And Change Or Variability (Ng/µl), On The Five MWs Of Interest, $\Delta[P]_{\text{After-Before (Experimental Procedure)}}$; B) The Difference $\Delta(T\text{-}1)_{\text{[Before And After Experimental Procedure]}}$ And C) Protein Average Concentration Of The 5 Preliminary Subjects (Ng/µl) $(T\text{-}1_{\text{[Before(In Black) And After(In White) Experimental Procedure]}})$

5.B. Expected Protein Profile

These are the results collected in the full study for the 92 subjects in the experimental and control groups. The protein profile was obtained by Experion™ Automated Electrophoresis System (Biorad®) in whole saliva samples. The total protein profile is shown both in capillary gel and quantitative electrophoretogram from [1.2;150]kDa.

The electrophoretogram range was limited to [1.2;150]kDa, because this is the migration interval of proteins present in saliva (Rosa et al., 2012; Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017; Cruz et al., 2018) and relevant to our study. The electrophoretogram refers to a cluster of proteins that migrate with the same MW, forming molecular bands. Please note that the presence of proteins in a single peak is a consequence of the low resolution of the instrument, viz. Experion™ Automated Electrophoresis System (Biorad®). A molecular band, therefore, does not refer to a specific protein, but to possible proteins that migrate to the same MW. They may or may not have specific and isolated biological functions, but during capillary electrophoresis they are separated according to their MW and polarity.

In the quantitative electrophoretograms, the fluorescence of the migration is also shown, i.e., the concentration (ng/µl) of a specific molecular band. In our study, for our sample of neurotypical young adults, concentration ranged from [-100;500] ng/µl.

In **Figure 45**, it is shown the Expected Protein Profile in T0, before the Intervention Protocol (Hipólito and Martins, 2017), of the best 40 subjects (T0).

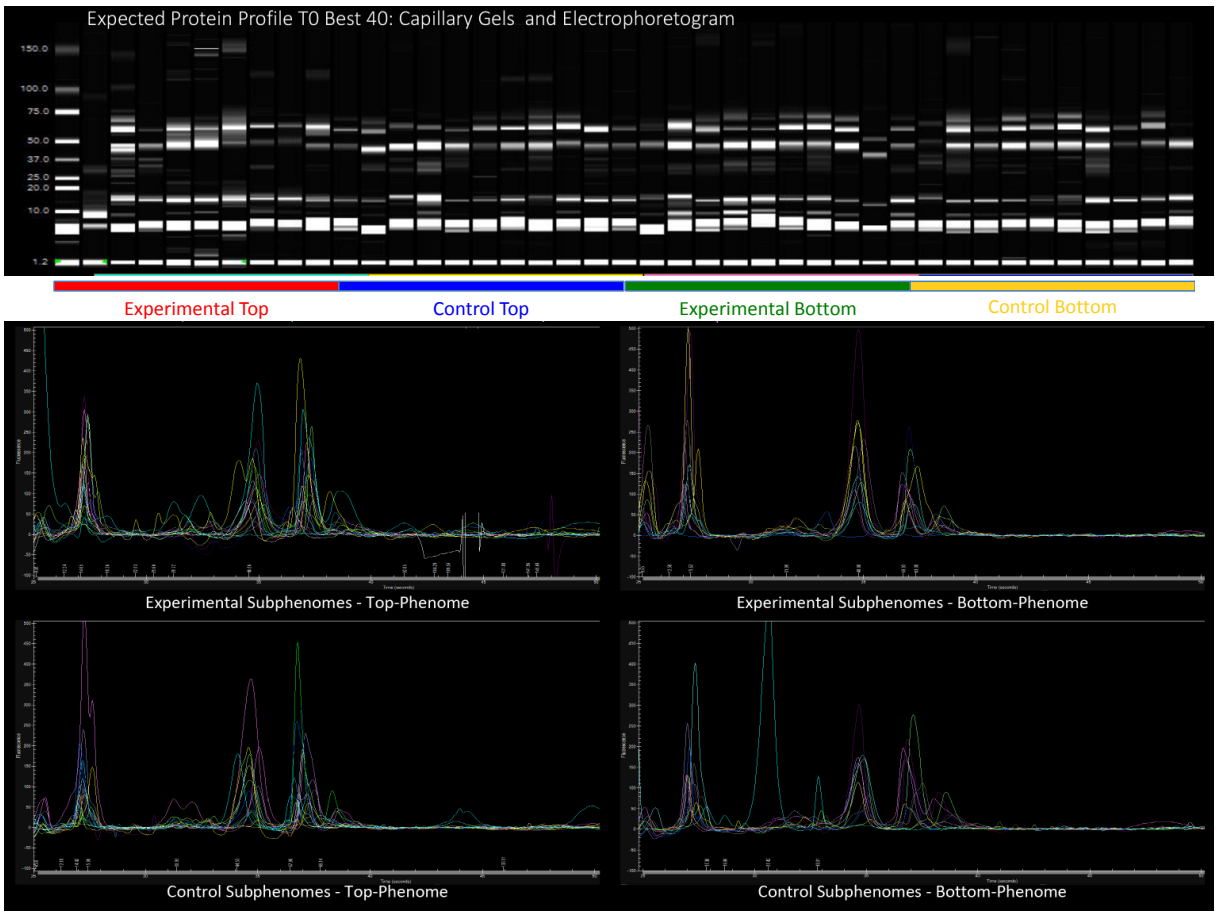


Figure 45. Capillary Gels And Electrophoretogram Profile Of The Selected Best 40 Subjects In T0
For the expected protein profile in T0, before the intervention protocol, a graphical representation is presented showing the best 10 capillary gels and quantitative electrophoretograms of each subgroup.

The best 10 capillary gels and quantitative electrophoretograms of each subgroup are presented: (1) Experimental Top (total = 23 subjects), (2) Control Top (total = 23 subjects), (3) Experimental Bottom (total = 23 subjects), and (4) Control Bottom (total = 23 subjects) Subgroups. Those best 40 subjects have scored higher in the clinical assessment (chapter 3).

Thus, by having a more significant clinical profile, they probably have, likewise, a more relevant molecular profile, i.e., a total protein profile. As shown in the first capillary gels of each group, a more heterogenous protein migration is presented, which better differentiates those first subjects from normality. I.e., those subjects with a more significant clinical profile have a distribution with a nearer [protein] of that specific group. Specifically, in the [10;30]kDa and [50;70]kDa ranges, the first subjects showed a specific and characteristic profile of protein migration.

Alternatively, in the quantitative electrophoretogram, the complexity and the difficulty to acquire protein profiles in general are shown. However, it is possible to observe: (a) a major density of protein peak dispersion in the [12;18]kDa and the [43;66]kDa MW ranges; and (b) a minor density of protein peak dispersion in the [20;40]kDa and the [70;145]kDa MW ranges. Those MW ranges show some intergroup variability and some intragroup homogeneity. Yet, with a simple visual plotting and analysis, it is impossible to advance with an objective discussion.

In **Figure 46**, the Expected Protein Profile in T1 is shown and the same best 40 subjects are presented after the Intervention Protocol, instead of before.

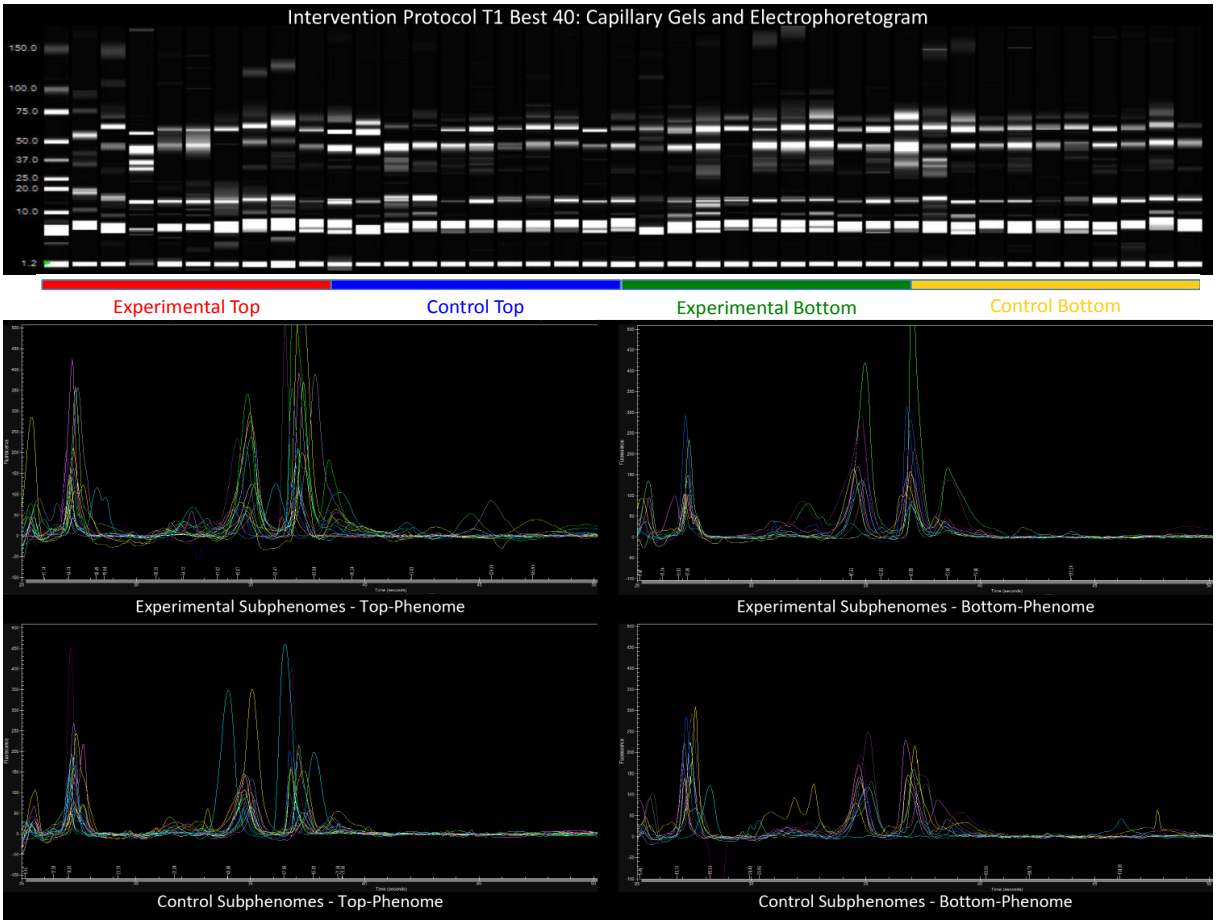


Figure 46. Capillary Gels And Electrophoretogram Profile Of The Selected Best 40 Subjects In T1
For the intervention protocol in T1, a graphical representation is presented showing the best 10 capillary gels and quantitative electrophoretograms of each subgroup.

In the 4 subgroups of study, a higher difference can be observed in the molecular profile on the (a) major and (b) minor protein peaks. However, this intergroup difference and intragroup homogeneity is more significant in **Figure 47** and **Figure 48**, where both T0 and T1 are plotted.

Figure 47 and **Figure 48** present the overall distribution of the total protein profile of our sample, the neurotypical young adults.

In **Figure 47**, the protein bands in the electrophoretic gels of the more representative subjects of the 4 subgroups in T0 and T1 are shown.

Protein profiles of 60 subjects are plotted:

(1) Experimental Top (total = 18 subjects);

(2) Control Top (total = 12 subjects);

(3) Experimental Bottom (total = 18 subjects);

(4) Control Bottom (total = 12 subjects).

Likewise, and in **Figure 48**, the electrophoretogram or the quantification graph of protein migration of the same subjects are correspondingly shown.

In **Figure 47** (on the right) and in **Figure 48** (on the bottom), a sample of proteins detected in saliva by Rosa and colleagues (2012) is, additionally, presented. Those proteins were previously identified by mass spectrometry.

Then, a sum of 55 proteins were characterized and listed in the OralCard (Arrais et al., 2013). **Figure 47** and **Figure 48** demonstrate the complexity of each molecular peak and show that a protein peak is a conjunction of several proteins that migrate in the same MW and an error correspondent to the lack of accuracy offered by Experion™ analysis.

In **Figure 47**, proteins that have a lower MW, e.g., Histatin-1, 7 kDa, migrate less and appear in the inferior zone of the capillary gel, whereas proteins with a higher MW, e.g., α-2-macroglobulin-like protein, 161 kDa, migrate more and appear in the superior zone.

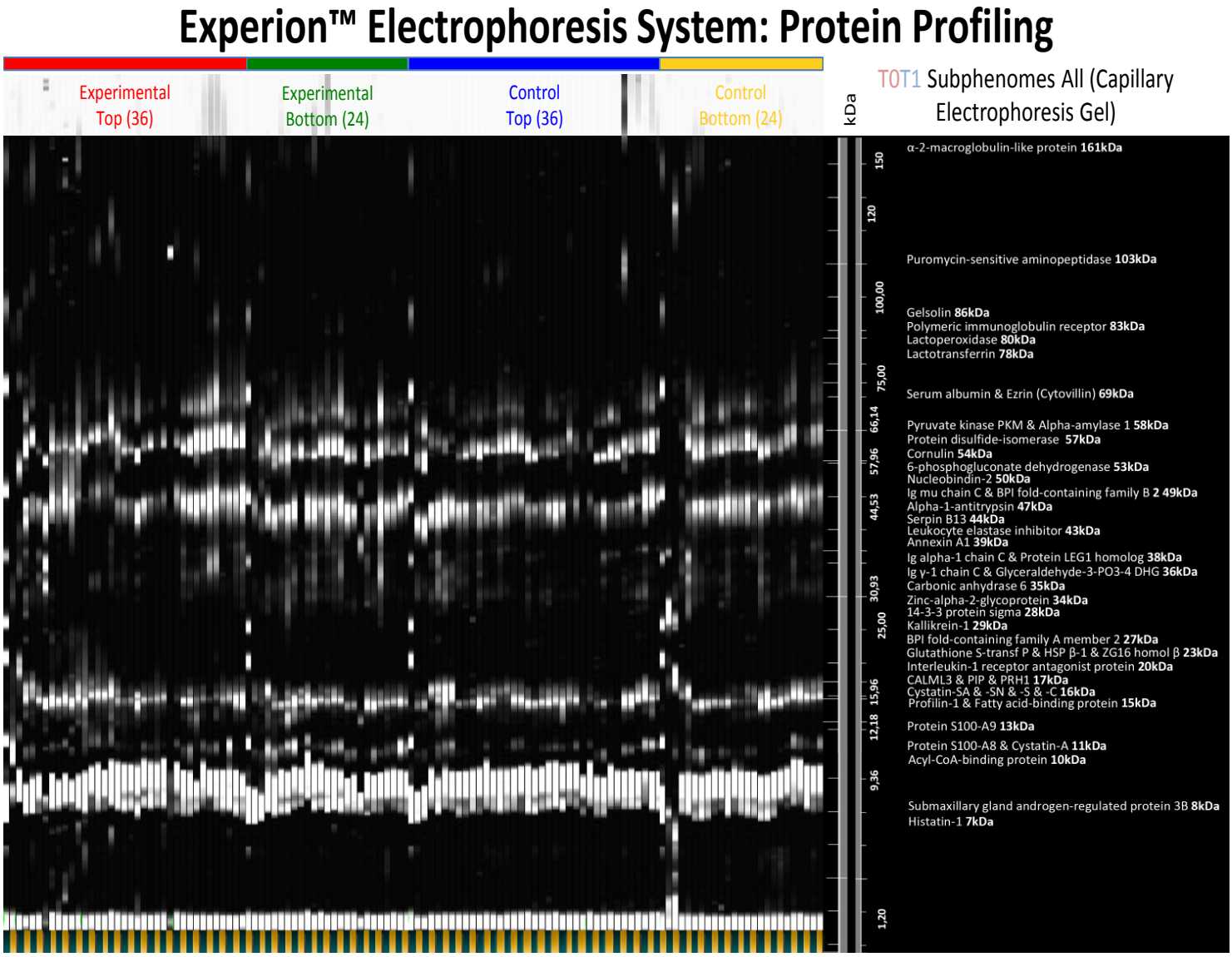


Figure 47. Experion™ Electrophoresis System: Protein Profiling (Bands) Of All Subjects In T0 And T1
Both T0 and T1 are plotted, presenting the overall distribution of the total protein profile of the neurotypical young adults. The protein bands in the electrophoretic gels of the more representative subjects of the 4 subgroups in T0 and T1 are shown. Protein profiles of 60 subjects are plotted: (1) Experimental Top (total = 18 subjects); (2) Control Top (total = 12 subjects); (3) Experimental Bottom (total = 18 subjects); (4) Control Bottom (total = 12 subjects). A sample of proteins detected in saliva by Rosa and colleagues (2012) is presented on the right. Those proteins were previously identified by mass spectrometry.

Figure 48 shows the same distribution but quantitatively plotted in an electrophoretogram, where the difference between T0 and T1 for all the subjects of each subgroup is significantly visible.

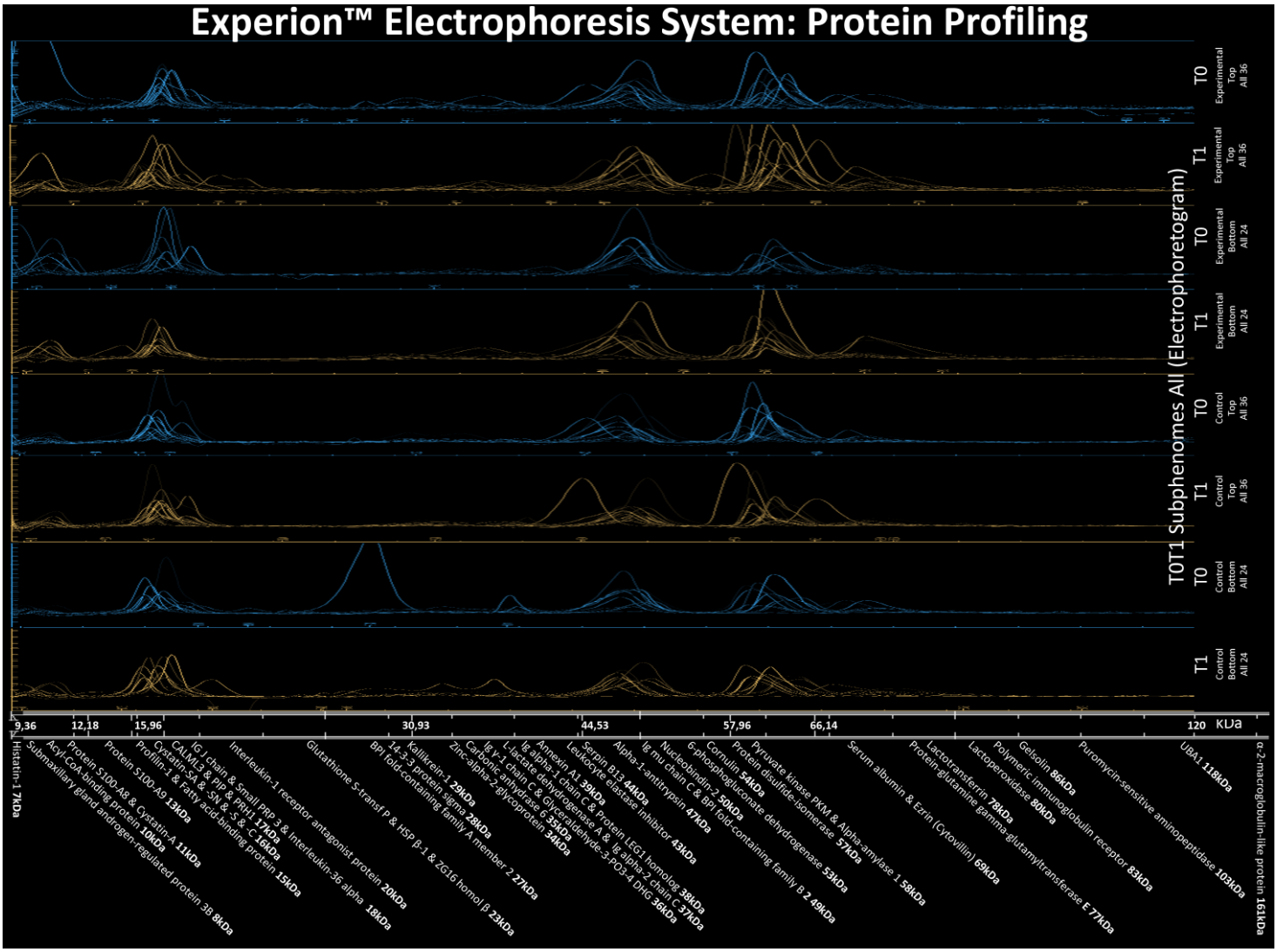


Figure 48. Experion™ Electrophoresis System: Protein Profiling (Graph) Of All Subjects In T0 And T1 Both T0 and T1 are plotted, presenting the overall distribution of the total protein profile of the neurotypical young adults. The electrophoretogram of the more representative subjects of the 4 subgroups in T0 and T1 are shown. Protein profiles of 60 subjects are plotted: (1) Experimental Top (total = 18 subjects); (2) Control Top (total = 12 subjects); (3) Experimental Bottom (total = 18 subjects); (4) Control Bottom (total = 12 subjects). A sample of proteins detected in saliva by Rosa and colleagues (2012) is presented on the bottom. Those proteins were previously identified by mass spectrometry.

This fluorescence difference, i.e., concentration (ng/µl) of a specific MW, is more meaningful for the (a) major density of protein peak dispersion in the [12;18]kDa and [43;66]kDa MW ranges.

As displayed in **Figure 45** and **Figure 48**, and more explicitly in **Figure 49**, specific protein profiles can be suggested for the 4 subgroups and for T0 and T1.

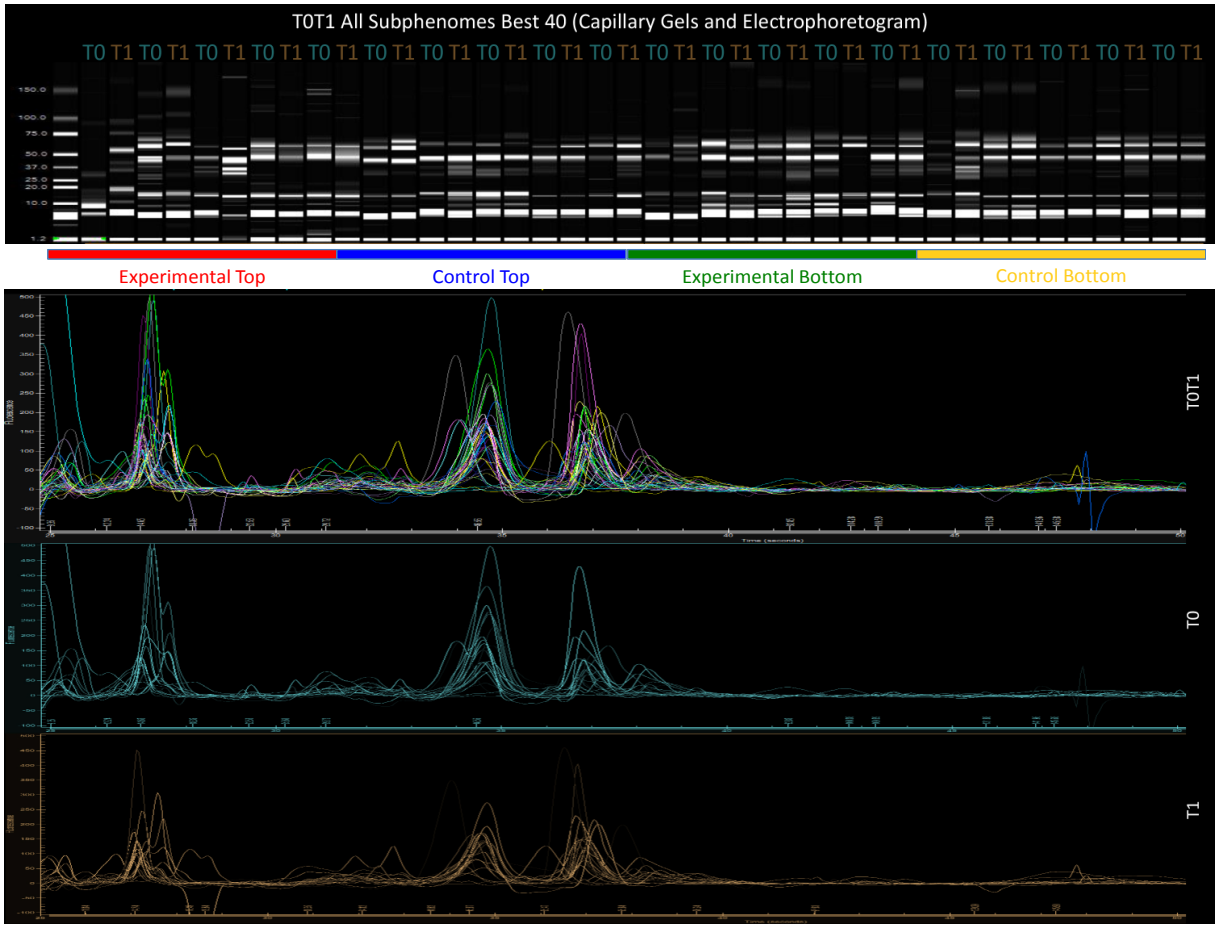


Figure 49. Capillary Gels And Electrophoretogram Profile Of The Selected Best 40 Subjects In T0, T1 And T0T1 For both the expected protein profile in T0, after the intervention protocol in T1, and the combined T0T1, a graphical representation is presented showing the best 5 capillary gels and quantitative electrophoretograms of each subgroup. Through this figure, the intergroup difference and the protein distribution are demonstrated and they can be better visualized.

Figure 49 presents the 5 best characterizing subjects of each subgroup altogether in T0, T1 and T0+T1. Like before, the capillary gels and electrophoretogram are shown.

Interestingly, in the 1st electrophoretogram, the 20 most characteristic subjects altogether (5 of each subgroup in T0+T1) have a very heterogenous protein profile.

However, when the electrophoretogram of T1 was equated against T0, the same difference in the (a) major and (b) minor density of protein peak dispersion is, similarly, observed. Nonetheless, and intriguingly, that difference is even more significant. This difference is also visible in the capillary gel (on the top), for each subgroup.

In **Figure 50**, like in **Figure 49**, the same subjects are equally plotted, but they are shown in the electrophoretogram subgroups, instead. Only the 5 more significant cognitive profiles are plotted, for both T0 and T1 together. In this figure, the intergroup difference and the protein distribution can be better visualized.

In the Experimental Top group, a more heterogenous dispersion is obtained. However, in the Experimental Bottom subgroup the dispersion is more homogenous and the peak concentration (ng/µl) in the range of [12;18]kDa and [43;66]kDa is higher.

Yet, in the Control Top group, in the [12;18]kDa range, the concentrations are higher; and lower in the [43;66]kDa MW range. Contrastingly, in the Control Bottom subgroup the concentrations are lower for both the (a) major and (b) minor density protein peaks.

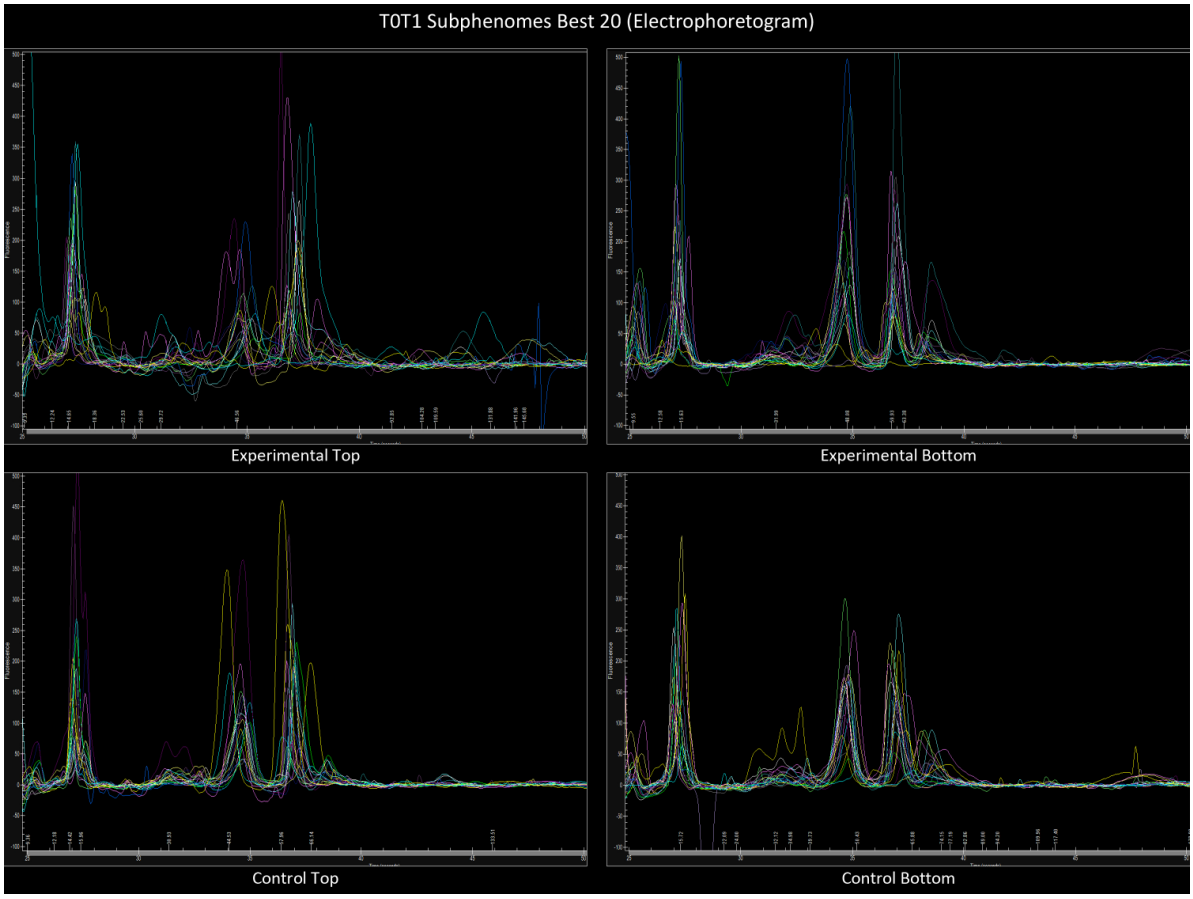


Figure 50. Electrophoretogram Profile Of The Selected Best 20 Subjects In T0T1
For the combined T0T1, a graphical representation is presented showing the best 5 quantitative electrophoretograms of each subgroup: Experimental Top, Control Top Experimental Bottom and Control Bottom. Through this figure, the intergroup difference is demonstrated and can be better visualized.

In **Figure 51**, the electrophoretograms of the same 5 subjects of the Experimental Top, Control Top, Experimental Bottom and Control Bottom subgroups are shown but separately for T0 and T1. In this figure, it is important to highlight, the intragroup difference between T0 and T1.

In the Experimental Top Subphenome, it is observed a heterogenous decrease of the concentration (ng/µl) in:

i. the (a) major [12;18]kDa and [43;66]kDa density protein peaks;

ii. the (b) minor [20;40]kDa and [70;145]kDa density protein peaks.

Likewise, in the Experimental Bottom Subphenome a decrease in the concentration is observed, but in a homogenous manner and shape.

However, in the Control Top Subphenome, an increase of the concentration in the [43;66]kDa and [20;40]kDa density protein peaks, and a decrease in the [12;18]kDa density protein peak is observed.

In the Control Bottom Subphenome, an increase of the [12;18]kDa MW range is observed.

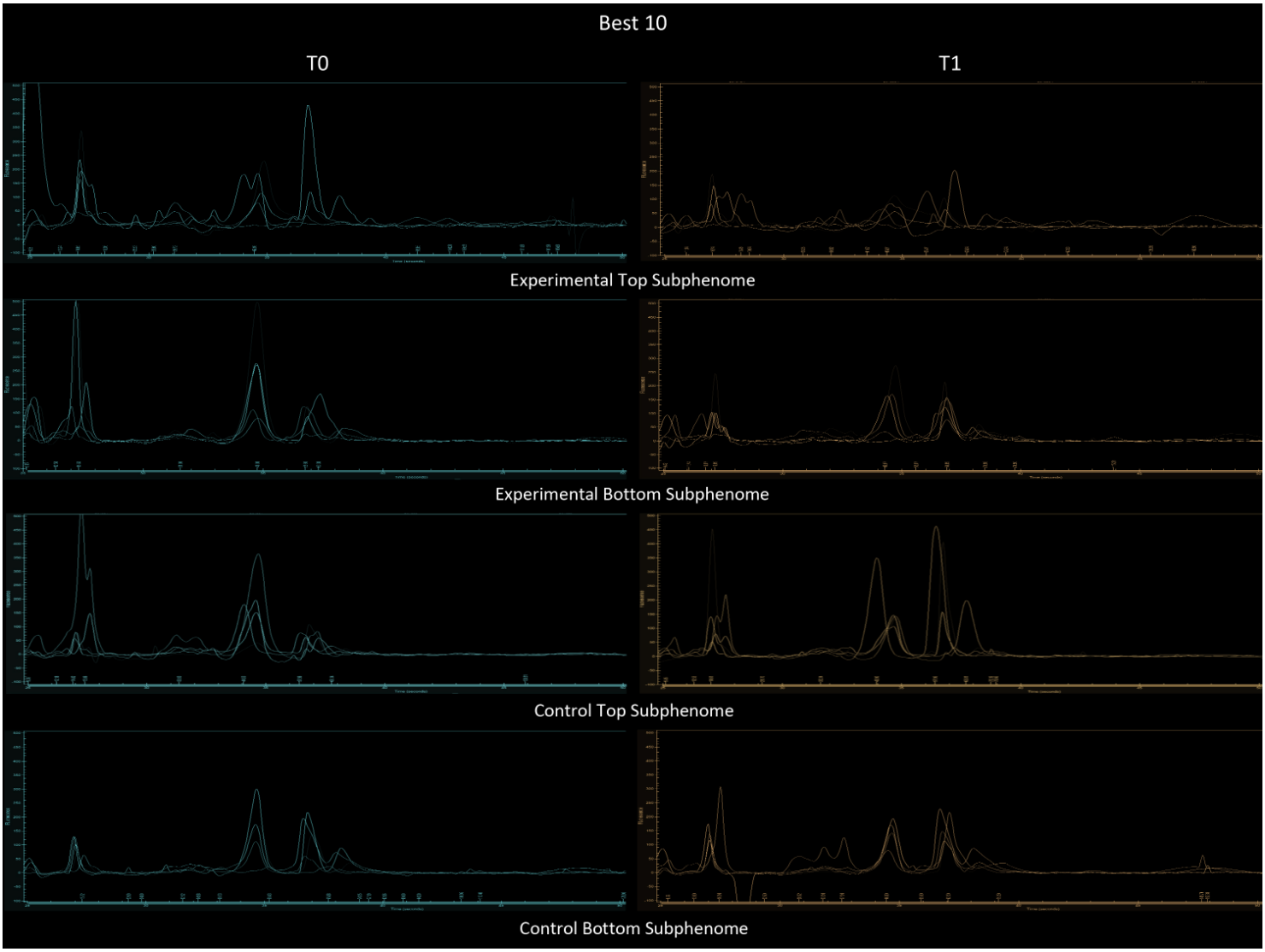


Figure 51. Electrophoretogram Profile Of The Selected Best 10 Subjects
For both the expected protein profile in T0, and after the intervention protocol in T1, a graphical representation is presented showing the best 5 quantitative electrophoretograms of each subgroup (Experimental Top – Experimental Bottom – Control Top – Control Bottom). Through this figure, the intragroup difference between T0 and T1 is demonstrated.

In **Figure 52**, the expected protein profile of the best 4 subjects, separately, is shown. Thus, individually, the most significant cognitive profiles are chosen and the Total Protein Profile, in T0 in T1, is plotted for each subgroup.

The protein profile is shown in red for the Experimental Top group, in blue for the Control Top group, in green the Experimental Bottom subgroup and in yellow the Control Bottom group.

In this figure, it is relevant to see (i) the same intergroup variability and (ii) Δ (T1-T0) peak distribution and concentration (ng/µl) difference.

From this figure, a tendency that is consistent with the previous analysis is observed. However, this visual exploration of the data offers only the opportunity to write hypotheses.

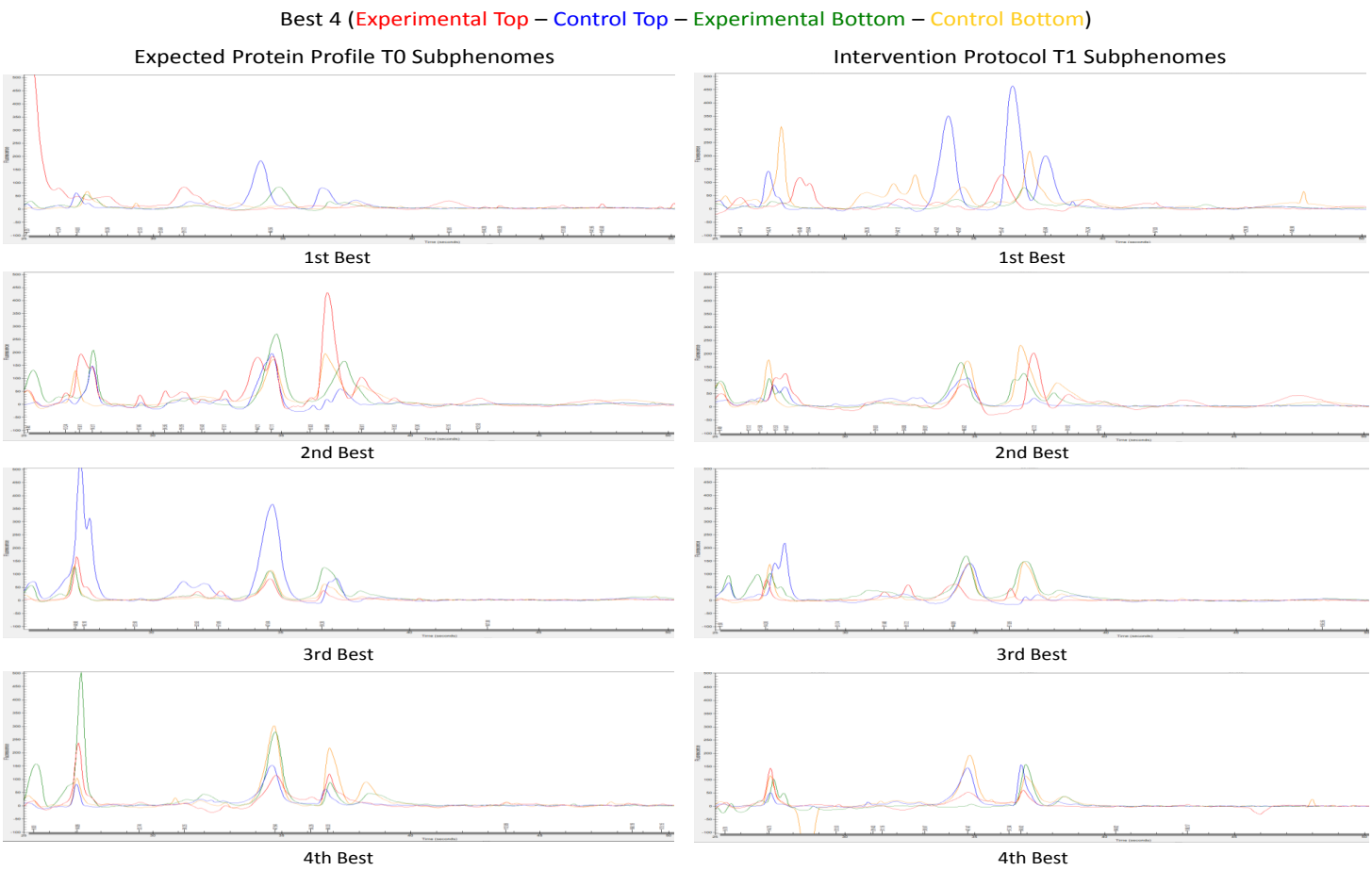


Figure 52. Electrophoretogram Profile Of The Selected Best 4 Subjects
For both the expected protein profile in T0, and after the intervention protocol in T1, a graphical representation is presented showing the best 4 quantitative electrophoretograms of each subgroup separately in a different color: Experimental Top (in red), Control Top (in blue), Experimental Bottom (in green), and Control Bottom (in yellow).
The intergroup variability and the Δ (T1-T0) peak distribution and concentration (ng/µl) difference are demonstrated.

To better understand the visual data of the capillary gel and the quantitative data of the electrophoretogram, we proceeded with the creation of a toolbox for exploration and visualization of this data, comprising the development of specific algorithms.

SalivaTec lab has already developed a strategy for the visualization of electropherogram profiles in salivary samples (Cruz, Esteves, Fernandes, Martins, Silva, Sousa, Rosa, Correia, Arrais and Barros, 2017; Cruz et al., 2018), which we complemented further with MODeLING.Vis.

5.C. MODeLING.Vis: Development Of A Protein Visualization Tool

Are there categorical differences in the protein profiles matching our mental health strata?
Could an unsupervised learning analysis find corresponded electrophoretic signatures?
Could MODeLING.Vis cluster proteins with a high discriminative power?

MODeLING.Vis is a GUI toolbox created to analyze electrophoretic data. Firstly, the full raw electrophoretogram of the Expected Protein Profiles of the 92 neurotypical

young adults was obtained. Then Experion™ Imaging software exported it to a comma-separated values file, ".csv". This exported raw profile was treated with the same preliminary strategy as in the Pipeline Oral Protein Profile Study, with the raw data of the 22 control subjects (T-1).

This raw data, a preliminary Expected Protein Profile workbook, can be consulted in: https://tinyurl.com/EPPStrategyDataExport (retrieved July 21, 2020; *in* Annex K: External Support).

This workbook is consisted of 6 worksheets, which demonstrates the preliminary strategy applied to the database – 6 stages were executed.

The first worksheet (1st stage: Total) shows the total raw data of the 12 electrophoretic runs performed for all the samples of neurotypical young adults.

The second worksheet (2nd stage: Total Reviewed) reviewed the previous one, showing only the Mol. Wt. (kDa) and Concentration (ng/µl) for each sample.

The third worksheet (3rd stage: Total Rounded) rounded the previous variables into decimals, as we did in the Pipeline Oral Protein Profile Study, and added the new variable "Order" to help sorting the samples.

In the fourth worksheet (4th stage: Total Subgroups Sorted) the following variables were added: Subphenome, Mol. Wt. (kDa) Color and Molecular Band. A Subphenome is a variable used to define which subgroup the sample belonged to: ES = (i), the top phenome of the Experimental group; CS = (ii) the top phenome of the Control group; EI = (iii) the bottom phenome of the Experimental group; and CI = (iv) the bottom phenome of the Control group. Mol. Wt. (kDa) Color is a variable showing the respective RGB color correspondent to each group. Molecular Bands is a variable that (i) sorted the rounded Mol. Wt. (kDa) according to a crescent kDa and (ii) showed the respective RGB color correspondent, from which electrophoretic run was executed.

In the fifth worksheet (5th stage: Total Clustered), in the 1st part, the variable MolecularBands was repeated according to the number of electrophoretic runs it detected (MolecularBandsRep). Then, in the 2nd part, the variable MolecularBandsRep was colored according to the subgroup the sample belonged to, using the algorithm "Excel VLOOKUP Function". This 5th stage originated the variable MolecularBandsSubgroups.

In the sixth worksheet (6th stage: EPPStrategyDataForMOdeLINGVis), the preliminary final database is shown, which is a triple entry table. In this worksheet, the previously acquired variables (Sample Number; Molecular Bands Subgroups; Concentration (ng/µl)) were used for the creation of the final table.

Subsequently, this preliminary final database: "EPPStrategyDataExport" database was treated to be imported to MATLAB.

First, the section: "Present in the following subphenomes" was added, which comprised binary variables (present/absent) to identify in which subgroup the Molecular Bands were present.

Secondly, the previously obtained preliminary triple entry table was added to the Molecular Bands Summary for each Subject (ex: D01309).

This final full raw electrophoretogram was named "ExportForMOdeLINGVis" and can be consulted in: https://tinyurl.com/ExportForMOdeLINGVis (retrieved August 08, 2020; *in* Annex K: External Support). Lastly, the database was implemented into the MODeLING.Vis toolbox and the variables were imported into arrays.

Those arrays indexed a linear matrix of the variables: Molecular Bands Subgroups (kDa) and Concentration (ng/µl) of each sample (subject).

One of the limitations of the exploration of the data with Experion Imaging software (Biorad®, Hercules, CA) was its incapacity for generating MW intervals and clustering the subjects according to those intervals.

Therefore, we have generated a toolbox for unsupervised/supervised machine learning. The GUI MATLAB code is accessible online (https://www.limmit.org/uploads/2/6/8/4/26841837/modeling.vis.zip, retrieved August 08, 2020; *in* Annex K: External Support), in the LIMMIT Lab, Faculty of Medicine, University of Lisbon website, as a free and open-source MATLAB toolbox, and it is shown in **Figure 53**.

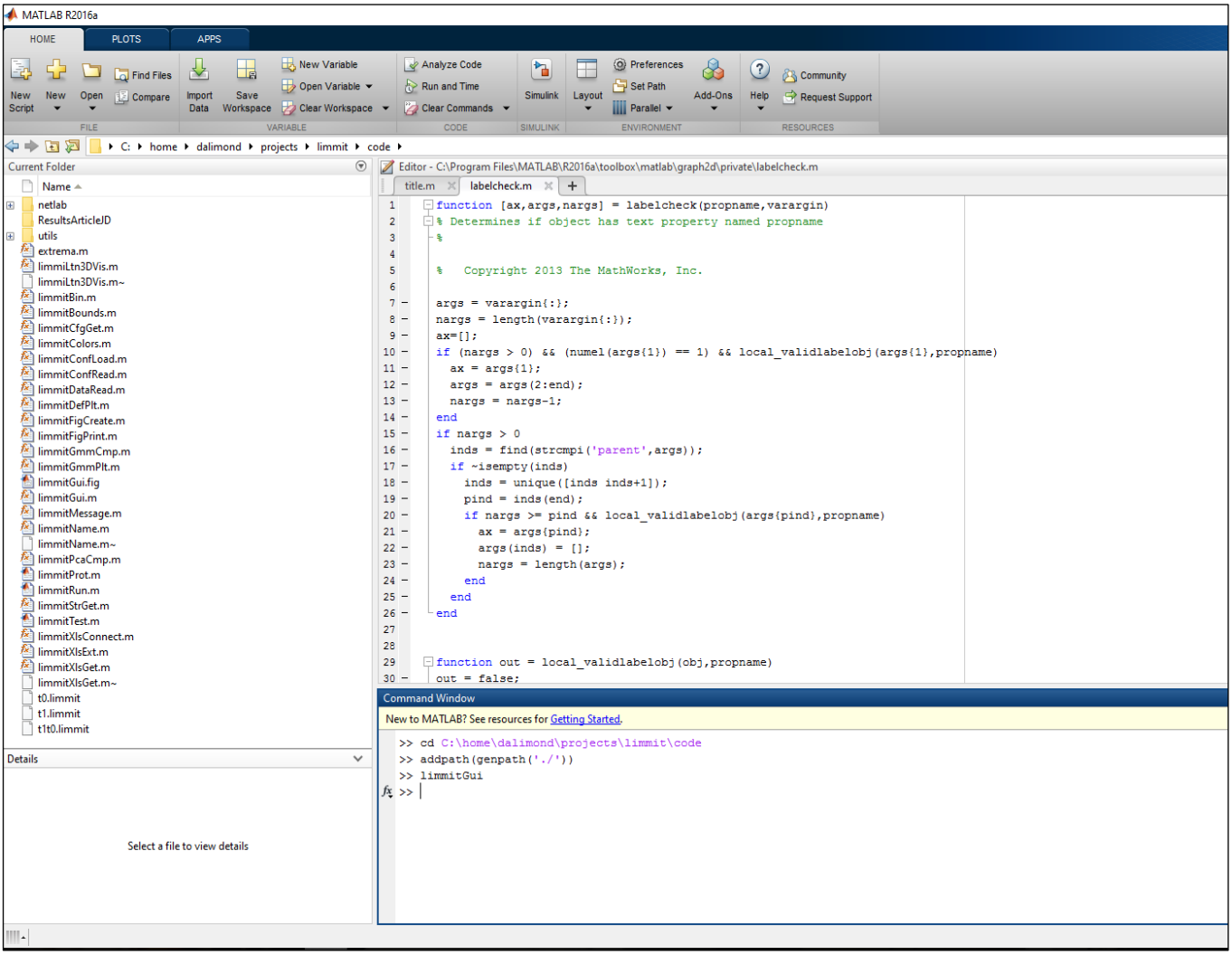


Figure 53. MODeLING.Vis Code MATLAB R2016a. Sample of the code created to analyze electrophoretic data, which is fully accessible online (https://www.limmit.org/uploads/2/6/8/4/26841837/modeling.vis.zip).

MODeLING.Vis comprehends 3 phases:
(i) Data Visualization;
(ii) Data Exploration;
(iii) Data Mining.

The first objective of (i) Data Visualization was transforming the independent continuous variable of MWs into molecular intervals through an algorithm based on the Expectation Maximization (EM) iteration. This method aims to find maximum likelihood or maximum *a posteriori* estimates of parameters in statistical models. These models generally depend on unobserved latent variables (Do, Chuong and Batzoglou, 2008).

The algorithm comprehended not only the EM iteration, but a definition of other functions that allowed the definition of concentration (ng/µl) thresholds and the quantity of MW intervals (kernels) we wanted to discover. As shown in **Figure 54**, the number of kernels was set to 13 because it was the best algorithm to treat our protein profile and the dispersion of our MW.

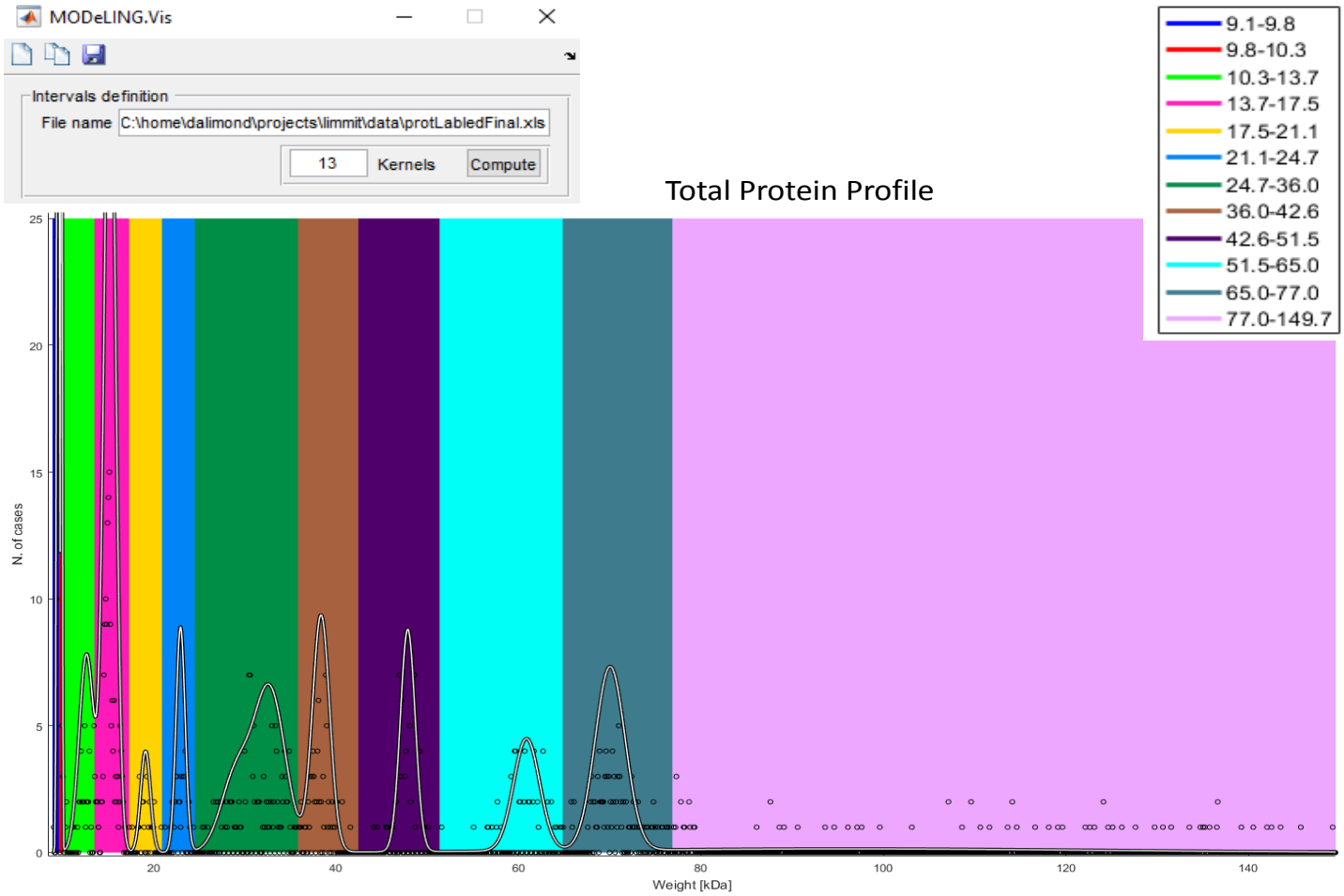


Figure 54. MODeLING.Vis Data Visualization
Use of the MODeLING.Vis with EM iteration, delimiting the concentration (ng/µl) thresholds and the quantity of MW intervals (kernels = 13). It provided the identification of the following significant ($p<0.05$) intervals of MW: A) [9.1;9.8]kDa, B) [9.8;10.3]kDa, C) [10.3;13.7]kDa, D) [13.7;17.5]kDa, E) [17.5;21.1]kDa, F) [21.1;24.7]kDa, G) [24.7;36]kDa, H) [36;42.6]kDa, I) [42.6;51.5]kDa, J) [51.5;65]kDa, K) [65;77]kDa, and L) [77;149.7]kDa.

Furthermore, this application was designed to import from other databases - other than human salivary electrophoretograms - and it had already been positively tested. The possibility of defining the number of kernels gives the researcher the control over the data exploration of his specific dataset and does not limit it to the constraints of a restricted unsupervised machine learning.

For our specific data, and for the number of fixed intervals we wanted to find and to compare between the 4 subgroups, we defined it to 13 kernels. This decision provided us with the following significant ($p<0.05$) intervals of MW: A) [9.1;9.8]kDa, B) [9.8;10.3]kDa, C) [10.3;13.7]kDa, D) [13.7;17.5]kDa, E) [17.5;21.1]kDa, F) [21.1;24.7]kDa, G) [24.7;36]kDa, H) [36;42.6]kDa, I) [42.6;51.5]kDa, J) [51.5;65]kDa, K) [65;77]kDa, and L) [77;149.7]kDa, as shown in **Figure 53**.

Similarly, these intervals are consistent with the ones discovered by the visual analysis of the capillary gels and quantitative electrophoretograms. And these intervals are equally also consistent with those discovered in the preliminary analysis of the 22 control subjects.

Hence, (a) the major density of protein peak dispersion - [12;18]kDa and [43;66]kDa - was statistically and relevantly subdivided into: C) [10.3;13.7]kDa, D) [13.7;17.5]kDa, E) [17.5;21.1]kDa, I) [42.6;51.5]kDa, and J) [51.5;65]kDa.

Similarly, (b) the minor density of protein peak dispersion - [20;40]kDa and [70;145]kDa - was statistically and relevantly subdivided into: F) [21.1;24.7]kDa, G) [24.7;36]kDa, H) [36;42.6]KDa, K) [65;77]kDa, and L) [77;149.7]kDa.

From this analysis, a new range of density of protein peak dispersion was discovered in the lower molecular range, which offered significant relevance to our specific molecular data dispersion - (c) the lower MW density protein peaks: A) [9.1;9.8]kDa and B) [9.8;10.3]kDa.

All data visualization and analysis are offered as an easy access tool for the researcher, who may simply update his proteomic dataset and evaluate how the offered solution reflects his data and his hypothesis, as shown in **Figure 54**.

Subsequently, our GUI provided us (ii) Data Exploration for hypothesis setting and testing. The toolbox was designed to explore not only one type of dataset, but to integrate other datasets acquired for the same sample of subjects. As it is, the researcher can feed multiple clinical and molecular datasets: e.g., clinical evaluations, genomic data, immune detection data, etc.

The main objective of this toolbox is to be a work in progress, which permits (i) the integration of multiple omics datasets and (ii) the visual access to explore all the information of the subject, in particular, and of the whole sample, in general. This approach (a) gives the researcher the opportunity to better conduct his multimolecular approaches in datasets (which tend to be multiple) and (ii) addresses a possible solution for the increasingly big data characteristics of omics methods.

Moreover, as shown in **Figure 55**, the researcher can define: a) colors, b) type of symbol and c) size for the clustering of his subgroups. This configuration eases the identification of specific clusters and makes hypothesis testing more visible.

In our study, we created a solution to import T0, T1 and Δ (T1-T0) datasets, and defined the supervised search of the 4 subgroups. In this part of the data mining, we wanted to feed the algorithm with a specific classification in order to learn and recognize the 4 specified labels which are our subgroups.

Moreover, we created the threshold variable for the independent variable (in the case of the electrophoretic data: concentration in ng/µl). The threshold offers the researcher the possibility to define how many subjects he wants to plot according to the T0, T1 and ((T1-T0) intergroup variability or effect size.

Intergroup statistical testing is performed by simple PCA analysis, as the data that is routinely fed into the toolbox and its algorithms needs an orthogonal linear transformation, which transforms the data into a new coordinate system.

In **Figure 55**, the data exploration of our electrophoretic dataset for T0 in MODeLING.Vis is shown. As mentioned, we had the option to define the threshold to 2500 ng/µl, because it better fits our data.

Then, we set the analysis to T0 and chose the Data Visualization of our electrophoretic data ("ExportForMOdeLINGVis") and the interval definition acquired before. Finally, we defined colors and symbols for our subgroups.

In this analysis, it is shown that data clusters in only 2 PCA components and that there is a small, but not very significant, separation of the Experimental Top (red square) and Control Bottom (yellow circle) subgroups.

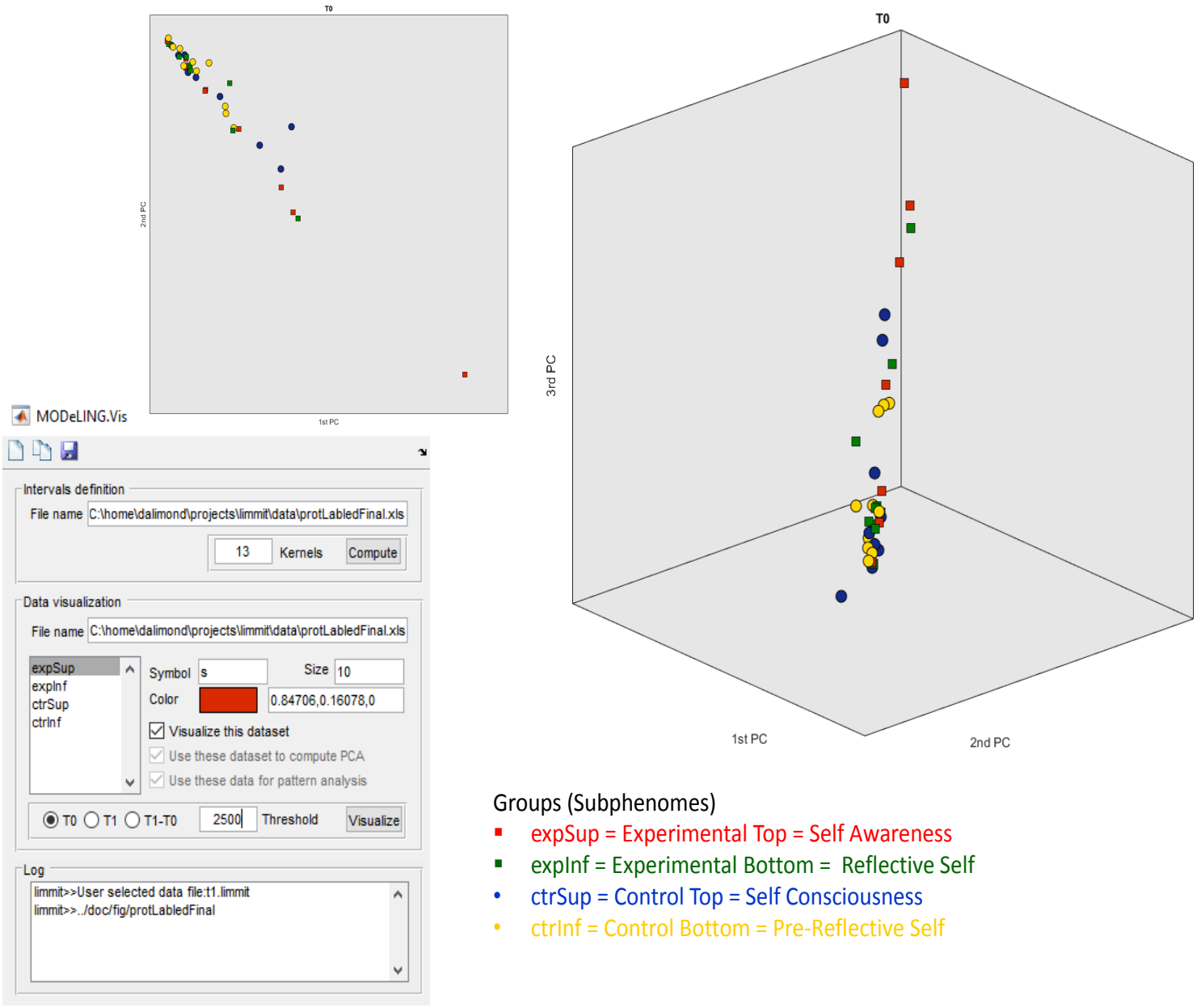


Figure 55. MODeLING.Vis Data Exploration T0
Exploration of the electrophoretic dataset for T0, defining the threshold to 2500 ng/µl. Experimental Top is shown in red; Experimental Bottom is shown in green; Control Top is shown in blue, and Control Bottom is shown in yellow. Data clusters in only 2 PCA components are represented. A small, but not significant ($p>0.05$), separation of the Experimental Top (red square) and Control Bottom (yellow circle) subgroups is presented.

In **Figure 56**, the data exploration of our electrophoretic dataset for T1 in MODeLING.Vis is shown.

The same parameters were set. This data exploration presents data clustering in 3 PCA components, and there is a more relevant separation of the Experimental Top (red square) and Control Bottom (yellow circle) subgroups (when compared to T0), which is not visually perceived.

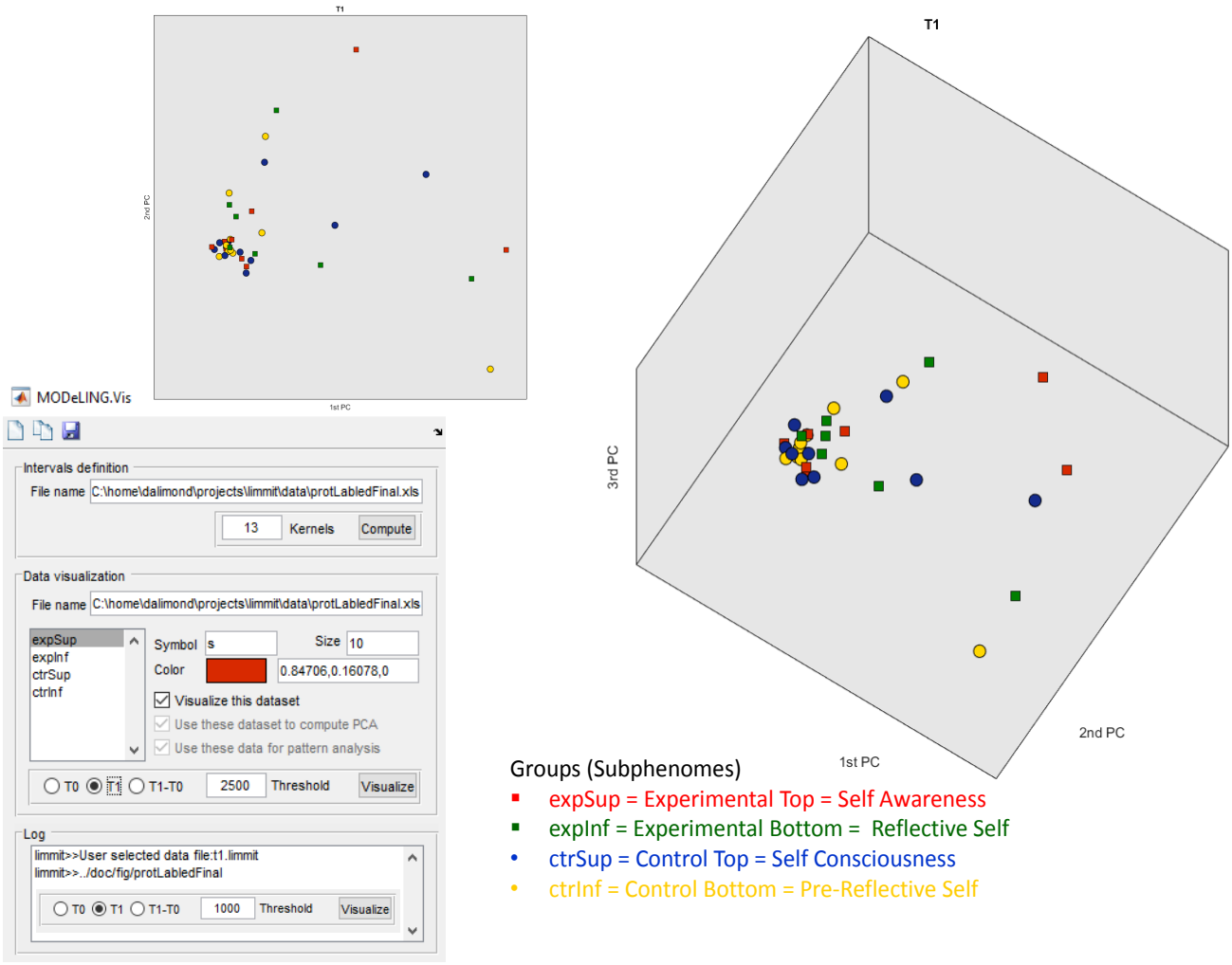


Figure 56. MODeLING.Vis Data Exploration T1
Exploration of the electrophoretic dataset for T1, defining the threshold to 2500 ng/µl. Experimental Top is shown in red; Experimental Bottom is shown in green; Control Top is shown in blue, and Control Bottom is shown in yellow. Data clusters in 3 PCA components are represented. A more relevant separation of the Experimental Top (red square) and Control Bottom (yellow circle) subgroups (when compared to T0) is presented.

However, in **Figure 57**, which shows the data exploration of our electrophoretic dataset for Δ (T1-T0), this separation is more evident. Likewise, the same parameters were set. Nevertheless, in this analysis, data clusters in 3 PCA components and there is a significant separation of our cluster of subjects, i.e., clustering in subgroups. The square symbols (Experimental subgroups), distributed along the top 2PCA and 3PCA axis, are separated from the circle symbols (Control subgroups), distributed along the bottom 2PCA and bottom 1PCA axis, with statistical relevance. This offers some consistency for a possible electrophoretic profile separation inter Experimental and Control groups.

Additionally, but not so significantly, there is a separation of the red squares (Experimental Top group) and green squares (Experimental Bottom group), alongside PCA1, and of the blue circles (Control Top group) and yellow circles (Control Bottom group), also alongside PCA1. This offers some consistency for a possible electrophoretic profile separation intra Experimental and Control groups, but it is not statistically relevant for the threshold ((T1-T0) and effect size of 2500 ng/µl.

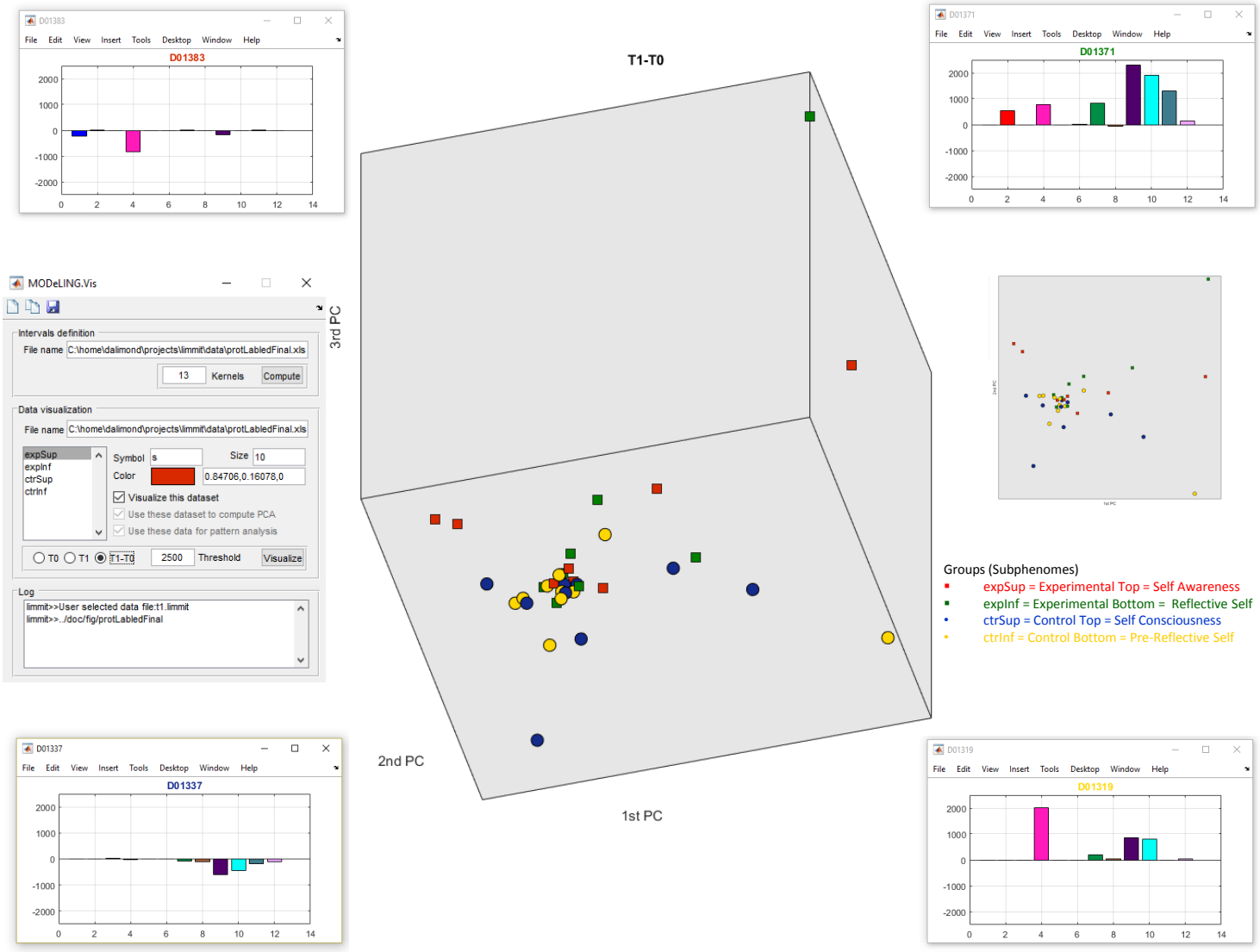


Figure 57. MODeLING.Vis Data Exploration T1-T0
Exploration of the electrophoretic dataset for T1-T0, defining the threshold to 2500 ng/µl. Experimental Top is shown in red; Experimental Bottom is shown in green; Control Top is shown in blue, and Control Bottom is shown in yellow. Data clusters in 3 PCA components are represented. A significant separation ($p<0.05$) of the square symbols (Experimental subgroups), distributed along the top 2PCA and 3PCA axis, and the circle symbols (Control subgroups), distributed along the bottom 2PCA and bottom 1PCA axis, is presented with statistical relevance.
As an example of this separation, it is shown a comparison of the electrophoretic profiles of the subject D01383 (Experimental Top subgroup (1)), subject D01371 (Experimental Bottom subgroup (3)), subject D01337 (Control Top subgroup (2)) and subject D01319 (Control Bottom subgroup (4)).

More significant is the separation and clustering, both alongside the 3PCA components, between (i) the red squares (Experimental Top group) vs. the blue circles (Control Top group), and (ii) the green squares (Experimental Bottom subgroup) vs. the yellow circles (Control Bottom subgroup). This data exploration offers some consistency for a possible electrophoretic profile separation between (i) the Experimental Top and Control Subphenomes, and (ii) the Experimental Bottom and Control Subphenomes.

This clustering lacks proper hypothesis testing to evaluate the exact concentration (ng/µl) of the Δ (T1-T0), which is the main limitation of this analysis. Notwithstanding,

it may offer the opportunity to define a consequent hypothesis, i.e., to better profile and stratify substrata in our total electrophoretic data. Therefore, and as a conceivable solution for this limitation, the subsequent data analysis was executed. The toolbox has the objective of (iii) Data Mining the individual molecular profile (subject to subject/sample to sample) and comparing it to the whole sample (neurotypical young adults). As referred, it was designed to integrate multiple clinical and molecular datasets.

As such, in **Figure 57**, we present an example of the comparison of 4 subjects after the 3 phases of the GUI toolbox: (i) Data Visualization, (ii) Data Exploration and (iii) Data Mining. We show, fittingly, subject D01383 (Experimental Top subgroup (1)), subject D01371 (Experimental Bottom subgroup (3)), subject D01337 (Control Top subgroup (2)) and subject D01319 (Control Bottom subgroup (4)).

These 4 subjects, also plotted in **Figure 52** (with the same colors), are the most significant subjects of each subgroup and represent the specific and characterizing stratum of the electrophoretic profile of their subgroup.

From the molecular intervals found, those which are more relevant are the red (No. 2) and the pink (No. 4) ones, which correspond to B) [9.8;10.3]kDa and D) [13.7;17.5]kDa in the lighter MW range (**Figure 54**). Also, with a correspondent relevance are the purple (No. 9) and light blue (No. 10) ones, which correspond to I) [42.6;51.5]kDa and J) [51.5;65]kDa in the heavier MW range (**Figure 54**).

The Δ (T1-T0) ng/µl of the B) [9.8;10.3]kDa and D) [13.7;17.5]kDa molecular weight range is: (1) for subject D01383 (representing the Experimental Top group) ≅ 50 ng/µl and -900 ng/µl; (3) for subject D01371 (representing the Experimental Bottom group) (600 ng/µl and 800 ng/µl; (2) for subject D01337 (representing the Control Top group) ≅ -10 ng/µl and -30 ng/µl; and (4) for subject D01319 (representing the Control Bottom group) ≅ 0 ng/µl and 2000 ng/µl.

Those MW ranges [B) [9.8;10.3]kDa and D) [13.7;17.5]kDa] are characteristic of molecules which have been documented to cross the blood-brain barrier (Banks, 2009). Please note that an error variable should be considered correspondent to the lack of accuracy offered by Experion™ analysis and the identified MW ranges, and that this consideration should take this inaccuracy in account but also the process of protein degradation observed and well documented in saliva.

Different molecular characteristics are associated with the capacity of crossing the blood-brain barrier, which is a major field of study in neuropharmacology (Pardridge, 2007; Salameh and Banks, 2014). However, in the interest of molecular biology, it is very important to understand the physiology and biological function of those small molecules.

Banks (2015) has described the biological characteristics of those small peptides crossing the blood-brain barrier and correlated them to the neuropeptide response. Likewise, this light MW [B) and D)] range was earlier associated with neuroinflammatory response (de Vries et al., 1997), but, more recently Erickson and Banks (2018) described it as part of the neuroimmune axes of the blood-brain barriers and blood-brain interfaces.

Please note that an error variable should be considered and correspondent to the lack of accuracy offered by Experion™ analysis and the identified MW ranges. These considerations should take this inaccuracy in account but also the process of protein degradation observed and well documented in saliva and fully detailed in chapter 4.

Hence, the importance of these small peptides, detected by capillary electrophoresis in this light MW [B) and D)] range, for the physiological and pathological regulation of neurotypical/atypical subjects.

The Δ (T1-T0) ng/µl of the I) [42.6;51.5]kDa and J) [51.5;65]kDa molecular weight range is: (1) for subject D01383 (representing the Experimental Top group) ≅ -100 ng/µl and 0 ng/µl; (3) for subject D01371 (representing the Experimental Bottom

group) ≅ 2200 ng/µl and 2000 ng/µl; (2) for subject D01337 (representing the Control Top group) ≅ -600 ng/µl and -400 ng/µl; and (4) for subject D01319 (representing the Control Bottom group) ≅ 900 ng/µl and 800 ng/µl.

Those MWs are characteristic of a group of bigger systemic molecules, which have not been documented to cross the blood-brain barrier (Banks, 2009). Hence, they are not directly relevant for our study as they are not brain produced proteins, but are indirectly important as systemic protein expression. In another oriented type of study design, they could be interesting for heavier protein molecular profiling of the subjects with systemic produced proteins. Specifically, this heavier MW range is important for the comprehension of the role of bigger proteins and protein complexes in non-neuropsychiatric diseases. As an example, shown in **Figure 47** and **Figure 48**, proteins, such as alpha-1-antitrypsin, 47 kDa (Fregonese and Stolk, 2008), pyruvate kinase PKM, 58 kDa (Desai et al., 2014) and serum albumin, 69 kDa (Sun et al., 2016), are important markers for hereditary, metabolic, and cardiovascular diseases, respectively.

As a hypothesis for better conduction of our study and better statistical generalization power, it is important to quantify those lighter MW ranges, i.e., B) [9.8;10.3]kDa and D) [13.7;17.5]kDa with more accurate sensibility and sensitivity.

The quantification and identification of those lighter MW ranges is imperative to better understand what is affecting this electrophoretic profile. However, the acquisition of data with the Experion™ automated electrophoresis system (Biorad®) offers low capacity to discriminate which proteins are reflecting that stratum. This low capacity is explained because electrophoretic patterns refer to a conjunction of proteins that migrate to the same MW and not a specific and single protein migration, and an error correspondent to the lack of accuracy offered by Experion™ analysis.

As such, a better acquisition method, with higher sensitivity, sensibility and discrimination, is necessary to explain which proteins are changing.

The identification of those specific proteins can improve our understanding of how they are influencing the total protein profile in those light molecular ranges: [9.1;30]kDa. This specific molecular range reflects the whole spectrum of peptides, peptide complexes, and small proteins that migrate in electrophoresis in (c) the lower MW density protein peaks.

For that purpose, simultaneous immune detection, with specific antibodies for specific peptides in those MW ranges, is mandatory for an adequate quantification and discrimination. Moreover, this quantification offers a suitable possibility for multivariate hypothesis testing of the identified peptides and proteins, by immune detection.

Following the work of Banks and colleagues (2009, 2014, 2015, 2018), and the objective of our study design, immune detection of the peptides and the small proteins implicated in the neuropeptide and the neuroinflammatory response should be addressed. Not only is this identification important for better characterization of the protein strata in this light MW range, but also for the better understanding of how they affect neurotypical young adults.

5.D. Neuroinflammatory And Neuropeptide Panel Choice

MODeLING.Vis analysis addressed the hypothesis of better understanding which proteins are responsible for the changes observed in the MW range of [9.1;30]kDa.

The [9.1;30]kDa MW interval corresponds to small proteins like the ones already identified in saliva by Rosa and colleagues (2012), and listed in the OralCard by Arrais and colleagues (2013) (**Figure 47** and **Figure 48**): Histatin-1, 7 kDa; Submaxillary gland androgen-regulated protein 3B, 8 kDa; Acyl-CoA-binding protein, 10 kDa; Protein S100-

A8, 11 kDa; Cystatin-A, 11 kDa; Protein S100-A9, 13 kDa; Profilin-1, 15 kDa; Fatty acid-binding protein, 15 kDa; Cystatin-SA, 16kDa; Cystatin-SN, 16 kDa; Cystatin-S, 16 kDa; Cystatin-C, 16 kDa; CALML3, 17 kDa; PIP, 17 kDa; PRH1, 17 kDa; Interleukin-1 receptor antagonist protein, 20 kDa; Glutathione S-transf P, 23 kDa; HSP β-1, 23 kDa; ZG16 homol β, 23 kDa; BPI fold-containing family A member, 27 kDa; 14-3-3 protein sigma, 28 kDa; and Kallikrein-1, 29 kDa.

The listed proteins are small enough either (i) to pass the blood-brain barrier or (ii) to be detected in saliva. Those proteins have not only well-known neurological functions, for instance, Cystatin-C in amyotrophic lateral sclerosis (Wilson, Boumaza and Bowser, 2013), but may also be altered in neurodevelopmental conditions, for instance, Interleukin-1 receptor antagonist protein (part of the neuroimmune system) in intellectual disability (Aureli et al., 2014).

Please note that an error variable should be considered and correspondent to the lack of accuracy offered by Experion^{TM} analysis and the identified MW ranges. These considerations should take this inaccuracy in account but also the process of protein degradation observed and well documented in saliva and fully detailed in chapter 4.

In **Figure 52**, the best 4 subjects of the (1) Experimental Top, (2) Control Top, (3) Experimental Bottom, and (4) Control Bottom subgroups are plotted. These 4 subjects represent the molecular profile that has bigger intergroup variability and intragroup homogeneity.

Therefore, they are the subjects that are more characteristic of each group, and that have a more representative molecular profile. Henceforth, we have chosen those 4 subjects, of each 4 subgroups, to perform the following analysis. Additionally, we have chosen 1 control subject for each group. As the objective of the following analysis was (i) to study the MW range considered for particles passing the blood-brain barrier and (ii) to probe into the neuroinflammatory and neuropeptide system, we have chosen one control subject of each subgroup with an inflammatory disease, undergoing the same cognitive load and task.

The subjects followed the analysis already discussed in (ii.) the Expected Protein Profile Results. A repetition of the Experion^{TM} Automated Electrophoresis System (Biorad®) analysis was performed, but in this case for those 5 subjects (4 best + 1 control), of each group. The final objective of this analysis was to evaluate if those 5 subjects should advance for simultaneous immune detection and quantification.

We would like to warn about the limitations of carrying out such an analysis and hypothesis testing. For the researcher, this non-blind analysis lacks the statistical power for generalization and it would be a type 1 statistical error to act as such.

However, as an exploratory study aiming at the sole understanding of the protein expression in this small MW range, such an exploration is valid. Therefore, the most significant protein profiles were selected. This (c) lower MW range [9.1;30]kDa was one of the protein density peaks with more intergroup concentration (ng/µl) difference and intragroup curve similarity. This [9.1;30]kDa interval is known as characterizing neuroinflammatory response (Suzumura and Ikenaka, 2013), as well as neuropeptide response, as published in NeuroPep database (Wang et al., 2015).

The cytokines, interleukins and neuropeptides are small proteins, which migrate in the electrophoresis in this molecular range. Hence, the important role that those small neuroimmune molecules (Young et al., 2016) and small neuropeptides (Lim, Bielsky and Young, 2005) may have in neurodevelopmental conditions; e.g., autism spectrum disorder (Hipólito and Martins, 2018).

In the following pictures, there are two molecular ranges that should be separated. From the [9.1;30]kDa range studied, interval I. [9.1;17]kDa, correspondent to the first

peaks of the electrophoretogram, is associated to the smallest molecules of neuropeptide response. Complementarily, interval II. [17;30]kDa corresponds to slightly bigger molecules associated with the neuroinflammatory response. For easier display, on the x-axis we added two markers indicated in the figures as Bioplex Th17 (Start and End). From the beginning of the x-axis to Bioplex Th17 Start, the interval is associated with the neuropeptide response. From the Bioplex Th17 [Start;End], the interval is associated with the neuroinflammatory response. We named the markers as indicative and referring to a possible Bioplex Th17 immunodetection panel, which would be a good panel for understanding the peptides involved in this MW range.

In **Figure 58**, the capillary gels and electrophoretograms of the 5 chosen subjects for the neuroinflammatory and neuropeptide panel, of all the 4 subgroups, are shown. All subjects (from all the subgroups) in T0+T1 are plotted together, and in T0 (before) and T1 (after) the Intervention Protocol. Likewise, the capillary gel from the total 4 subgroups, separately, in T0+T1, is shown.

In the interval I. [9.1;17]kDa, the total 4 subgroups of study show two protein peaks of a considerable high heterogeneity, both in the MW range as in fluorescence (concentration (ng/µl)), which need further investigation. Likewise, in interval II. [17;30]kDa, the total 4 subgroups of study show one protein peak of a considerable high heterogeneity, more in the MW range variable than in the concentration variable. This heterogeneity can also be observed in the capillary gels.

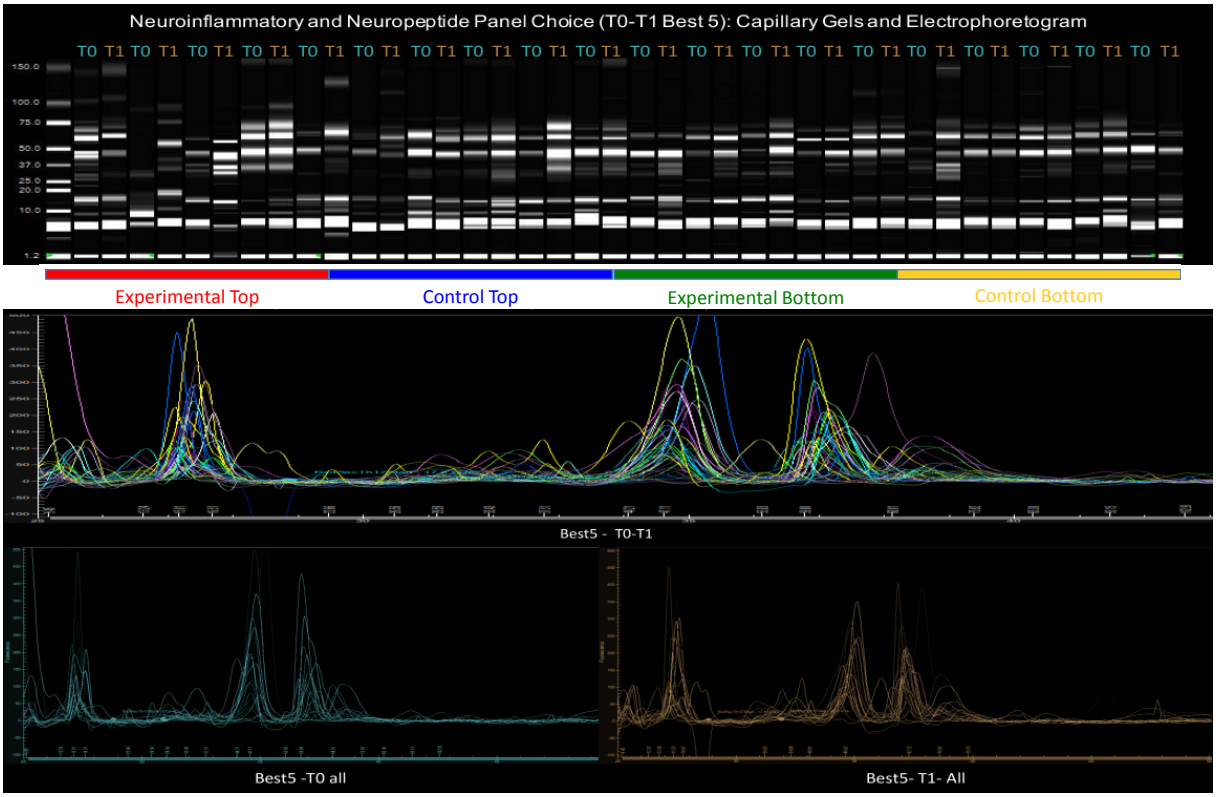


Figure 58. Capillary Gels And Electrophoretogram Profile Of The Selected Best 5 Subjects In All T0-T1, All In T0 And All In T1, For The Neuroinflammatory And Neuropeptide Panel
From each subphenome, for both the expected protein profile in T0, after the intervention protocol in T1, and the combined T0T1, a graphical representation is presented showing the best 5 capillary gels and quantitative electrophoretograms for the study of the neuroinflammatory and neuropeptide panel. The intergroup difference and the protein distribution are represented.

In **Figure 59**, it is possible to see the electrophoretograms, separately in T0 and T1, of the subjects belonging only to the (1) Experimental Top Subphenome. 5 subjects were plotted. Moreover, they were also charted together in T0+T1 without the positive control for that group. The 4th graph shows T0+T1 for the positive control, which was a subject with an ICD-10 classification: J30.1 – Allergic rhinitis due to pollen, and medicated with the antihistaminergic Zyrtec.

As it is shown in the first two graphs, there is a considerable variability from the T1 to the T0, specifically a slight increase of the concentration (ng/µl) in the interval I. [9.1;17]kDa and a substantial decrease of the concentration (ng/µl) in the interval II. [17;30]kDa.

These results confirm the hypotheses made previously in the MODeLING.Vis for the Δ (T1-T0) ng/µl of the B) [9.8;10.3]kDa and D) [13.7;17.5]kDa MW range. In the 3rd graph, we can see the overall intragroup homogeneity of the concentration (ng/µl) in the [9.1;30]kDa (the (c) lower MW range), contrastingly to the positive control, plotted in the 4th graph, showing a significant increase of the concentration in this MW range. Specifically, this augmentation is visible in the interval I. [9.1;17]kDa, this augmentation is visible in the interval I. [9.1;17[kDa.

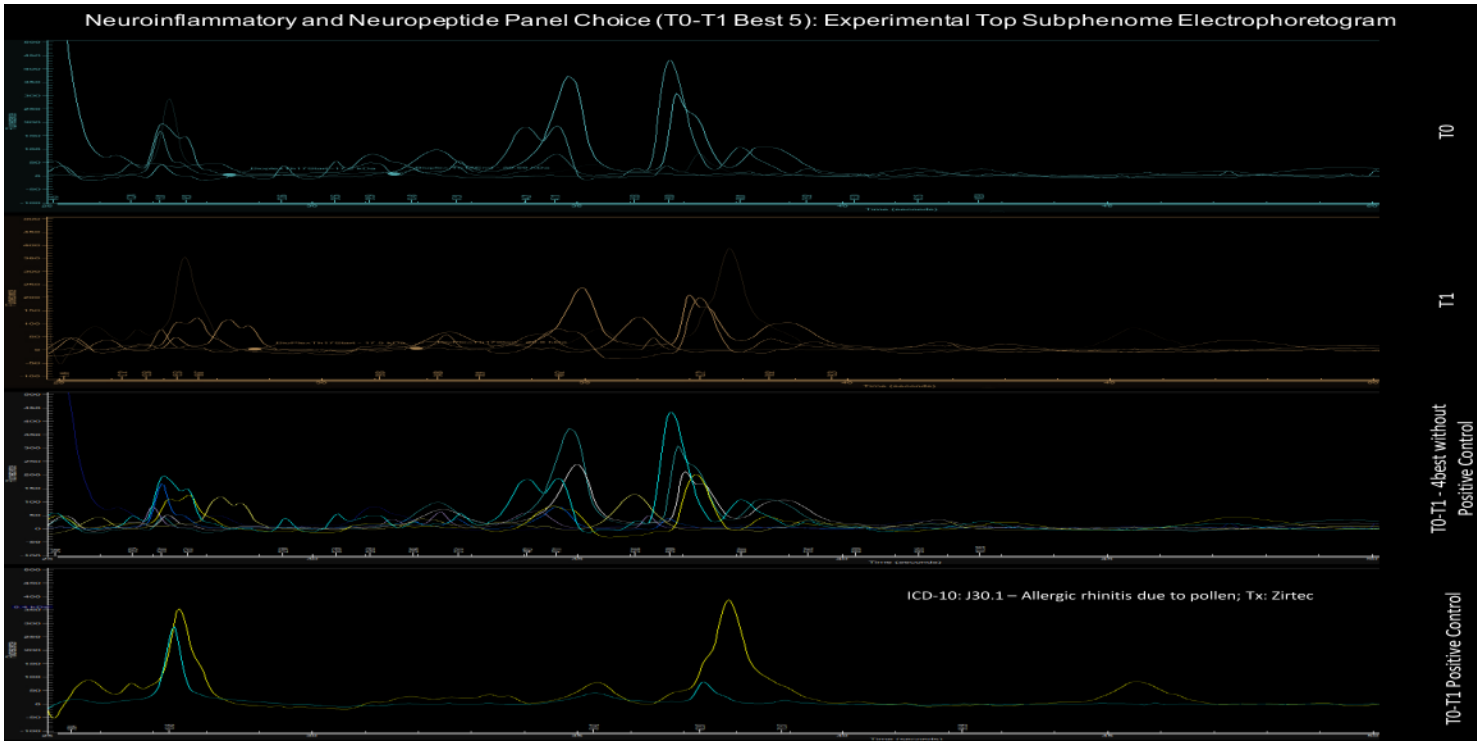


Figure 59. Electrophoretogram Profile Of The Selected Best 5 Subjects From The Experimental Top Subgroup, In T0, In T1, In T0-T1 Without Positive Control And In T0-T1 Only Positive Control, For The Neuroinflammatory/Neuropeptide Panel
For the expected protein profile in T0, after the intervention protocol in T1, the combined T0T1 without the positive control, and the combined T0T1 of only the positive control, a graphical representation is presented showing the best 5 quantitative electrophoretograms of the Experimental Top subgroup to study the neuroinflammatory and neuropeptide panel.
Through this figure, the intragroup difference between T0 and T1 is demonstrated.

In **Figure 52**, the same electrophoretograms are equally shown, but for the 5 subjects belonging to (3) the Experimental Bottom Subphenome.

In this case, the 4th graph shows T0+T1 for the positive control, which was a subject with an ICD 10: J30.9 – Allergic Rhinitis, unspecified, and non-medicated.

As it is shown in the first two graphs, there is a considerable variability from T1 to T0, specifically a significant increase in the concentration (ng/µl) of both intervals I. [9.1;17]kDa and II. [17;30]kDa. In this electrophoretogram, the atypical protein profile is due to the positive control in the [9.1;17]kDa, and better demonstrated in the 4th graph, showing a significant decrease in the concentration in this MW range.

These results also confirm the hypothesis made previously in the MODeLING.Vis for the Δ (T1-T0) ng/µl of the B) [9.8;10.3]kDa and D) [13.7;17.5]kDa MW range. In the 3rd graph, we can also see the overall intragroup homogeneity of the concentration in the [9.1;30]kDa, contrasting with the positive control, plotted in the 4th graph.

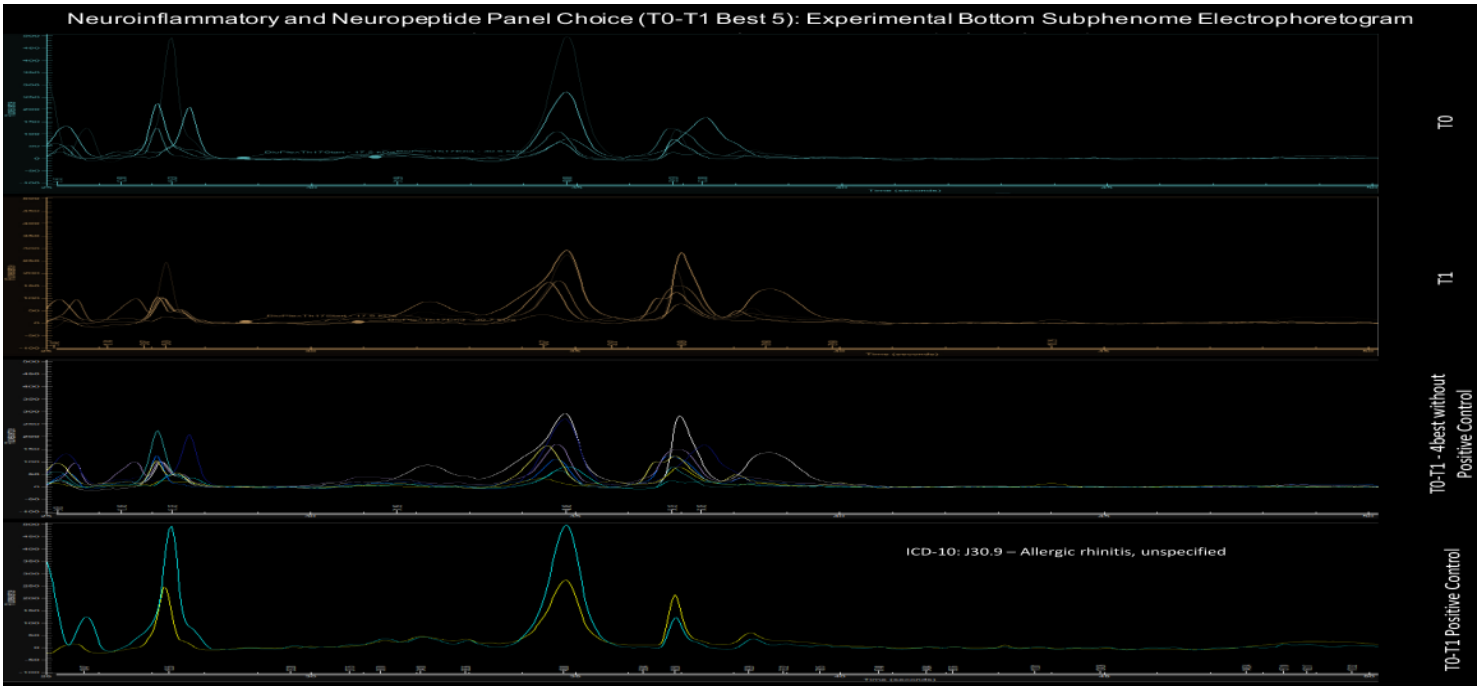


Figure 60. Electrophoretogram Profile Of The Selected Best 5 Subjects From Experimental Bottom Subgroup, In T0, In T1, In T0-T1 Without Positive Control And In T0-T1 Only Positive Control, For The Neuroinflammatory/Neuropeptide Panel
For the expected protein profile in T0, after the intervention protocol in T1, the combined T0T1 without the positive control, and the combined T0T1 of only the positive control, a graphical representation is presented showing the best 5 quantitative electrophoretograms of the Experimental Bottom subgroup to study the neuroinflammatory and neuropeptide panel.
Through this figure, the intragroup difference between T0 and T1 is demonstrated.

In **Figure 61**, the 5 subjects belonging to the (2) Control Top Subphenome are specifically shown. In this situation, the 4th graph shows T0+T1 for the positive control, which was a subject with an ICD-10: J45 – Asthma, non-medicated.

As it is shown in the first two graphs, there is a considerable variability from T1 to T0, specifically a slight decrease of the concentration (ng/µl) in both intervals I. [9.1;17]kDa and II. [17;30]kDa.

These results also confirm the hypothesis made previously in the MODeLING.Vis for the Δ (T1-T0) ng/µl of the B) [9.8;10.3]kDa and D) [13.7;17.5]kDa MW range. In the 3rd graph, we can see the overall intragroup homogeneity of the concentration in the [9.1;30]kDa.

Additionally, the electrophoretogram of the positive control, plotted in the 4th graph, shows a baseline control concentration in interval II. [17;30]kDa, characteristic of the neuroinflammatory response.

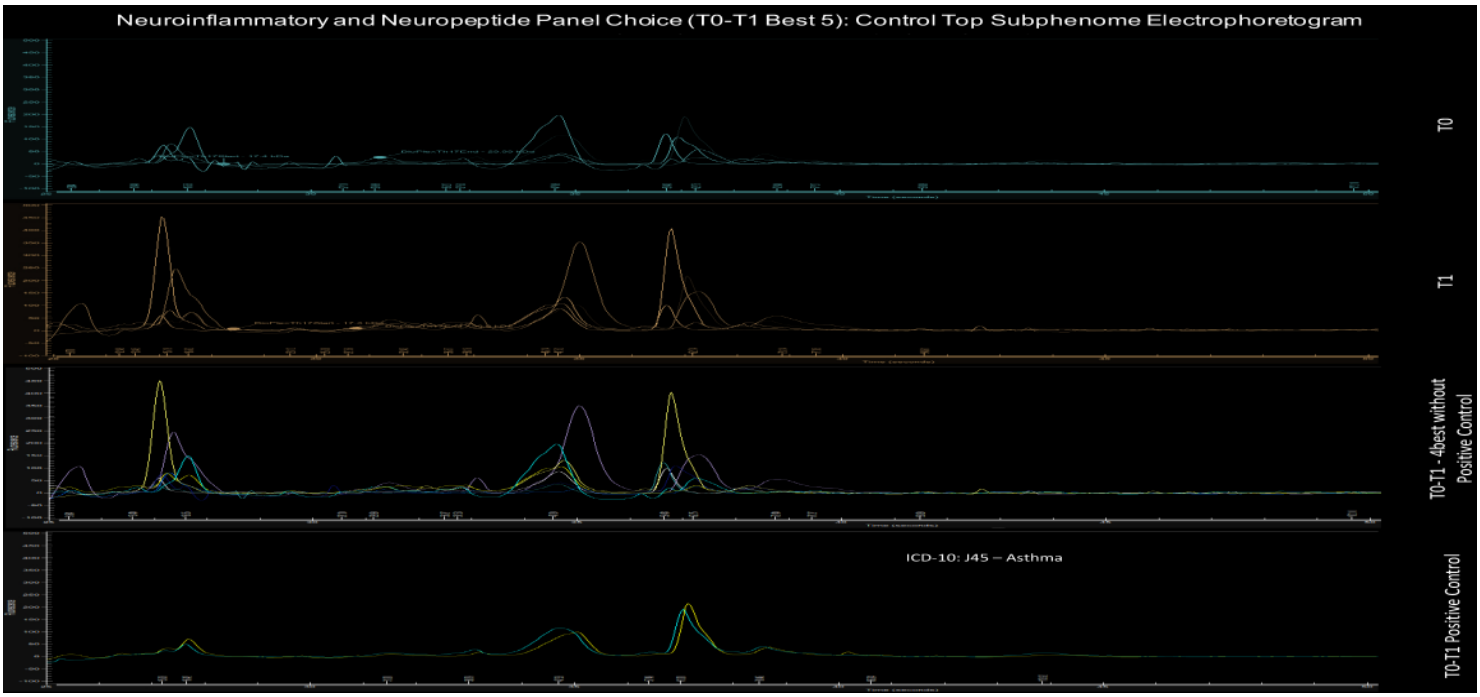


Figure 61. Electrophoretogram Profile Of The Selected Best 5 Subjects From Control Top Subgroup, In T0, In T1, In T0-T1 Without Positive Control And In T0-T1 Only Positive Control, For The Neuroinflammatory/Neuropeptide Panel
For the expected protein profile in T0, after the intervention protocol in T1,
the combined T0T1 without the positive control, and the combined T0T1 of only the positive control,
a graphical representation is presented showing the best 5 quantitative electrophoretograms
of the Control Top subgroup
to study the neuroinflammatory and neuropeptide panel.
Through this figure, the intragroup difference between T0 and T1 is demonstrated.

In **Figure 62**, the 5 subjects belonging to the (4) Control bottom Subphenome are finally shown. In this condition, the 4th graph shows T0+T1 for the positive control, which was a subject with an ICD-10: J30.9 – Allergic Rhinitis, unspecified, and medicated with a leukotriene receptor antagonist (Singulair®), a corticosteroid (Pulmicort®) and a long-acting β2-agonist (Simbicort®).

As it is shown in the first two graphs, there is a considerable variability from T1 to T0, specifically the significant increase of the concentration (ng/µl) in interval II. [17;30]kDa.

These results also confirm the hypothesis made previously in the MODeLING.Vis for the Δ (T1-T0) ng/µl of the B) [9.8;10.3]kDa (which remains constant) and D) [13.7;17.5]kDa (which augments considerably) MW range. In the 3rd graph, we can see a slight overall intragroup heterogeneity in the [9.1;30]kDa MW range, compared to the other subgroups. This heterogeneity is due to the lower clinical score characterizing this subgroup (Hipólito and Martins, 2017), and therefore an also lower specific molecular print in this MW range.

The electrophoretogram of the positive control, plotted in the 4th graph, shows a baseline control concentration in interval II. [17;30]kDa, also characteristic of the neuroinflammatory response.

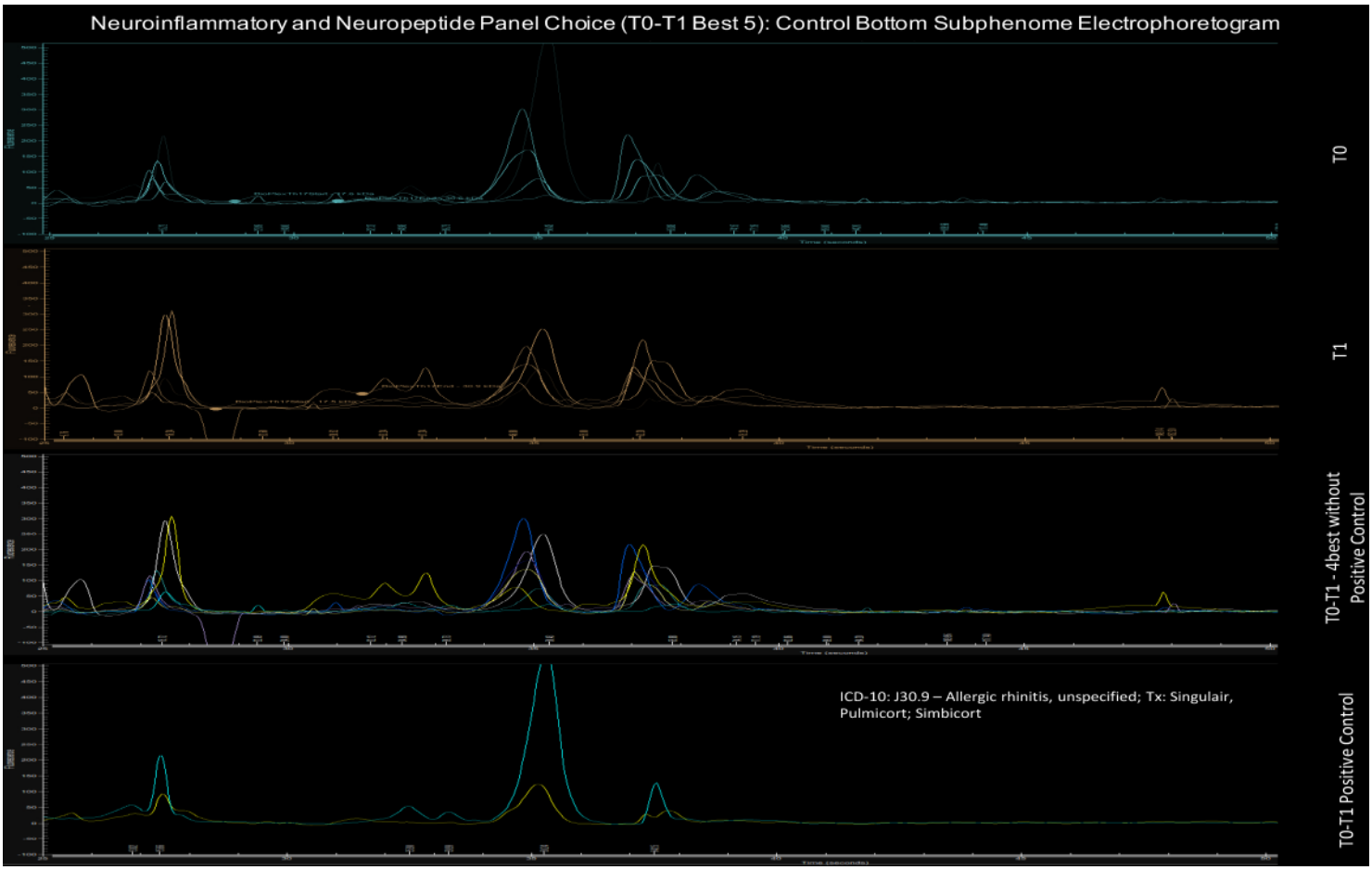


Figure 62. Electrophoretogram Profile Of The Selected Best 5 Subjects From Control Bottom Subgroup, In T0, In T1, In T0-T1 Without Positive Control And In T0-T1 Only Positive Control, For The Neuroinflammatory/Neuropeptide Panel
For the expected protein profile in T0, after the intervention protocol in T1, the combined T0T1 without the positive control, and the combined T0T1 of only the positive control, a graphical representation is presented showing the best 5 quantitative electrophoretograms of the Control Bottom subgroup to study the neuroinflammatory and neuropeptide panel. Through this figure, the intragroup difference between T0 and T1 is demonstrated.

After this analysis, we postulate that those 5 subjects should be chosen to advance for a sequential phase of our molecular screening: simultaneous immune detection.

The excerpt of the research outline (**Figure ζ**) systemizes the Section II: Molecular Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 5 in the overall experiment.

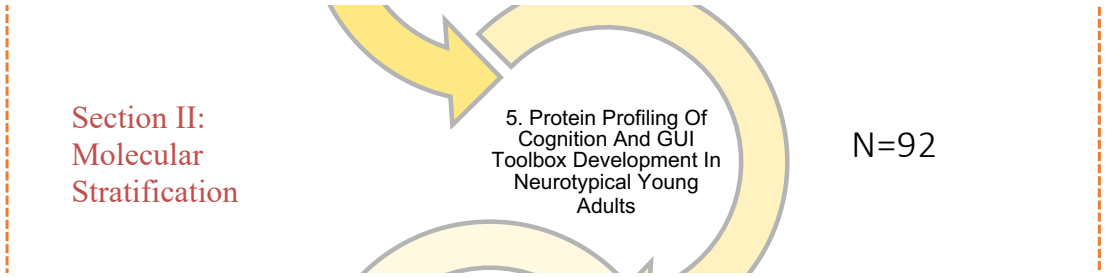


Figure ζ. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section II: Molecular Stratification
The chapter 5 (Protein Profiling Of Cognition And GUI Toolbox Development In Neurotypical Young Adults) is displayed, which comprehended the methodology that led to the establishment of the Neuro.SalivaPrint. It comprised a stratification stage with total protein profiling, data visualizing and data mining of 92 stratified subjects.

Chapter 6. Functional Profiling Of Neurotypical Subjects: Neuropeptide Response And Anti/Pro T-helper Cell Type 17 Equilibrium

Preamble ("How to quantify the neuropeptide and neuroimmune response?")
Cognitive-immune coupling is a possible low-level biomolecular structure of high-level neuronal cognition. Neuropeptide and immune response have been linked to cognitive states and stimuli, as part of the neuro-immune interface and characterized by a small molecular weight range of approximately [9.1;30]kDa. Moreover, its homeostasis has been studied as a physiological evidence of neurological and mental health (Pacheco, Martins and Simões, 2019) and well-being, which is achieved, on the one hand, by regulation of small peptides like Orexin, β-Endorphin, Oxytocin and Substance P, and on the other hand, by Th17 cell type response and biochemical messengers. In this descriptive study, we continued the profiling of a sample of 128 young neurotypical adults through a combined multiplex panel: the *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the *Bio-Plex Pro™ Human Th17 Cytokine Assay*. Saliva proteomics, through capillary electrophoresis and multiplexed simultaneous immunodetection mixed analysis, was used as a technique to evaluate neuropeptide response and anti/pro Th17 equilibrium. The results were the acquisition of 20 stratified subjects, corresponding to 4 mental health profiles. The stratified subjects had a reasonable distinct neuropeptide response and anti/pro T-helper cell type 17 equilibrium. As a milestone, we developed a molecular functional clustering of the immune interaction networks: the Neuro-Immune Network and the Human Th17 Cytokine Database. This molecular profiling illustrates, to a certain degree, cognitive-immune coupling in a neurotypical sample and opens the path for causalistic analysis.

Materials And Methods

Chapter 6 comprises the methodology that led to quantification of the neuropeptide and neuroimmune response in young neurotypical adults. This methodology required a previous sample selection and stratification, which was already described in the previous chapters.

6.1. Sample Collection And Biobanking

Unstimulated whole saliva (WS) was collected and processed using Rosa and colleagues' (2016) *standard operating procedure* (SOP). SOP is composed of:

(i) sampling of the subjects with saliva collection through the cotton roll-based method;

(ii) collection and transport process of the sample aliquots were made on -4ºC;

(iii) saliva extraction from the cotton rolls through centrifugation;

(iv) total volume collection and protein concentration measurement;

(v) aliquotation of the total volume of each sample;

(vi) sample storage and preservation at -80ºC at the biobank.

The sampling and biobanking procedures are fully detailed in chapter 5, and the samples were requested from Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal.

6.2. Sample Characterization

The sample is fully characterized in chapters 2 and 3. For this neuroimmune quantification, 128 neurotypical young adults were previously stratified by total protein profiling (chapter 5).

This stratification resulted in the selection of 20 subjects with a more significant and relevant protein signature in the [9.1;30]kDa molecular weight range. Those were the subjects we selected to study the neuropeptide and immune response (chapter 4).

The 20 subjects belonged to 4 different and separate subgroups, previously identified and stratified in chapters 2 and 3 of this thesis. Each subgroup is constituted by 4 subjects and 1 control subject, for the neuropeptide and immune response:

(i) the top phenome of the Experimental group;
(ii) the top phenome of the Control group;
(iii) the bottom phenome of the Experimental group;
(iv) the bottom phenome of the Control group.

In this study we specifically named:

(i) the top phenome of the Experimental subgroup ⇒ Self Awareness;
(ii) the top phenome of the Control subgroup ⇒ Self Consciousness;
(iii) the bottom phenome of the Experimental subgroup ⇒ Reflective Self;
(iv) the bottom phenome of the Control subgroup ⇒ Pre-Reflective Self.

This denomination was used only to facilitate subgroup identification and is explained, comprehensively, in **Supplementary Figure 1**.

6.3. Experion™ Automated Electrophoresis System (Biorad®)

Total protein profiling was executed by electrophoretic stratification. This preceding profiling selected 20 neurotypical subjects (chapter 5). They were verified/measured through capillary electrophoresis to see if they had an adequate protein distribution in the [9.1;30]kDa molecular weight range.

This analysis helped to visualize if the selected subjects had salivary samples with significant proteomic features to be assessed in the multiplex-biometric immunoassay. Two molecular weight ranges were detailed and investigated: (i) the [9.1;17]kDa associated with the neuropeptide response, and (ii) the [17;30]kDa associated with the immune response.

These two molecular weight ranges were then assessed by the Bio-Plex® MAGPIX™ Multiplex Reader. This multiplex-biometric immunoassay analyzed:

(i) the [9.1;17]kDa, with the *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Magnetic Bead Panel - Neuroscience Multiplex Assay*;

(ii) the [17;30]kDa, with the *Bio-Plex Pro™ Human Th17 Cytokine Assays*.

However, it is important to emphasize that the detection sensitivity of Experion™ is ng/µl, whereas the sensitivity of Bio-Plex® is pg/ml. Therefore, the sensitivity of MAGPIX™ Multiplex is significantly higher and, as a limitation, there is an inter instrument measurement error when correlating these two assessments.

6.4. Bio-Plex® MAGPIX™ Multiplex Reader

To detect and quantify the neuropeptide and immune response, we used a multiplex-biometric immunoassay.

(i) Neuropeptides (Orexin, β-Endorphin, Oxytocin and Substance P) and (ii) cytokines (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α) were measured, separately, in a multiplex-biometric immunoassay containing fluorescent microspheres conjugated with target-specific monoclonal antibodies. The quantification was performed according to the manufacturer's instructions. We followed:

(i) https://tinyurl.com/Merck-Millipore-HNPMAG-35K Protocol (retrieved July 22, 2020): Human Neuropeptide Magnetic Panel, for the *HNPMAG-35K* assay, Merck Millipore®, used to study the neuropeptide response;

(ii) Geng, Ma and Roen's (2012) guidelines for *Human Th17 Cytokine* assay, Bio-Rad®, used to study the immune response.

6.5. Data Analysis

The data about the subjects was used as input for the Laboratory Information Management: LabWare™ LIMS and biobanking software Quartzy™ (Rosa et al., 2016).

Then, a clinical profiling was performed in chapters 2 and 3 of this thesis.

Subsequently, a proteomics screening (chapter 5) of the salivary samples, with Experion™ Automated Electrophoresis System (Biorad®) was executed.

The samples were, then, ready for the analysis of this study.

First, data acquisition was executed and, secondly, data processing was implemented, as follows.

6.6. Data Acquisition

Supernatants from whole saliva samples were evaluated using a multiplex-biometric immunoassay. In total, 19 soluble factors were measured using (i) Merck Millipore® and (ii) Bio-Rad® assays, according to the manufacturer's instructions.

As protocolized, the samples were diluted in 25 µl (sample) to 50 µl (assay buffer), in a total volume equal to 75 µl. Then, to explain it briefly, duplicate supernatant samples were incubated with microbeads coated with antibodies specific for the above-mentioned neuropeptides and cytokines, for 16 hours, at 4ºC.

After washing, the beads were incubated with the detection antibody cocktail, which included a biotinylated antibody specific for each single analyte. After another wash step, the beads were incubated with streptavidin-phycoerythrin for 10 minutes and washed again. Finally, fluorescence was measured using a Bio-Plex® MAGPIX™ Multiplex Reader.

(a) Bio-Plex Manager™ and (b) Bio-Plex Data Pro™ Software (Bio-Rad®, Hercules, California, USA) were used for data visualization and hierarchical cluster analysis.

The former enables data analysis and curve optimization for data generated from the Luminex instrument, and the latter provides tools to organize and visualize multiple data sets generated from xMAP multiplex experiments: combining multiple results files, performing basic statistical analysis, and generating graphs and charts.

The data was then calibrated and normalized as shown in **Supplementary Figure 2**, using Bio-Plex Manager™. As a limitation in our study, we only had 20 samples (in duplicate) for each analyte; hence the importance of a good calibration. Calibration curves were obtained with different regression types, e.g., Logistic 4PL and Logistic 4PL 5PL, defined by us to better fit our data.

Then, we calculated the calibration curve for each of the 15 analytes (pg/mL). We defined the standards, and the positive and negative controls, according to Geng, Ma and Roen's (2012) guidelines for *Human Th17 Cytokine* assay (Bio-Rad®).

The upper limit of quantification (ULOQ) and the lower limit of quantification (LLOQ) were set by us, according to the guidelines for salivary samples. The outliers were automatically defined by the algorithm curve optimization of the Luminex instrument.

6.7. Data Processing

A post-analysis was performed using the statistical and data management software: IBM®SPSS®ver.22 – significance level = 5%, as the Bio-Plex Data Pro™ Software only performs a basic analysis.

For the descriptive and exploratory analysis, frequency tables (simple/double entries) and boxplots were used as analytical/graphic exploratory tools.

Scale variables were summarized as mean, standard deviation, median, quartiles, minimum, maximum and/or other order statistics, whenever the sample distribution justified it.

Categorical variables were summarized using frequency and percentages. When the scale of measure was ordinal, order statistics were considered. Besides that, Spearman's rank correlation coefficient was applied.

For the inferential analysis, T-student for two independent samples and T-student for two paired samples were used to compare medians. One-way analysis of variance (ANOVA), parametric and non-parametric, was used when comparing more than two means, i.e., the mean difference 4 subgroups in study, for (i) the Human Neuropeptide and (ii) the Human Th17 Cytokine assays.

Moreover, correlations between scores of the neuropeptide and the cytokine assays were calculated. To test the conditions of application of the T-student test and one-way ANOVA, the following tests were applied: Normality, Kolmogorov, with Lilliefors correction, $n \geq 50$, or Shapiro Wilks, $n < 50$, and Levene's equality of variances.

When the conditions were not met, non-parametric tests were used. When appropriate, and to indicate a significant difference, results were complemented with confidence intervals at 95% and a significance level = 5%.

To minimize error associated with the measurement instrument Bio-Plex® MAGPIX™ Multiplex Reader, we used duplicate sampling for each 4 neuropeptides and 15 cytokine analytes. Instrument associated error bars were added to the results, when appropriate. The Error Bar is equal to the SD (rep)/(√ (No. rep)), rep being the duplicate sampling.

For data clustering, we used an unsupervised learning algorithm, the Principal Component Analysis (PCA). We wanted to find patterns and regularities without a direct supervision and labeling of the immune analytes. PCA was used to find mutually orthogonal directions of greatest variance both in (i) the 20 neurotypical subjects and in (ii) the 15 analytes of the Human Th17 Cytokine Assay.

This analysis permitted data visualization and exploration of the neuropeptide and cytokine profiles. Double and triple entry charts (x: analyte; y: [Obs]) and (x: sample; y: [Obs]), 2 component PCA, boxplots, pie charts and network modeling were used as graphic and visual exploratory tools.

6.8. Software

The following pieces of software were used:

(i) IBM SPSS Statistics 22 (with a significance level = 5%) for statistical and data management;

(ii) LabWare™ LIMS and Microsoft Excel for laboratory information management;

(iii) Bio-Plex Manager™ and Bio-Plex Data Pro™ Software (Bio-Rad®, Hercules, California, USA) were used for data visualization and hierarchical cluster analysis;

(iv) Prezi Inc. infographic for data visualization;

(v) PANTHER v.15.0 and Cytoscape v.3.6.0, as bioinformatics tools used for Functional analysis of the immune response.

Results And Discussion

This set of results was obtained in lab rotations of the student in the following laboratories and specifically in collaboration with the following researchers[78]: Martins, J. E.[a,b,c,d], Sousa, S.[a,b], Simões, J. F.[a,b,c], Esteves, E.[a,c], Rosa, N.[b], Correia, M. J.[b], Simões, M.[a,c], Barros, M.[b].

The results, and their discussion, comprehend the data acquired in:

(6.A.) the *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Magnetic Bead Panel - Neuroscience Multiplex Assay*;

(6.B.) the *Bio-Plex Pro™ Human Th17 Cytokine Assays*.

The variables analyzed are quantitative/continuous.

6.A. Human Neuropeptide Assay: *Quantification In Saliva*

Orexin, β-Endorphin, Oxytocin and Substance P were the neuropeptides we analyzed for the profiling of our neurotypical sample. We used the FI (median fluorescent intensity of the analyte), in cps (counts per second), as a unit of measurement.

FI was a better unit for plotting the data of the neuropeptide response, due to the high analyte dispersion.

Figure 63 shows a boxplot and a dispersion representation of the neuropeptide dataset in Log_2FI. It is possible to observe that the data is widely dispersed along the 1st and 3rd percentile. However, the median FI for all the 4 analytes tends to be in similar and in the same range of [8;10]cps.

Oxytocin is the analyte with the most florescence, then Orexin, followed by Substance P, and finally β-Endorphin.

We chose to use Log2FI also because of the quantification limits, i.e., the Lower Limit Of Quantitation (LLOQ) and Upper Limit Of Quantitation (ULOQ) of these analytes are low in salivary samples.

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[d]Lisbon Mind and Cognition Group, Institute for Philosophy of Nova, New University of Lisbon, Lisbon, Portugal

Therefore, by using the median fluorescent intensity of each analyte, we acquired better detection and quantification, as more magnetic beads were detected.

For Orexin, 56 magnetic beads with the corresponding antibody were detected; for β-Endorphin, 20 magnetic beads; for Oxytocin, 65 magnetic beads; and for Substance P, 75 magnetic beads. In the dispersion representation, we can observe the FI of all the 20 neurotypical subjects, with duplicate sampling in T0 and T1.

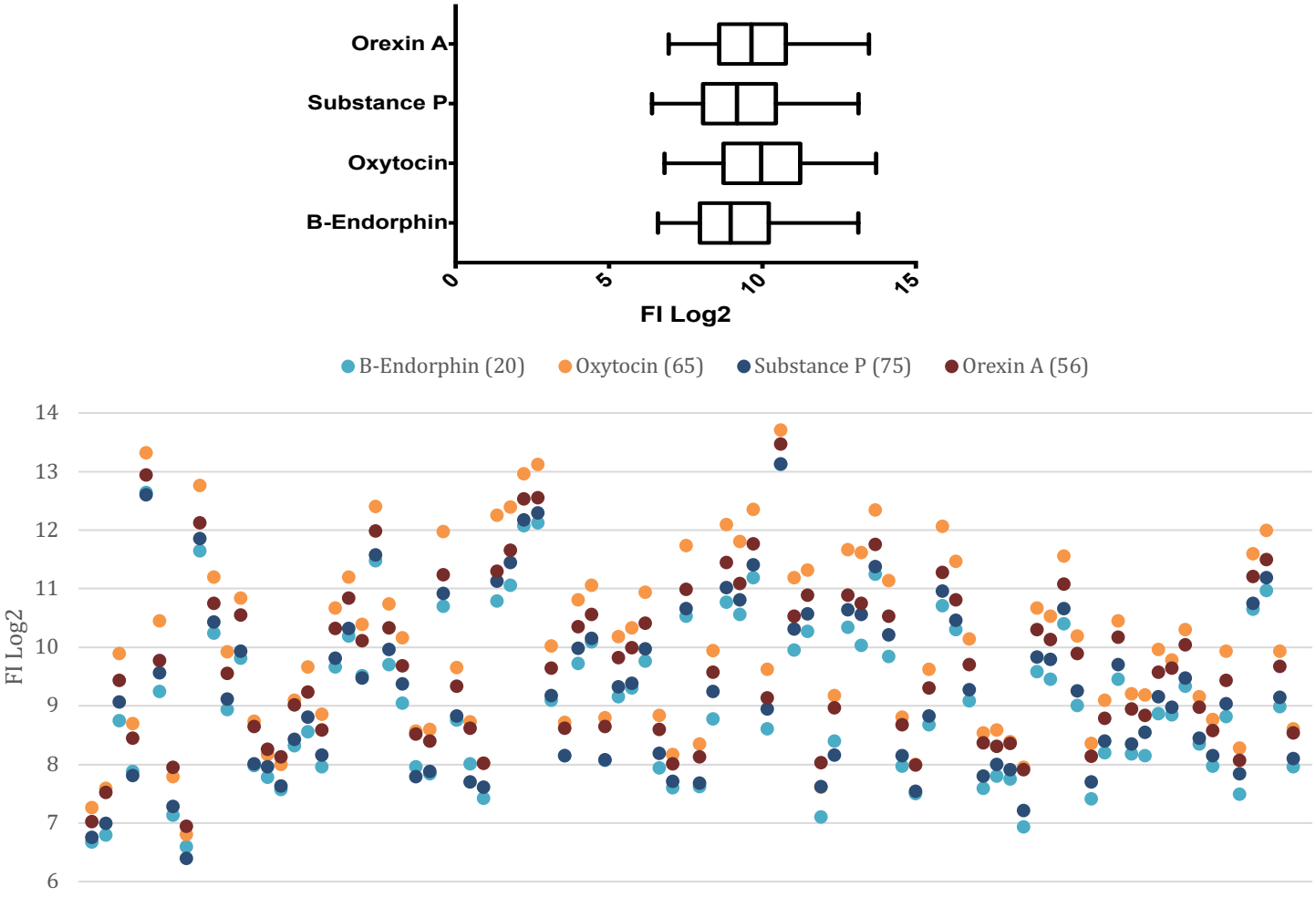


Figure 63. Data Dispersion (Log_2fi) Of The Human Neuropeptide Assay: Boxplot And Dispersion Representation
Two plots are presented to show the log_2FI of Orexin, β-Endorphin, Oxytocin and Substance P. The total number of magnetic beads detected in the 20 neurotypical subjects (duplicate sampling) are shown for T0 and T1. The upper boxplot shows the median FI for all the 4 analytes tends to be in similar and in the same range of [8;10]cps. The dispersion representation (bottom plot) shows the dispersion of the neuropeptide data: in light blue (B-Endorphin), in orange (Oxytocin), in dark blue (Substance P), and in red (Orexin A).

Figure 63 identified the detection values (log_2FI) in saliva for the Human Neuropeptide Assay with a sample of young neurotypical adults. It shows thus the physiological window of Orexin, β-Endorphin, Oxytocin and Substance P in this population without neuropsychiatric conditions.

Notwithstanding, we wanted to understand the specific neuropeptide response in our 4 subgroups:

Self Awareness;
Self Consciousness;
Reflective Self;
Pre-Reflective Self.

The objective was to test if a neuropeptide stratification could be verified in our 4 mental health profiles. Henceforth, we executed a labeled and supervised analysis, with the already obtained mental health profiles (Hipólito and Martins, 2017).

Based on the literature revision of the neuropeptide response, we postulated the following hypothesis for each of the neuropeptides.

These hypotheses were tested to understand if neuropeptide strata were present and correspondent to the 4 subgroups.

a) there is intergroup variability of the median FI of the total 4 analytes;

b) there is intergroup variability of the median FI in the neuropeptide trait profile (T0);

c) there is intergroup variability of the median FI in the neuropeptide state profile ($\Delta_{T0\text{-}T1}$);

d) there is low intragroup variability of the median FI in the neuropeptide trait profile (T0);

e) there is low intragroup variability of the median FI in the neuropeptide state profile ($\Delta_{T0\text{-}T1}$).

For better understanding of those differences in the Human Neuropeptide Assay, in our neurotypical sample, we separated the analysis in two time points: T0 and T1.

On the one hand, T0 reflects the baseline concentration of all neuropeptides for the subjects in the 4 subgroups.

T0 is the timepoint before the Intervention Protocol (Hipólito and Martins, 2017; chapter 3).

Thus, T0 expresses the neuropeptide trait of our neurotypical sample.

On the other hand, T1 reflects the concentration of those analytes for the subjects in the 4 subgroups after the Intervention Protocol.

It was calculated by:

$$\Delta_{T0\text{-}T1} = FI(analyte)_{T1} - FI(analyte)_{T0}$$

, of each 4 neuropeptides present in the assay.

Thus, T1 expresses the neuropeptide state of our neurotypical sample.

This neuropeptide profile characterization is further investigated and tested both for the trait (T0) and state ($\Delta_{T0\text{-}T1}$) response.

Table 8 summarized the descriptive analysis of the neuropeptide profile characterization.

The table has information concerning the N (number of subjects); mean and medium FI (according to the central limit theorem) of the 4 analytes, minimum/maximum, interquartile range and the corresponding standard deviation.

Moreover, the neuropeptide profiles were systematized in the total (N20) neurotypical sample and in each of the 4 subgroups (subphenomes). The working hypothesis of this analysis was that the distinctive mental health phenomes (Hipólito and Martins, 2017; chapter 3) had different neuropeptide profiles.

Table 8. Descriptive Analysis Of The Neuropeptide Response
Table summarizing the mean, median, standard deviation, minimum, maximum and interquartile range for the neuropeptide profiles in FI (cps), in the neurotypical sample (N20) and in the 4 mental health profiles (N5 for each subphenome).

HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Magnetic Bead Panel			Trait Profile (T0)				State Profile Δ[T0;T1]			
			Orexin	β-Endorphin	Oxytocin	Substance P	Orexin	β-Endorphin	Oxytocin	Substance P
Total Phenome (N=20)		μ	959,1	635,9	1363,3	731,6	205,9	140,7	251,9	163,2
		σ	713,89	497,61	1261,1	608,1	437	272,7	577,3	315,5
Groups (Subphenomes)	*Self Awareness* (N=5)	μ	1413,0	956,6	2048,0	1107,8	642,2	402,0	806,0	470,8
		Median	1485,0	989,0	2329,0	1269,0	518,0	397,0	855,0	425,0
		σ	896,2	618,9	1406,5	707,7	413,2	263,2	584,5	305,5
		Min	281,0	190,0	256,0	199,0	238,0	131,0	232,0	146,0
		Max	2365,0	1606,0	3585,0	1796,0	1305,0	826,0	1664,0	919,0
		IQR	1754,0	1217,0	2727,5	1380,0	700,5	427,5	1064,5	562,5
	Self Consciousness (N=5)	μ	1109,0	735,4	1723,6	861,8	421,4	259,6	524,0	332,6
		Median	1020,0	633,0	1285,0	671,0	507,0	303,0	674,0	371,0
		σ	883,5	633,5	1846,0	823,2	276,0	137,8	299,7	186,8
		Min	330,0	193,0	372,0	223,0	-13,0	31,0	12,0	34,0
		Max	2516,0	1773,0	4881,0	2246,0	713,0	364,0	718,0	549,0
		IQR	1530,5	1061,0	2849,5	1325,0	468,0	228,5	460,0	281,0
	Reflective Self (N=5)	μ	489,4	309,8	602,4	347,4	-196,8	-110,4	-280,4	-121,0
		Median	505,0	326,0	572,0	350,0	-125,0	-76,0	-138,0	-72,0
		σ	172,5	119,2	301,2	146,8	133,7	97,4	237,2	111,5
		Min	248,0	141,0	221,0	156,0	-422,0	-272,0	-667,0	-295,0
		Max	690,0	453,0	978,0	524,0	-97,0	-33,0	-109,0	-10,0
		IQR	321,0	217,5	572,0	279,5	208,5	161,0	387,0	190,5
	Pre-Reflective Self (N=5)	μ	825,4	541,8	1079,2	609,4	-43,2	11,6	-41,8	-29,6
		Median	799,0	549,0	1039,0	580,0	-87,0	-86,0	-89,0	-13,0
		σ	472,8	304,4	709,8	393,3	257,3	230,0	396,6	175,4
		Min	328,0	215,0	336,0	208,0	-406,0	-265,0	-618,0	-295,0
		Max	1304,0	844,0	1798,0	1010,0	205,0	251,0	332,0	126,0
		IQR	944,0	608,0	1417,5	785,5	474,5	430,0	724,0	319,5

In **Figure 64**, an interpretative preliminary schematic of our results for the trait (T0) and state ($\Delta_{T0\text{-}T1}$) interleukin profiles is presented. This preliminary schematic is based on the state of the art and bibliography already explored in the introduction of this thesis. In darker gray, the higher $\Sigma\ [FI]_4$ analytes are represented; and in lighter gray, the contrary, i.e., the lower analyte levels. This grading offered a scale that is based on the total neuropeptide fluorescence of all the subjects in the 4 subgroups.

On the left side, **Figure 64.A**, the trait is plotted from [1;4], while on the right side (**Figure 64.B**) the state is plotted from [-1;2]. Therefore, the FI of the following subgroups are graded from higher to lower in the following profiles:

a. In the Orexin trait profile (**Figure 64.A**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Hence, an Orexin trait, e.g., reward function associated, might be higher in the Self Awareness subgroup and marginal in the Reflective Self group;

b. In the Orexin state profile (**Figure 64.B**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Thus, an Orexin state, or a neuropeptide response associated with arousal, might be higher in the Self Awareness subgroup and negative in the Pre-Reflective Self group;

c. In the β-Endorphin trait profile (**Figure 64.A**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Hence, a β-Endorphin trait, e.g., reward system and associative learning related, might be higher in the Self Awareness subgroup and minor in the Reflective Self group;

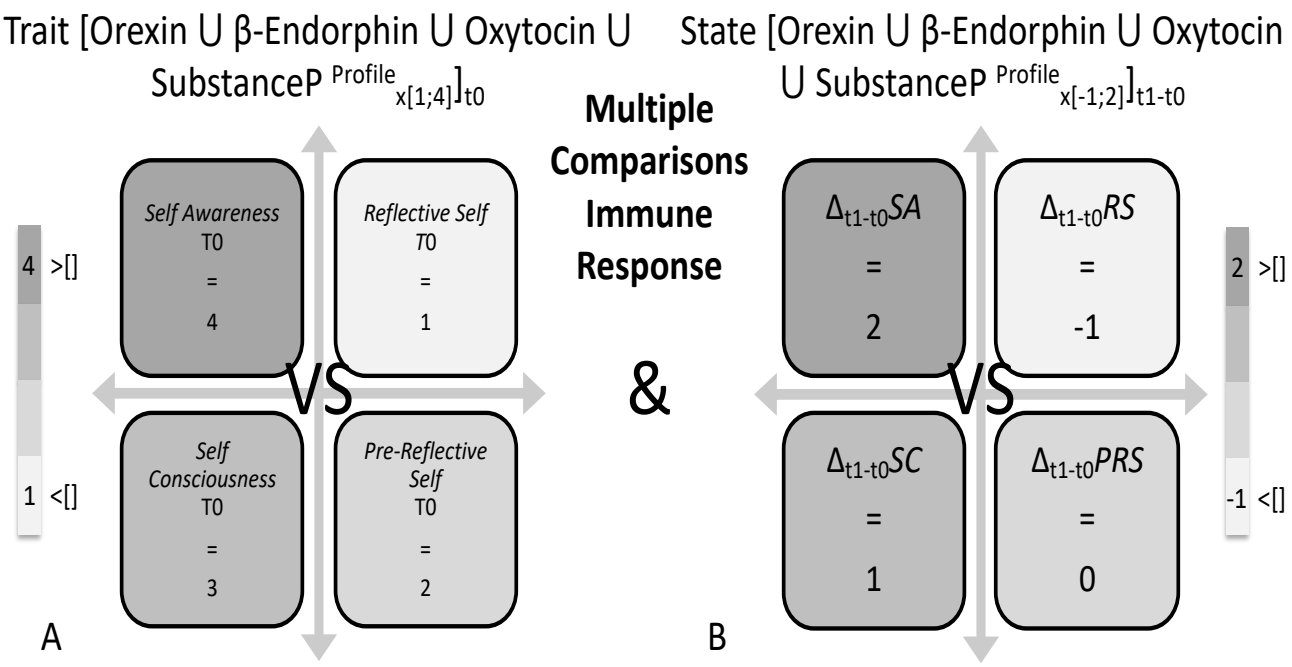


Figure 64. Interpretative Preliminary Schematic For The Trait (T0) And State (ΔT0-T1) Neuropeptide Profiles
A 2-axis representation of the multiple comparisons of the FI (median fluorescent intensity of the analyte) in cps (counts per second) is presented. Orexin, β-Endorphin, Oxytocin and Substance P are plotted in the 4 subphenomes (N5), for the trait (T0) and state ($\Delta_{T0\text{-}T1}$) profile.
In the vertical axis, the Σ [FI]4 analytes are represented. The darker the gray, the higher the concentration.

d. In the β-Endorphin state profile (**Figure 64.B**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Thus, a β-Endorphin state, or a neuropeptide response induced by incentive salience and positive emotions like pleasure, might be higher in the Self Awareness subgroup and negative in the Reflective Self group;

e. In the Oxytocin trait profile (**Figure 64.A**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Hence, an Oxytocin trait, e.g., reduced fear and anxiety, might be higher in the Self Awareness subgroup and marginal in the Reflective Self group;

g. In the Oxytocin state profile (**Figure 64.B**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Thus, an Oxytocin state, or a neuropeptide response related to increased social interaction, might be higher in the Self Awareness subgroup and negative in the Reflective Self group;

h. In the Substance P trait profile (**Figure 64.A**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Hence, a Substance P trait, e.g., a crosstalk between endocrine cells and peptidergic nerves, might be higher in the Self Awareness subgroup and minor in the Reflective Self group;

i. In the Substance P state profile (**Figure 64.B**): Self Awareness subgroup > Self Consciousness subgroup > Pre-Reflective Self subgroup > Reflective Self group. Thus, a Substance P state, or neuropeptide response related to the augmenting of autonomic nervous system functioning, might be higher in the Self Awareness subgroup and marginal in the Reflective Self group.

6.A.1. Intergroup And Intragroup Variability

In the first phase of the descriptive analysis, explored in **Table 8**, we wanted to test:

(i) intergroup variability, assumed by the variable mean;

(ii) intragroup variability, given by the variable SD.

(i) intergroup variability was calculated comparing the mean difference between each subgroup (N=5) and the mean of the complete sample (N=20).

(ii) intragroup variability was calculated comparing the standard deviation difference between each subgroup (N=5) and the total (N=20) neurotypical sample. Hence: $\Delta[\sigma_{group}; \sigma_{totalsample}]$ of the neuropeptide profiles.

Eight variables were used to characterize the 4 subgroups:

Orexin Trait;

β-Endorphin Trait;

Oxytocin Trait;

Substance P Trait;

Orexin State;

β-Endorphin State;

Oxytocin State;

Substance P State;

However, some variables had higher discriminative power than others, as described below.

In (a) trait (T0) profile, all the variables had (i) medium high intergroup variability and (ii) medium intragroup variability.

In (b) state $\Delta_{T0\text{-}T1}$ profile, all the variables had (i) high intergroup variability and (ii) medium low intragroup variability.

Intergroup variability is higher in the state profile and intragroup variability is lower. We postulate that the Intervention Protocol used from $\Delta_{T0\text{-}T1}$ might be important for subject stratification.

The Self Awareness subgroup is the one with the highest intragroup variability, possibly due to the higher cognitive load demanded by the Intervention Protocol.

The analysis of the intergroup and intragroup variability was later complemented by the inferential analysis.

Furthermore, for better data visualization of the neuropeptide profiles, we also plotted the descriptive analysis in boxplots (**Figure 65**).

These boxplots summarized the distribution of the Human Neuropeptide Assay and provided a more straightforward way to compare the neuropeptide response in the 4 subgroups.

They present the P25, the medians (according to the central limit theorem), the P75, the minimum and the maximum for the FI.

We wanted to understand if there is a difference in the Orexin, β-Endorphin, Oxytocin and Substance P responses, in the 4 subphenomes or subgroups of this study:

Self Awareness;

Self Consciousness;

Reflective Self;

Pre-Reflective Self.

Accordingly, **Figure 65** presents the characteristic and distinct neuropeptide stratum of the phenotypic trait (T0) and state $\Delta_{T0\text{-}T1}$ profiles.

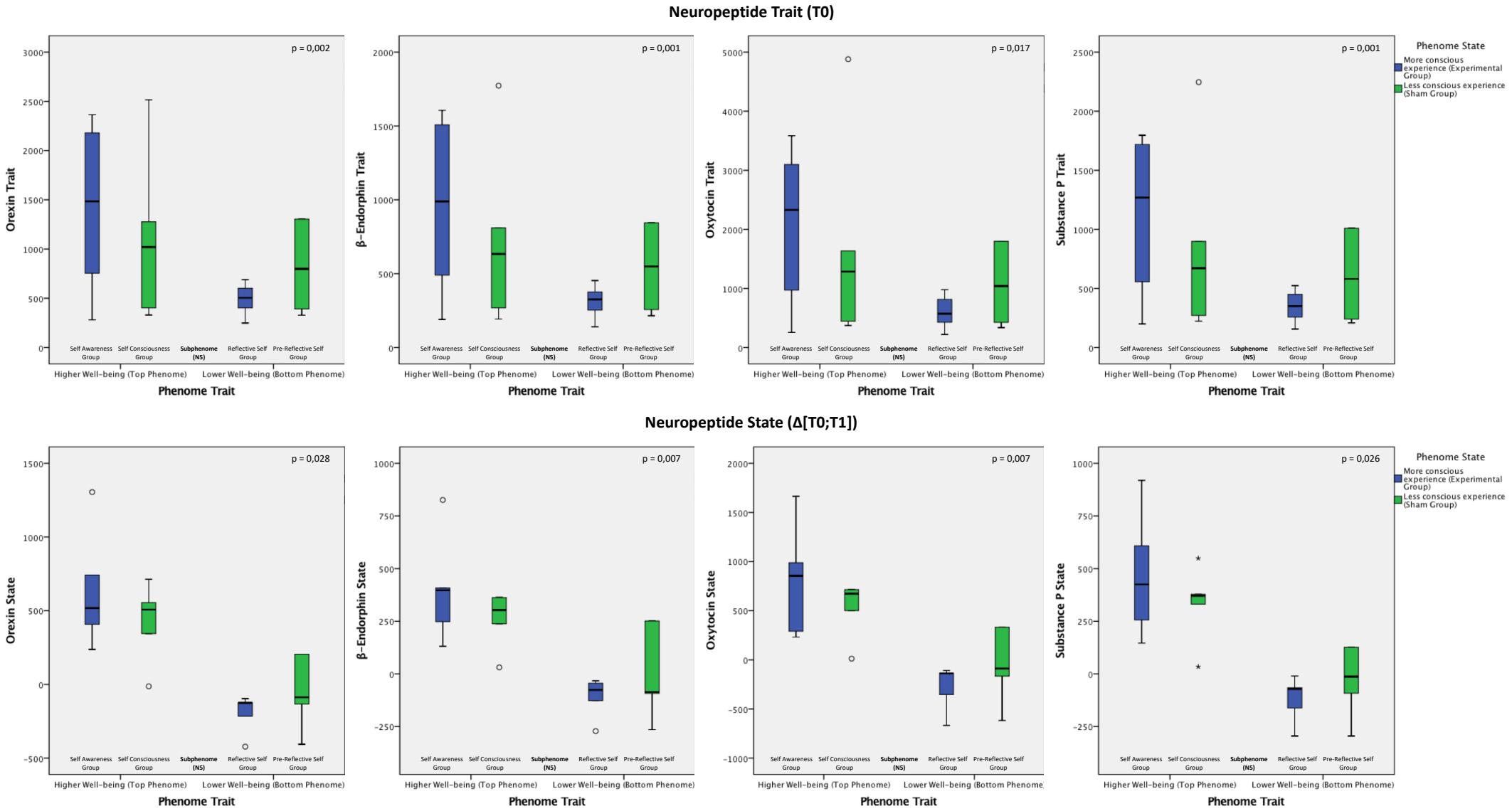


Figure 65. Inferential Analysis Of The Neuropeptide Profiles

The P25, medians, P75, minimum and maximum for the neuropeptide response in FI (cps) is presented in 8 boxplots. Both the Trait (4 boxplots) and State (4 boxplots) profiles are plotted for each of the neuropeptides: Orexin, β-Endorphin, Oxytocin and Substance P, in the 4 subphenomes.

6.A.2. Strata Characterization

In the second phase of the descriptive analysis, shown in **Figure 65**, we wanted to perform group, or strata, characterization, given by the investigation of each of the 8 variables. The objective was to test if there was a constant tendency present in all the variables. Our results followed our expectations and hypotheses addressed in **Figure 64**.

In (a) trait (T0) profile, the median FI of Orexin, β-Endorphin, Oxytocin and Substance P followed the same tendency: median FI (analyte) Self Awareness subgroup > median FI (analyte) Self Consciousness subgroup > median FI (analyte) Pre-Reflective Self subgroup > median FI (analyte) Reflective Self group.

In (a) state (Δ_{T0-T1}) profile, the median FI of Orexin, β-Endorphin, Oxytocin and Substance P followed, likewise, the same tendency: median FI (analyte) Self Awareness subgroup > median FI (analyte) Self Consciousness subgroup > median FI (analyte) Pre-Reflective Self subgroup > median FI (analyte) Reflective Self group.

The Self Awareness subgroup had the highest neuropeptide response, but more specifically an increase in Orexin, β-Endorphin and Oxytocin levels; followed by the Self Consciousness group, and more specifically, an increase in Oxytocin levels. The Pre-Reflective Self subgroup had marginal neuropeptide response for all analytes. The Reflective Self subgroup had a negative neuropeptide response for all analytes, but more importantly a decrease in Orexin and Oxytocin levels.

Moreover, in the state (Δ_{T0-T1}) profile, intergroup variability is higher. We conclude, then, that the Intervention Protocol used from Δ_{T0-T1} was important for a better stratification and, hence, for the distinct separation of the neuropeptide response in the 4 subgroups.

We conclude, thus, that for each analyte there is a distinct response in the 4 subgroups. This trait and state response is better characterized in the inferential analysis.

This stratification showed the presence of a statistical tendency. To better understand this neuropeptide statistical tendency in the 4 subgroups, we performed the following inference analysis.

This inferential analysis was used for hypothesis testing of the multiple comparisons we wanted to make between [all the analytes; all the samples] in our Human Neuropeptide Assay. Software Bio-Plex Data Pro™ has reserved capabilities to comply with this analysis. Consequently, we performed the statistical inference with SPSS Statistics 22.

6.A.3. Analysis Of Inferences And Multivariate Factor Analysis

The Inferential Analysis examined the same 8 variables of the descriptive analysis. The results of the inferential analysis were obtained by parametric hypothesis testing. The mean difference was considered significant when $p < \alpha=0.05$.

The normality, homogeneity of variances, asymmetries in the distribution, sample dimension and scale of the variables determined a parametric type of testing.

First, we (i) rejected ($\alpha=0.05$) homoscedasticity (Levene Test) and (ii) confirmed Brown-Forsythe equality of means.

Consecutively, a) a One-way ANOVA (Parametric) test was performed, to reject ($\alpha=0.05$ mean difference) intergroup variable score equality in the (i) Self Awareness group; in the (ii) Self Consciousness group; in the (iii) Reflective Self subgroup and in the (iv) Pre-Reflective Self group. Afterwards, if rejected, a) ANOVA for multiple comparisons was also applied.

In the (a) trait (T0) profile, it was rejected with a very high significance, that the score was equal between subgroups for the analytes:

(i) Orexin (One-Way ANOVA α=0.002); (ii) β-Endorphin (One-Way ANOVA α=0.001); (iii) Oxytocin (One-Way ANOVA α=0.017); and (iv) Substance P (One-Way ANOVA α=0.001).

And equally in multiple comparisons (95% Confidence Interval) with Bonferroni correction.

In the (b) state $\Delta_{T0\text{-}T1}$ profile, it was rejected with a very high significance that the score was equal between subgroups for the analytes:

(i) Orexin (One-Way ANOVA α=0.028); (ii) β-Endorphin (One-Way ANOVA α=0.007); (iii) Oxytocin (One-Way ANOVA α=0.007); and (iv) Substance P (One-Way ANOVA α=0.026).

And similarly, in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Finally, a multivariate factor analysis was executed to explore if the descriptive and inferential analysis had the capacity (i) to represent and (ii) to discriminate the differences in the 4 analytes of the Human Neuropeptide Assay.

For this analysis, the N (number of samples) was equal to 40, as we used duplicate sampling for each of the 20 subjects. Initially, both an (i) factor and (ii) reliability analysis was conducted. The (i) factor analysis showed, in the initial extraction communalities, an adequate minimum of 0.983. The communalities in our variables are high, which indicates that the inferential analysis represented the 4 analytes well. All analytes, extracted by Varimax rotation method with Kaiser normalization, had a minimum of 0.894 contribution (i.e., the Substance P) for its components (**Figure 66**).

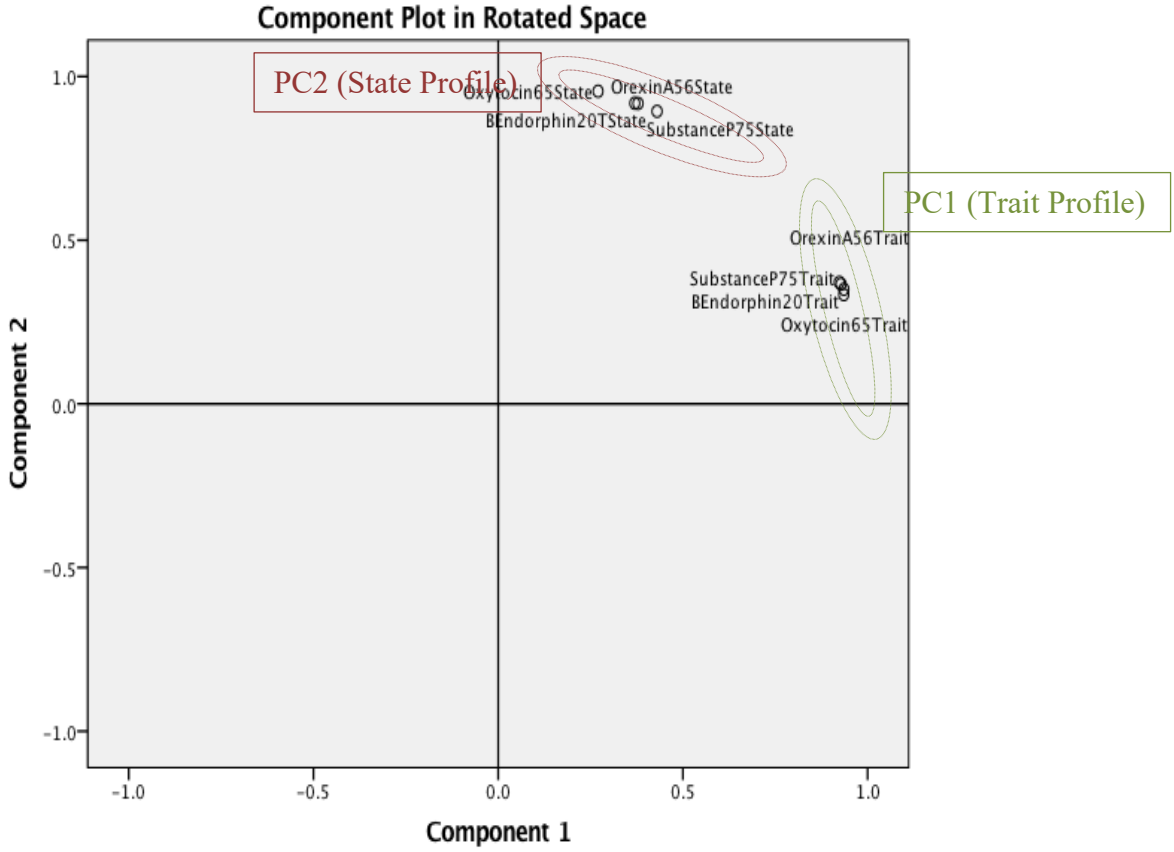


Figure 66. PCA Extraction Of The Neuropeptide Response In Young Neurotypical Adults
A 2-axis plot represents the (i) factor and (ii) reliability analysis of the Human Neuropeptide Assay. It is explained by two Eigenvalue components (98.7%), with a PC1 (0.714) and PC2 (0.700) contribution in the component matrix analysis.

Hence, the total variance in our Human Neuropeptide Assay was explained by two Eigenvalue components (98.7%), with a PC1 (0.714) and PC2 (0.700) contribution in the component matrix analysis. PC1 represented all the analytes of the trait profile, whereas PC2 the state profile. This analysis demonstrated that the 4 neuropeptides' assay was adequate to be applied to our sample of neurotypical subjects.

Afterwards, (ii) a reliability test of all the 4 analytes was performed to assess their internal consistency in our sample.

The reliability analysis, for a) the trait (T0) profile, showed an initial high Cronbach's α=0.948. The reliability analysis, for (b) the state $\Delta_{T0\text{-}T1}$ profile, showed an initial slightly bigger Cronbach's α=0.963.

Therefore, all analytes in our neurotypical sample had good discriminative power.

6.B. Human Th17 Cytokine Assay: *Quantification Of Interleukins In Saliva*

For better visualization of all the data of the Human Th17 Cytokine Assay, i.e., of the quantified proteins of the neuroimmune response, we created a Prezi infographic (Krum, 2013). It can be retrieved online with private access: https://tinyurl.com/Neuro-Immune-Network (retrieved July 23, 2020; *in* Annex K: External Support). This infographic clearly shows, with graphical zooming tools, the neuro-immune network. It comprehends 3 parts:

1. Graphical representation of all the samples across a single analyte;
2. Graphical representation of all the analytes across a single sample;
3. Functional analysis of the immune response in neurotypical subjects. It integrates the Bio-Plex® MAGPIX™ analysis, the anti-inflammatory and pro-inflammatory clustering of the Human Th17 Cytokine Assay, the PANTHER GO functional classification, and the Cytoscape functional clustering of the immune interaction networks.

This infographic is fully detailed and described along this section.

Figure 67 shows a boxplot as a dispersion representation of the Human Th17 Cytokine dataset in Log_2FI.

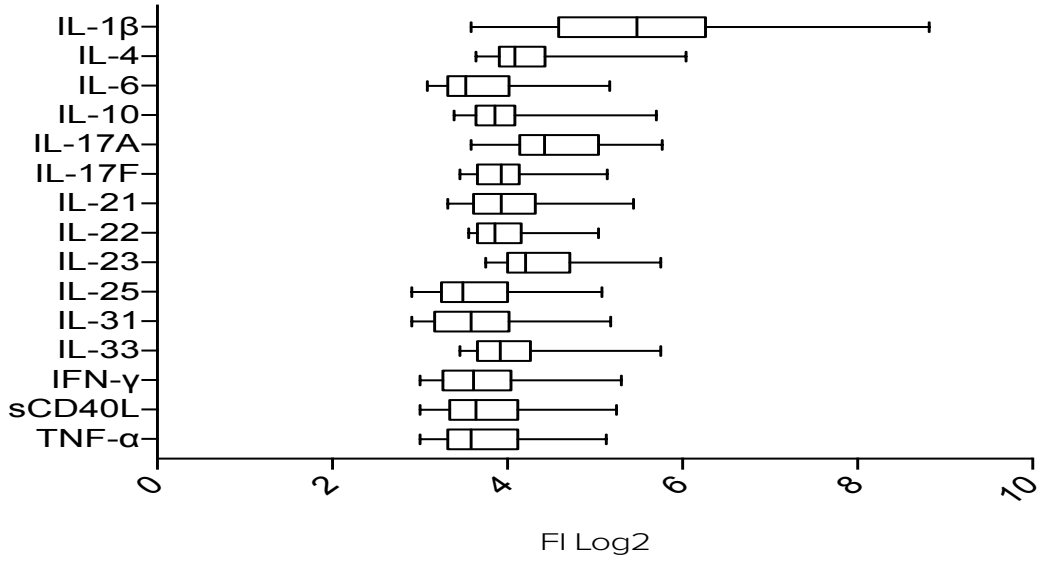


Figure 67. Data Dispersion (Log2fi) Of The Human Th17 Cytokine Assay
A boxplot of the 15 analytes studied is presented showing the P25, medians, P75, minimum and maximum, of the 20 neurotypical subjects (duplicate sampling) in T0 and T1

Figure 67 identified the detection values (log_2FI) in saliva for the Human Th17 Cytokine Assay with a sample of young neurotypical adults. It shows thus the physiological window of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α in this population without neuropsychiatric conditions.

It is possible to observe that the data is widely dispersed along the 1st and 3rd percentiles. However, the median FI for all the 15 analytes tends to be similar and in the same range [3.5;4.5]cps.

In the dispersion representation, we can observe the FI of all the 20 neurotypical subjects, with duplicate sampling in T0 and T1. IL-1β is the only analyte with a high florescence variability. Therefore, this should be carefully considered in future analysis.

However, we wanted to understand the immune response specifically in our 4 subgroups: Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self. The main objective was to test if an immune stratification could be verified in our 4 mental health profiles.

As shown in **Figure 68** (bottom), the Human Th17 Cytokine Assay had the following distribution.

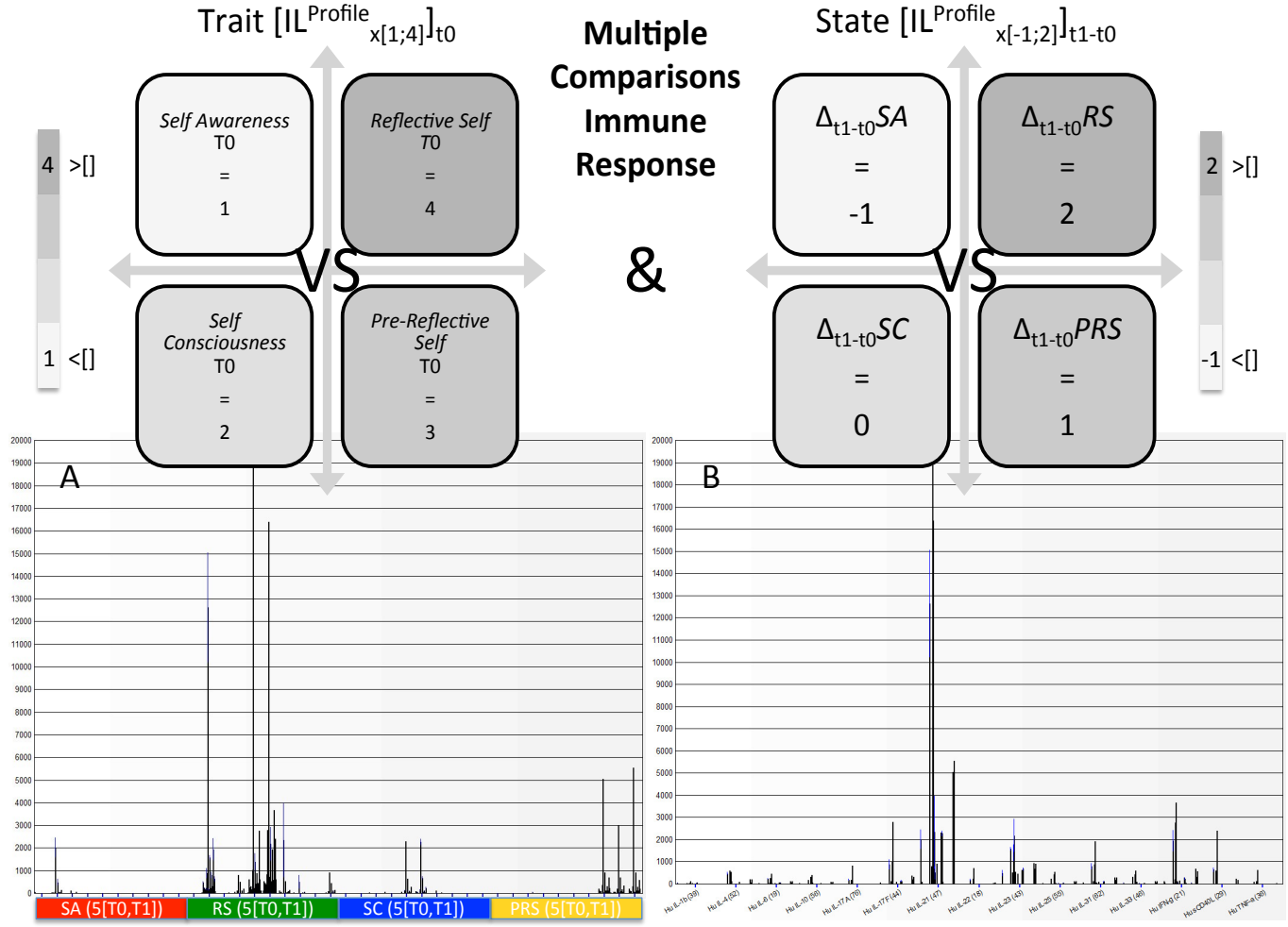


Figure 68. Interpretative Preliminary Schematic For The Trait (T0) And State (ΔT0-T1) Interleukin Profiles
A two-axis representation and two plots (A and B) are presented for the multiple comparisons of the observed concentration [Obs](pg/mL) w/ Error Bar (SD(rep)/√n.ºrep) of the analytes. In (A), the Human Th17 Cytokine Assay, in phenome (N20) and subphenomes (N5) (T0,T1), is represented (immune response analytes across multiple samples). In (B), all the samples are plotted across the multiple immune response analytes (15).

To plot the immune response in our 4 neurotypical subgroups, we used the observed concentration variable: [Obs](pg/mL) w/ Error Bar (SD (rep)/(√ (No. rep)). This variable is directly extracted with Bio-Plex Manager™.

In **Figure 68.A**, all the 15 analytes are plotted across the 20 samples (5 for each of the 4 subgroups). In **Figure 68.B**, all the samples are plotted across the multiple immune response analytes (15).

This extraction can be obtained by Bio-Plex Data Pro™.

In **Figure 68.A**, it is easily observed that the total cytokine or interleukin concentration, i.e., the $\Sigma\ [Obs]_{15\ analytes}$, is very different in the 4 subgroups.

This preliminary analysis shows that the Self Awareness subgroup had the lowest $\Sigma\ [Obs]_{15\ analytes}$, followed by the Self Consciousness group. The Pre-Reflective Self subgroup had higher analyte concentration and, lastly, the Reflective Self subgroup had the highest $\Sigma\ [Obs]_{15\ analytes}$.

In **Figure 68.B**, it is directly observed that some cytokines, e.g., IL-21, have a higher overall concentration, i.e., $[Obs]_{IL\text{-}21}$, than others, e.g., IL-4 or IL-6.

In neurotypical population, it has been documented that the serum levels of IL-21 do not follow a normal distribution and have a wide concentration range from [7.8;1621.3] pg/mL, with a median concentration of 66.33 pg/mL (Oliveira et al., 2015). This could be associated with the physiologic role of regulatory interleukins, such as IL-21, IL-22 and IL-33, and their combined anti&pro-inflammatory characteristics.

Contrastingly, the serum dosage of IL-4, a clearly anti-inflammatory cytokine, and IL-6, a clearly pro-inflammatory cytokine, follows a normal distribution and has lower concentrations. In young neurotypical adults, the mean $[IL\text{-}4]_{serum}$ is 7.5 pg/mL and the mean $[IL\text{-}4]_{serum}$ is 11.5 pg/mL (Kleiner et al., 2013).

This difference in the interleukin profile, in **Figure 68.A** and **Figure 68.B**, is relevant for the T0 + T1 time points, in a preliminary analysis.

However, for better understanding of those differences in the Human Th17 Cytokine Assay, in our neurotypical sample, we separated the analysis in two time points: T0 and T1.

On the one hand, T0 reflected the baseline concentration of all analytes for the subjects in the 4 subgroups: Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self.

T0 is the time point before the Intervention Protocol (Hipólito and Martins, 2017; chapter 3). Thus, T0 expressed the cytokine trait of our neurotypical sample.

On the other hand, T1 reflected the concentration of all analytes for the subjects in the 4 subgroups after the Intervention Protocol. It was calculated by: $\Delta_{T0\text{-}T1} = [analyte]_{T1} - [analyte]_{T0,}$ of each 15 cytokines present in the assay. Thus, T1 expressed a cytokine state of our neurotypical sample.

As shown at the top of **Figure 68**, we created a schematic of our expected results for the trait (T0) and state ($\Delta_{T0\text{-}T1}$) interleukin profiles. In darker gray, the highest $\Sigma\ [Obs]_{15\ analytes}$ are represented in lighter gray, the contrary, i.e., the lowest concentration.

This grading offered a scale that is based on the total cytokine or interleukin concentration of all the subjects in the 4 subgroups. On the left side (**Figure 68.A**), the trait profile is plotted from [1;4], while on the right side (**Figure 68.B**), the state profile is plotted from [-1;2]. Therefore, we expected the $\Sigma\ [Obs]_{15\ analytes}$ to be graded as following:

a. In the interleukin trait profile: Self Awareness subgroup < Self Consciousness subgroup < Pre-Reflective Self subgroup < Reflective Self group. Hence, the inflammatory trait, or pro-immune phenotype, would be higher in the Reflective Self subgroup and almost none existent in the Self Awareness group;

b. Likewise, in the interleukin state profile: Self Awareness subgroup < Self Consciousness subgroup < Pre-Reflective Self subgroup < Reflective Self group. Thus, the pro-inflammatory state, or immune response, would be higher in the Reflective Self group. However, we expected that the Self Awareness subgroup would have an anti-inflammatory response.

This interleukin profile characterization was further investigated and tested both for the trait (T0) and state ($\Delta_{T0\text{-}T1}$) immune response. Therefore, to understand if the subjects could be clustered in groups based on the Σ $[Obs]_{15\ analytes}$, we performed an unsupervised machine learning to reduce the dimensionality of our interleukin data set.

As shown in **Figure 67** and **Figure 68**, the data consists of several anti- and pro-inflammatory analytes (variables) specifically correlated with each other. As we wanted to retain the variation present in the dataset, up to the maximum extent, we used Principal Component Analysis (PCA) not to force, or label, specific subgroups.

6.B.1. Analyte Distribution

In **Figure 69**, with a general confidence interval equal to $\alpha<0,05$, we can observe that our 20 neurotypical subjects cluster in two main subgroups, in a two component PCA.

For the interleukin trait profile (T0), with a 68.6% eigenvalues variance, two separate groups are clustered: one with Self Awareness subjects and another with Pre-Reflective Self and Reflective Self subgroup subjects. Likewise, for the interleukin state profile (Δ[T0;T1), but with a higher 81.8% eigenvalues variance, two separate groups are clustered: one with Self Consciousness subjects and another with Pre-Reflective Self and Reflective Self subgroup subjects.

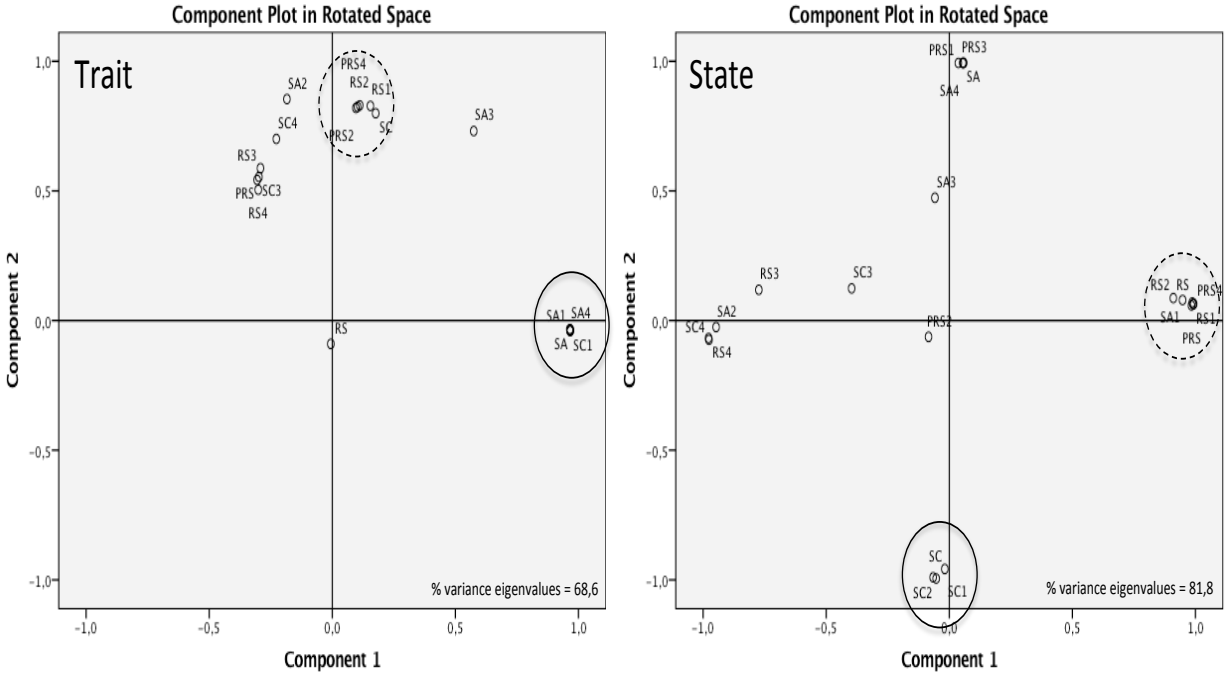


Figure 69. Subjects Clustering In The Human Th17 Cytokine Assay
The Total variance is explained by 2 components, with a PCA analysis of the 15 analytes. A 2-axis plot represents with a general confidence interval equal to $\alpha<0,05$, a cluster in two main subgroups for the Trait and State profiles.

This clustering reflects our Human Th17 Cytokine Assay dataset. To visualize how the data distributes (i) along the 15 cytokines and (ii) along the 20 neurotypical young adults, we propose a graphical representation shown in **Figure 70**.

In **Figure 70.B**, samples are plotted across multiple analytes. Likewise, for each analyte, its effect on the immune system is presented, i.e., the Anti (↑) / Pro (↓) Inflammatory Regulation.

This regulation is dependent on the physiological or pathological model that was reviewed in the Introduction.

Hence, the analytes are grouped as:

(i) anti-inflammatory: IL-4, IL-10 and IL-25;

(ii) pro-inflammatory: IL-1β, IL-6, IL-17A, IL-17F, IL-23, IL-31, INF-γ, sCD40L and TNF-α;

(iii) anti&pro-inflammatory: IL-21, IL-22 and IL-33.

The observed concentration is given by log[Obs](pg/mL) and the Error Bar by the SD (rep)/√ no. rep.

Figure 70 plots an overall distribution of the anti-/pro-inflammatory cytokines equilibrium, in T0 and T1, for the phenome (N20) and subphenomes (N5), i.e., the 4 subgroups.

In **Figure 70.A**, the distribution of all analytes is shown in different colors, e.g., IL-1β in light blue or TNF-α in light green. Il-4 and IL-6, in green and lighter blue respectively, are almost not visible due to their lower concentrations.

Both T0 and T1 concentrations are plotted, and it is observable that some analytes increase their concentration but others decrease it. For instance, the $\Delta_{T0\text{-}T1}$[IL-10] (in light brown) increases in the Self Awareness and Self Consciousness subgroups, whereas the $\Delta_{T0\text{-}T1}$[IL-1β] decreases in the Self Awareness subgroup and increases in the Reflective Self group.

On the contrary, IL-21 is an interleukin with an atypical profile in our sample, because its log[Obs](pg/mL) does not follow the exact tendency of the other 15 analytes, as further explained in the multivariate factor analysis. This is justified by both the anti- and pro-inflammatory roles of this analyte.

It is important to note that the analytes cluster in 4 different strata. These 4 distinct strata correspond to the 4 different subgroups in this study: Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self subgroups.

Therefore, those 4 separate immune profiles visually corresponded to the already stratified profiles of our neurotypical sample (Hipólito and Martins, 2017, chapter 3).

Summarizing, in a preliminary visual analysis, we could address the hypothesis that there is a correlation between these 4 immune strata and the mental health (chapter 3) and total protein profiles (chapter 5).

In **Figure 70.B**, the distribution of all 20 neurotypical samples across each analyte is shown. Some analytes, e.g., IL-21or IL-33, are more highly expressed than others, e.g., IL-1β or TNF-α.

Likewise, some analytes are ubiquitously or universally expressed in all the 20 subjects, e.g., IL-1β or TNF-α, while others are only expressed in some subjects, e.g., IL-17A or sCD40L.

That analysis is important to understand that the variability of the cytokine profile is high, but some patterns can be found and addressed with further hypothesis testing.

For instance, subject DO1296 (T0, in green) and DO1296R (T1, in brown), from the Reflective Self group, has a high immune response over all analytes. Contrastingly, subject DO1350 (T0, in light green) and DO1350R (T1, in dark yellow) has a low immune response over all analytes.

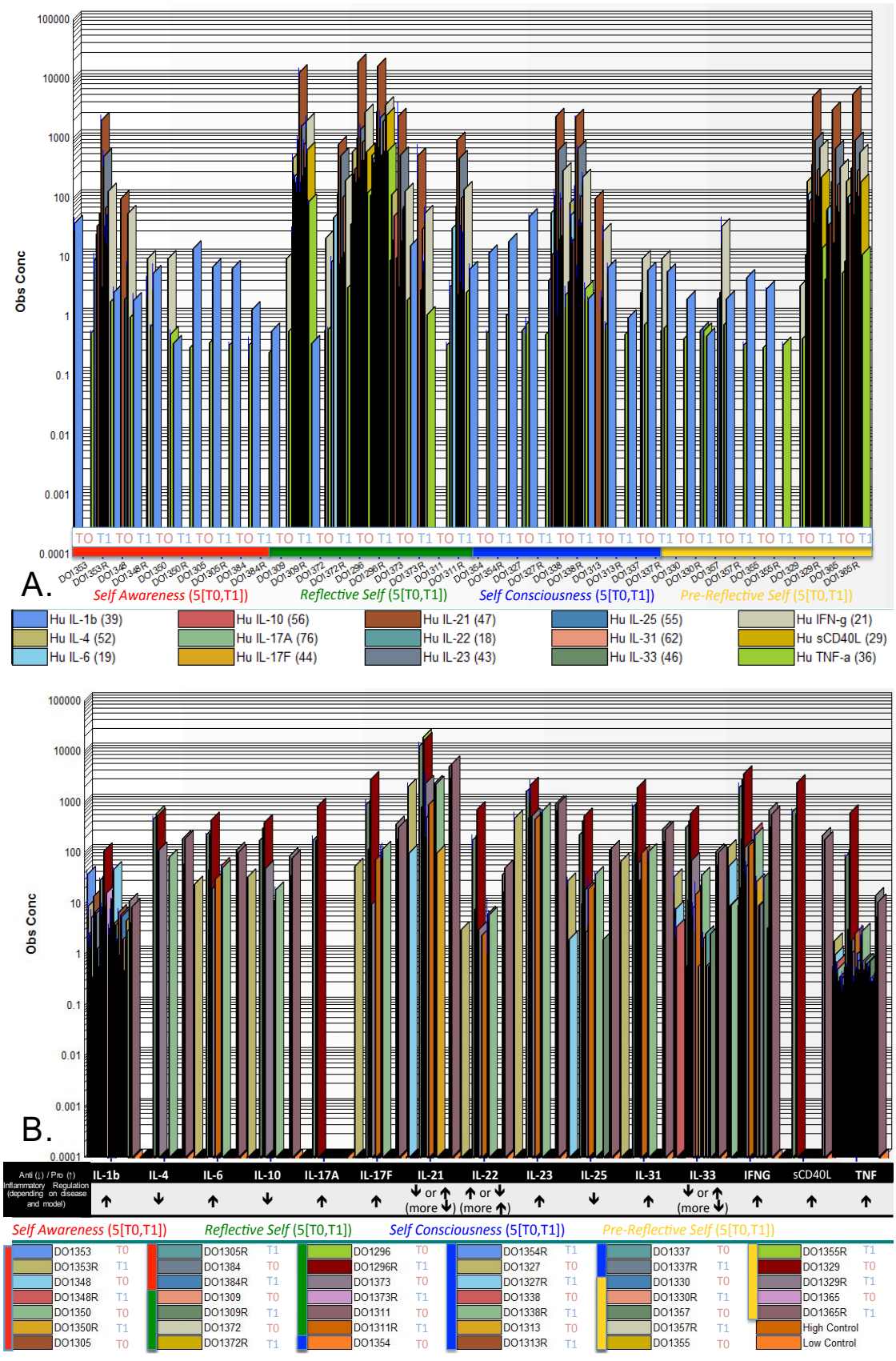


Figure 70. Descriptive Analysis Of The Anti-/pro-inflammatory Cytokine' Profiles
The observed concentration log[Obs](pg/mL) and Error Bar (SD(rep)/√no-rep) is presented for the Anti/pro-inflammatory cytokines equilibrium, in T0 and T1, for phenome (N20) and subphenomes (N5).
In A., the distribution of all analytes is shown in different colors (analytes across multiple samples).
In B., the distribution of all 20 neurotypical samples across each analyte is shown in different colors.

To better visualize this distribution, we created the previously referred infographic, and we plotted those results in two sets of graphics.

In the infographic https://tinyurl.com/Neuro-Immune-Network (retrieved July 24, 2020), the 15 analytes dataset is plotted in: Section 1 – showing a graphical representation of all the samples across a single analyte; Section 2 – showing a graphical representation of all analytes across a single sample.

This graphical representation better explores the complexity and variability of the cytokine assay, but analyte by analyte, in Section 1, and subject by subject, in Section 2.

Section 1 is shown, below, in **Figure 71**, and Section 2 in **Figure 72**.

In **Figure 71**, cytokines are divided according to the previous analysis in (i) anti-inflammatory, (ii) pro-inflammatory, and (iii) anti&pro-inflammatory; and plotted in a CMYK color scale. The cytokines are also grouped in correspondence with their subgroup of 5 subjects: Self Awareness subgroup (in red); Self Consciousness subgroup (in blue); Reflective Self subgroup (in green) and Pre-Reflective Self subgroup (in yellow).

The observed concentration is given by [Obs](pg/mL) and Error Bar by SD (rep)/√ no. rep, for time points T0 and T1.

In **Figure 72**, all subjects are plotted in T0 and T1, showing all the cytokines for that specific subject. Likewise, subjects are gathered in their subgroup.

Those subjects correspond to the 20 selected subjects stratified by total protein profiling (chapter 5). Therefore, the last subject of each subgroup corresponds to the controls for the immune response, i.e., the 5th line of graphs on the bottom of **Figure 71**. For better data visualization, in **Figure 72**, we used the observed concentration given by the log[Obs](pg/mL).

Figure 71 details the behavior of the interleukin profile in the 4 subgroups and shows that there is a tendency, both for T0 and T1 timepoints. This tendency differs if we consider only (i) anti-inflammatory cytokines, (ii) pro-inflammatory cytokines or (iii) anti&pro-inflammatory cytokines. Moreover, it is possible to observe that there is an intergroup significant variability and an intragroup consistency.

As generally described for the multiple subject view in **Figure 70**, but better detailed here, Self-Awareness subgroup subjects tend to have an anti-inflammatory response. Self-Consciousness subgroup subjects tend to have no immune response. Pre-Reflective Self subgroup subjects tend to have a light pro-immune response. Lastly, Reflective Self subgroup subjects tend to have a high immune response.

For instance, subject D01296, of the Reflective Self group, clearly has a pro-inflammatory immune response in all IL-1β, IL-6, IL-17A, IL-17F, IL-23, IL-31, INF-γ, sCD40L and TNF-α. Contrastingly, subject D01353, of the Self Awareness group, has no expression of pro-inflammatory cytokines and a positive expression of anti-inflammatory cytokines: IL-10 and IL-25.

Figure 72 details the same interleukin behavior, but better plots T0 and T1 time points. This analysis shows not only a tendency for (i) anti-inflammatory, (ii) pro-inflammatory and (iii) anti&pro-inflammatory cytokines, but also for the interleukin trait (T0) and state $\Delta_{T0\text{-}T1}$.

As an example, D01309, of the Reflective Self group, had a non-inflammatory trait (T0), but a highly inflammatory response, i.e., state $\Delta_{T0\text{-}T1}$. This state profile is due to the increase of all pro-inflammatory cytokines, but particularly IL-17F and IL-21. On the contrary, subject D01357, of the Self Consciousness group, had a slight inflammatory trait (T0), but an anti-inflammatory response, i.e., state $\Delta_{T0\text{-}T1}$. This state profile is due to the decrease of both pro-inflammatory cytokines: INF-γ and TNF-α, and IL-33, an anti&pro-inflammatory cytokine.

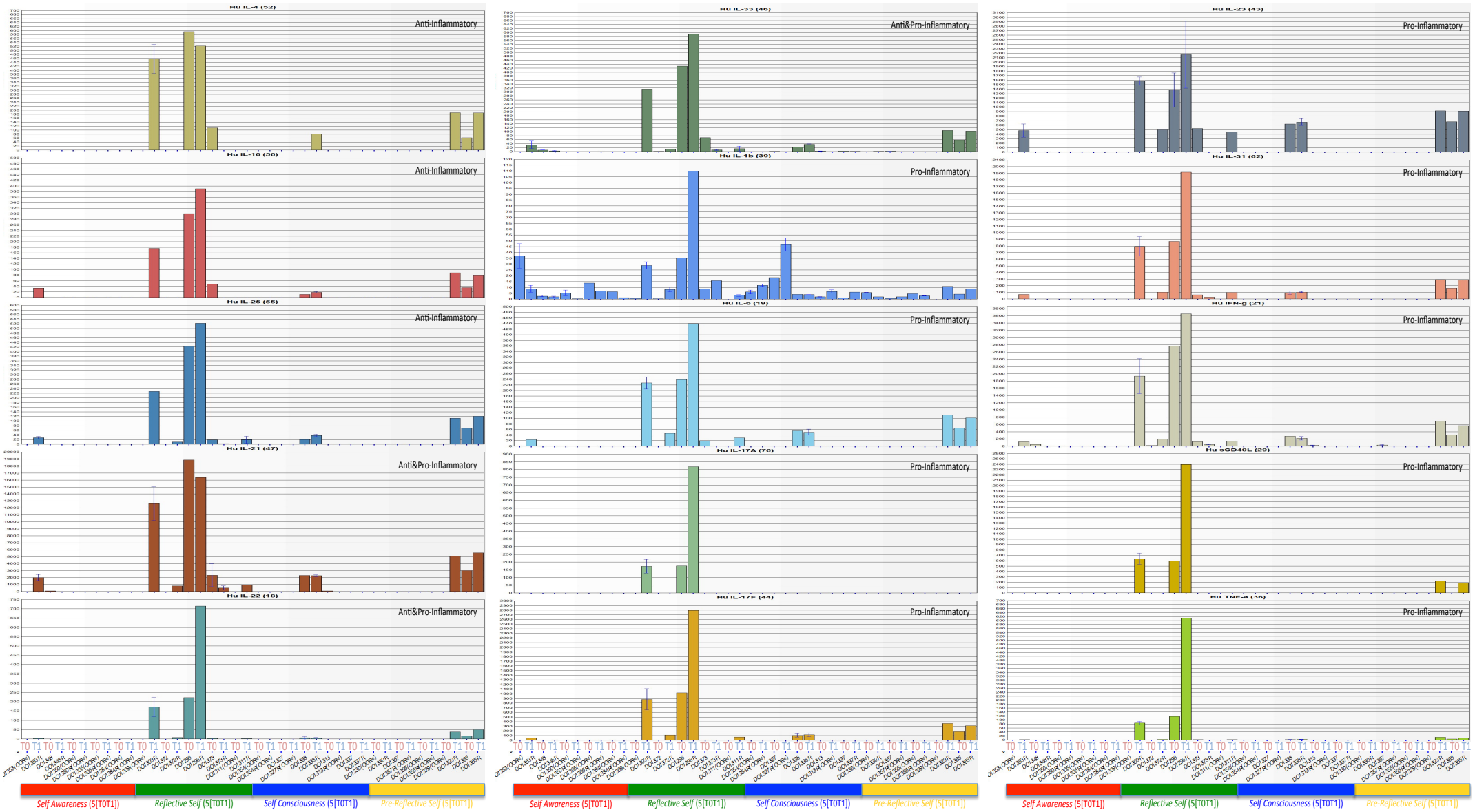


Figure 71. Descriptive Analysis Of The Human Th17 Cytokine Assay: Samples Across A Single Analyte
The Observed concentration [Obs](pg/mL) and Error Bar (SD(rep)/√n.ºrep) is presented, in T0 and T1, for the phenome (N20) and subphenomes (N5) for all analytes.

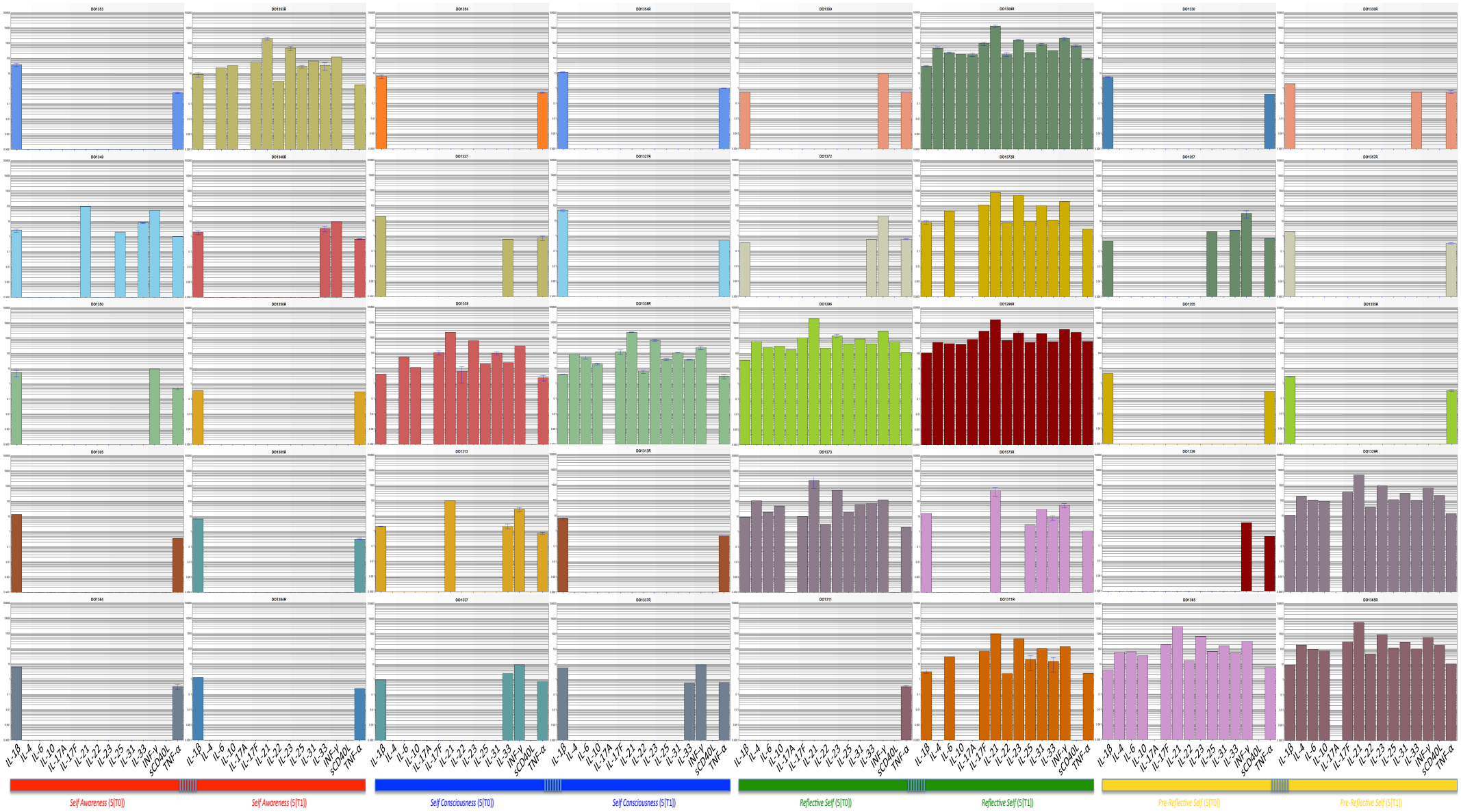


Figure 72. Descriptive Analysis Of The Human Th17 Cytokine Assay: Analytes Across A Single Sample
The Observed concentration [Obs](pg/mL) and Error Bar (SD(rep)/√n.ºrep) is presented, in T0 and T1, for the phenome (N20) and subphenomes (N5) for all analytes.

However, if we want to investigate if an interleukin profile is present, it is necessary to better test this tendency and the following hypotheses:

a) there is intergroup variability of the [Obs](pg/mL) of the total 15 analytes, clustered by their anti-inflammatory, (ii) pro-inflammatory or (iii) anti&pro-inflammatory response;

b) there is intergroup variability of the [Obs](pg/mL) in the interleukin trait profile (T0);

c) there is intergroup variability of the [Obs](pg/mL) in the interleukin state profile ($\Delta_{T0\text{-}T1}$);

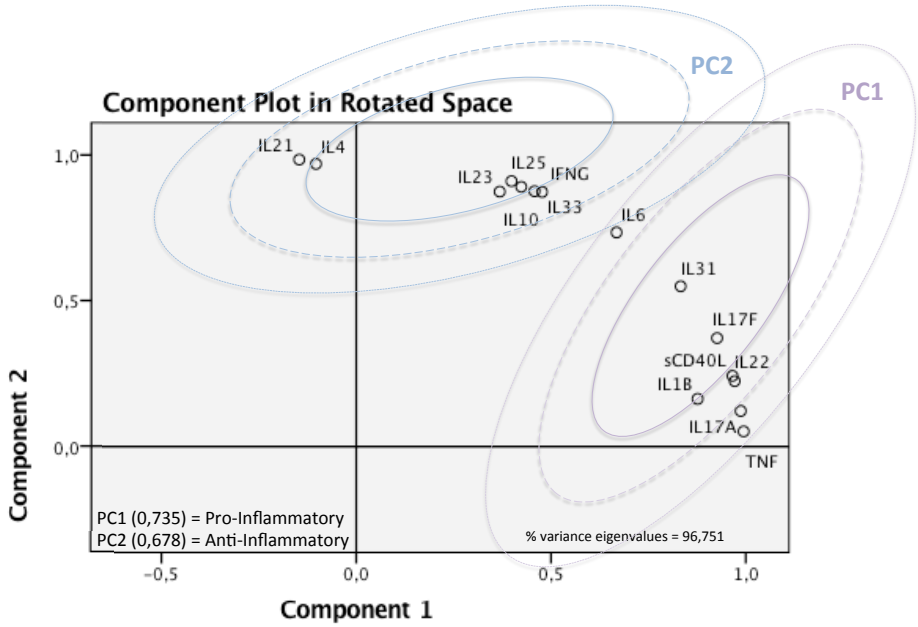
d) there is low intragroup variability of the [Obs](pg/mL) in the interleukin trait profile (T0);

e) there is low intragroup variability of the [Obs](pg/mL) in the interleukin state profile ($\Delta_{T0\text{-}T1}$).

6.B.2. Inflammatory Profiling By Unsupervised Learning

To test those hypotheses, we first visualized if there was an unsupervised clustering of the 15 cytokines in functional subgroups. This clustering would specifically reflect if, for our 20 neurotypical young adults' sample, there was a cytokine grouping. This grouping particularly reflects the Human Th17 Cytokine Assay, measured in our sample, and not the literature revision of the interleukin functions.

Therefore, we did not label or addressed it in a supervised machine learning. Instead, we used a PCA extraction, as shown in **Figure 73**.



Citokines, grouped by PCA, more expressed by subphenome

Anti-Inflammatory				Pro-Inflammatory			
SA	RS	SC	PRS	SA	RS	SC	PRS
		IL-4	IL-4	IL-1β	IL-1β	IL-1β	IL-1β
IL-10	IL-10	IL-10			IL-6		IL-6
IL-21	IL-21	IL-21	IL-21		IL-17A		
IL-23	IL-23	IL-23	IL-23		IL-17F		IL-17F
IL-25	IL-25	IL-25			IL-22	IL-22	IL-22
IL-33	IL-33	IL-33	IL-33		IL-31	IL-31	IL-31
IFN-γ	IFN-γ	IFN-γ	IFN-γ		sCD40L		sCD40L
					TNF-α		TNF-α

Figure 73. Human Th17 Cytokine Assay Clustering
A 2-axis plot represents the anti-inflammatory and pro-inflammatory strata, by PCA extraction of the Human Th17 Cytokine Assay. The total variance is explained by two Eigenvalue components (97%), extracted using component matrix method through Varimax rotation with Kaiser normalization.

Figure 73 shows that our immune data, in our neurotypical sample, had a tendency, which is uncovered by pattern recognition of the $[Obs]_{T0;T1}$. We observe that the Human Th17 Cytokine Assay dataset clusters in two main functional subgroups, or two components, with a 96,751% eigenvalues variance. These subgroups were obtained by the observed concentration ([Obs](pg/mL)) of all 15 analytes in the 20 subjects.

Those two components were first acquired by a factor analysis, showing an adequate minimum of 0.901 in the initial extraction communalities, except for the IL-1β (0.795).

Communalities indicate the amount of variance in each variable that is accounted. They are estimates of the variance in each variable accounted by all factors. The communalities in our two components are high, which indicates that the extracted components represent all the variables of the Human Th17 Cytokine Assay well, apart for IL-1β (0.795).

The total variance was explained by two Eigenvalue components (97%). These two components were extracted using the PCA component matrix method through Varimax rotation with Kaiser normalization.

The first PC1 component explains 73.5% of the distribution and comprehends the pro-inflammatory cytokines: IL-1β, IL-6, IL-17A, IL-17F, IL-31, sCD40L and TNF-α. It also includes IL-22, an anti&pro-inflammatory cytokine. Those are all analytes consistent with a pro-inflammatory profile. Thus, this PC1 reflects a specific clustering, in our neurotypical data of cytokines, which we consider having a pro-immune response.

The second PC2 component explains 67.8% of the distribution and comprehends the anti-inflammatory cytokines: IL-4, IL-10 and IL-25. It also includes IL-21 and IL-33 anti&pro-inflammatory cytokines. Those are all analytes consistent with an anti-inflammatory profile. Thus, this PC2 reflects a specific clustering, in our neurotypical data of cytokines, that we consider having an anti-immune response.

IL-23 and INF-γ were considered, by the PCA analysis of our 20 subjects' dataset, part of the PC2, and thus placed outside their correspondent physiological role.

This may be justified, as described in the Introduction, because IL-23 has an anti&pro-inflammatory function. Likewise, IFN-γ has not only a pro-inflammatory function, but also a social cognition function (Filiano et al., 2016). Therefore, they could have been statistically placed in PC2, because they are important in cognitive functions, like memory and learning (Litteljohn, Nelson and Hayley, 2014).

Hence, this unsupervised algorithm approximately follows our literature revision about the biological function of those 2 analytes. Nevertheless, in this machine learning we need to consider them as outliers, because they clearly have no anti-inflammatory function.

Afterwards, a reliability analysis of all the 15 analytes was performed to assess their internal consistency in the 4 subgroups, or subphenomes, i.e., to understand if those two components and their clustered analytes had the capacity to separate the Self Awareness group, the Self Consciousness group, the Reflective Self group, and the Pre-Reflective Self group.

The reliability analysis was obtained by final component contribution extracted by PCA with Varimax rotation method and Kaiser normalization. It showed an initial medium-low Cronbach's $\alpha=0.597$; but if IL-21 was discarded, it increased to 0.915. Therefore, IL-21 is the analyte that, in our neurotypical profiling, does not have good discriminative power.

The analytical difference between all the analytes, in the 4 factors, given by the ANOVA Cochran Q test, has a very high significance with a $p<0,001$, if IL-21 discarded. Henceforth, in the bottom of **Figure 73**, the clustered analytes in the two components, (i)

anti-inflammatory (PC2) and pro-inflammatory (PC1), were classified according to those 4 factors, i.e., according to their expression, the analytes were grouped in their specific subgroup. An unsupervised learning designated, as described below, which analytes were present, or more expressed, on the 4 subgroups: Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self.

In (i) the anti-inflammatory (PC2) dimension:

The Self Awareness subgroup is represented by the overexpression of IL-10, IL-21, IL-25 and IL-33;

The Reflective Self subgroup is represented by the overexpression of IL-10, IL-21, IL-25 and IL-33;

The Self Consciousness subgroup is represented by the overexpression of IL-4, IL-10, IL-21, IL-25 and IL-33;

The Pre-Reflective Self subgroup is represented by the overexpression of IL-4, IL-21 and IL-33.

In (ii) the pro-inflammatory (PC1) dimension:

The Self Awareness subgroup is represented by the overexpression of IL-1β;

The Reflective Self subgroup is represented by the overexpression of IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L and TNF-α;

The Self Consciousness subgroup is represented by the overexpression of IL-1β, IL-22, IL-31;

The Pre-Reflective Self subgroup is represented by the overexpression of IL-1β, IL-6, IL-17F, IL-22, IL-31, sCD40L and TNF-α.

The results of this machine learning not only (i) reflect the data distribution, of our sample, plotted on **Figure 70, Figure 71** and **Figure 72**, but are also (ii) consistent with the literature revision that we described in the Introduction, for the biological function of each of the 15 analytes.

Henceforth, we were able to create (i) anti-inflammatory, (ii) pro-inflammatory or (iii) anti&pro-inflammatory variables, with the correspondent cytokines.

The 15 analytes were assembled in those 3 variables and denominated as inflammatory profiles. Then, we carried out a descriptive analysis, of all the 15 analytes, classified in those 3 variables.

From the complete Human Th17 Cytokine Assay dataset, 24 variables were extracted. Twelve correspond to the physiological trait (T0) profile, while the other 12 correspond to the physiological state $\Delta_{T0\text{-}T1}$ profile. They were separated into absolute values: [analyte] ($\log_{10}$[Obs](pg/mL); and relative values: ([analyte] / [$analyte_{PositiveControl}$]) / [UpperLimitAnalyte] (%).

Moreover, the variables average of the [analyte] of the 5 subjects of each subgroup was calculated and it defined group variables.

To do so, the $analyte_{PositiveControl}$ was chosen from the 4 positive controls subjects expressing clinical evidence of a chronic inflammatory pathology (chapter 5), i.e., Asthma and Allergic Rhinitis. In each subgroup, those had *a priori* a positive pro-inflammatory [analyte].

The [UpperLimitAnalyte] was the subject that had the highest [analyte] expression for a specific analyte. When a specific analyte had an out of range (sensitivity) concentration, we opted for using the lowest limit of quantification (Bio-plex® Pro human Th17 cytokine panel) of that analyte, to calculate its relative value.

Therefore, we found the following variables (**Table 9**), which will be discussed subsequently, and integrated into the 4 subgroups previously established (chapter 3; chapter 5).

Table 9 summarizes the descriptive analysis. It has information regarding: N (number of subjects); mean concentration (log_{10}[Obs](pg/mL)) of the analytes in each of the 3 clustered variables (according to the central limit theorem), or inflammatory profiles; and the corresponding standard deviation.

In this analysis, we present the inflammatory profiles both in relative % and in absolute values. The relative % was calculated by: inflammatory profile divided by the total inflammatory load ([anti; pro; anti&pro]). Hence, relative % = inflammatory profile / [anti; pro; anti&pro]$_{total}$. The absolute values were given directly by log_{10}[Obs](pg/mL).

Moreover, the inflammatory profiles were systematized by subjects and by groups. In the subject analysis, we presented N, mean concentration and standard deviation for every 20 subjects in each 4 subgroups. On the contrary, in the group analysis, we directly compared the groups mean concentration. The working hypothesis of this analysis was that the distinctive mental health phenomes (chapter 3) had different inflammatory profiles. As the measurements were made in the time points T0 and T1, both a physiologic trait (T0) and a physiologic state (Δ_{T1-T0}) were tested (**Figure 68**).

Table 9. Descriptive Analysis Of The Neuroimmune Response
Table summarizing the N, mean and standard deviation for the inflammatory profiles in relative % and absolute values, by subjects and by groups.

Bio-plex Pro human Th17 cytokine panel, Bio-Rad®		Inflammatory Profile	% by Subject			% By Group			By Subject			By Group		
			Anti	Pro	Anti&Pro	Anti	Pro	Anti&Pro	Anti	Pro	Anti&Pro	Anti	Pro	Anti&Pro
		N	20	20	20	20	20	20	20	20	20	20	20	20
TRAIT	All	Mean	0,065	0,085	0,065	0,065	0,085	0,065	28,308	60,153	459,122	28,308	60,153	459,122
		σ	0,224	0,219	0,223	0,096	0,079	0,092	98,395	180,365	1458,831	42,569	70,542	598,792
		Mean	,000	,040	,002	,000	,040	,002	,129	2,900	6,953	,129	2,900	6,953
Self Awareness		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,001	,042	,003	,000	,000	,000	,289	2,318	15,547	,000	,000	,000
		Mean	,006	,042	,015	,006	,042	,015	2,090	27,437	162,047	2,090	27,437	162,047
Self Consciousness		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,013	,051	,030	,000	,000	,000	4,673	57,256	343,686	,000	,000	,000
		Mean	,226	,219	,220	,226	,219	,220	99,807	177,644	1461,877	99,807	177,644	1461,877
Reflective Self		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,436	,436	,438	,000	,000	,000	191,697	350,289	2841,911	,000	,000	,000
		Mean	,026	,038	,025	,026	,038	,025	11,207	32,630	205,613	11,207	32,630	205,613
Pre-Reflective Self		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,057	,066	,054	,000	,000	,000	24,699	70,016	459,296	,000	,000	,000
		N	20	20	20	20	20	20	20	20	20	20	20	20
STATE	All	Mean	,101	,099	,084	,101	,099	,084	26,763	107,407	345,458	26,763	107,407	345,458
		σ	,252	,231	,203	,088	,134	,096	71,474	243,253	1072,551	22,469	138,805	307,083
		Mean	,020	-,002	,017	,020	-,002	,017	4,000	14,538	129,457	4,000	14,538	129,457
Self Awareness		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,047	,013	,042	,000	,000	,000	9,310	37,007	308,607	,000	,000	,000
		Mean	,020	,012	,001	,020	,012	,001	7,081	,375	-8,014	7,081	,375	-8,014
Self Consciousness		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,044	,017	,007	,000	,000	,000	15,834	1,993	14,144	,000	,000	,000
		Mean	,222	,321	,237	,222	,321	,237	55,471	336,108	741,080	55,471	336,108	741,080
Reflective Self		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,464	,396	,368	,000	,000	,000	134,236	414,694	2079,024	,000	,000	,000
		Mean	,143	,065	,082	,143	,065	,082	40,500	78,607	519,309	40,500	78,607	519,309
Pre-Reflective Self		N	5	5	5	5	5	5	5	5	5	5	5	5
		σ	,212	,108	,122	,000	,000	,000	59,250	127,295	773,652	,000	,000	,000

For (a) the trait, the inflammatory profiling was made through the following analysis:

(i) the subject's relative variable was calculated by the subject's inflammatory profile divided by $\log_{10}$[Obs](pg/mL) of the positive control for the corresponding subgroup. Therefore, trait cytokine accounts (relative % of the anti-, pro- and anti&pro-inflammatory profiles) were studied to probe into the individual clinical inflammatory behavior;

(ii) the group's relative variable, on the contrary, was calculated by the subject's inflammatory profile divided by the positive control subject with the highest $\log_{10}$[Obs](pg/mL), regardless of the group. Therefore, average trait cytokine accounts (group relative % of the anti-, pro- and anti&pro-inflammatory profiles) were studied to probe into the relative immune response. This average immune response is necessary to validate distinct profiles in the 4 subgroups, and demonstrates a characteristic $\log_{10}$[Obs](pg/mL) of that stratum;

(iii) the subject's absolute variable was calculated with direct $\log_{10}$[Obs](pg/mL);

(iv) the groups' absolute variable was calculated directly with the average $\log_{10}$[Obs](pg/mL) of each of the 4 subgroups. This variable is important because it permits to study the effect (or ratio) that a cognitive trait has on the (i) anti-, (ii) pro- and (iii) anti&pro-inflammatory profiles, and, more specifically, the effect that the cognitive load has on the total inflammatory load. For Dantzer and colleagues (2008), there is a correlation between the total inflammatory and cognitive load, in neurological and mental health.

A high cognitive load could possibly increase the absolute expression ($\log_{10}$[Obs](pg/mL)) of both anti-, pro- and anti&pro-cytokines. This increase could possibly be justified by changing the inflammatory equilibrium or tendency to a higher metabolic state. When a task increases stress systems, cognition starts (Hipólito and Martins, 2018).

For the (b) state, the inflammatory profiling was made with the same analysis. Contrastingly, the same hypothesis testing was made but for the $\Delta_{T0\text{-}T1}$ difference.

The *rationale* of performing the measurements in the (a) trait [T0] and (b) state $\Delta_{T0\text{-}T1}$ profiles is because our neurotypical sample may have an *a priori* immune phenotype (T0); but also, a state change $\Delta_{T0\text{-}T1}$, induced by the Intervention Protocol, on the immune response.

6.B.3. Intergroup And Intragroup Variability

In the first phase of the descriptive analysis, explored in **Table 9**, we wanted to test (i) intergroup variability, assumed by the variable mean, and (ii) intragroup variability, given by the variable SD.

(i) intergroup variability was calculated comparing the mean difference between each subgroup (N=5) and the mean of the complete sample (N=20).

(ii) intragroup variability was calculated comparing the standard deviation difference between the total samples (N=20) systemized by subjects and the total samples (N=20) systemized by groups. Hence: $\Delta[\sigma_{subject}; \sigma_{group}]$ of the inflammatory profiles, both in relative % and absolute values.

From all 24 variables used to characterize the 4 subgroups, some variables had higher discriminative power than others, as described below.

In (a) the trait (T0) profile, for the variable:

(i) Relative percentage of the anti-inflammatory Trait, (i) intergroup variability is moderate-low and (ii) intragroup variability is low;

(ii) Relative percentage of the pro-inflammatory Trait, (i) intergroup variability is low and (ii) intragroup variability is low;

(iii) Relative percentage of the anti&pro-inflammatory Trait, (i) intergroup variability is moderate and (ii) intragroup variability is low;

(iv) Absolute anti-inflammatory Trait, (i) intergroup variability is moderate and (ii) intragroup variability is moderate;

(v) Absolute pro-inflammatory Trait, (i) intergroup variability is moderate and (ii) intragroup variability is high;

(vi) Absolute anti&pro-inflammatory Trait, (i) intergroup variability is very high and (ii) intragroup variability is very high.

In (b) the state $\Delta_{T0\text{-}T1}$ profile, for the variable:

(i) Relative percentage of the anti-inflammatory State, (i) intergroup variability is low and (ii) intragroup variability is low;

(ii) Relative percentage of the pro-inflammatory State, (i) intergroup variability is moderate and (ii) intragroup variability is very low;

(iii) Relative percentage of the anti&pro-inflammatory State, (i) intergroup variability is moderate-low and (ii) intragroup variability is low;

(iv) Absolute anti-inflammatory State, (i) intergroup variability is moderate-low and (ii) intragroup variability is moderate;

(v) Absolute pro-inflammatory State, (i) intergroup variability is moderate-high and (ii) intragroup variability is high;

(vi) Absolute anti&pro-inflammatory State, (i) intergroup variability is high and (ii) intragroup variability is very high.

For the variables Absolute anti&pro-inflammatory Trait and anti&pro-inflammatory State, we observed that there is elevated intragroup variability. This is justified by the more heterogenous type of response, i.e., a combined anti- and pro-inflammatory response, characterizing IL-21, IL-22 and IL-33. The analysis of the intergroup and intragroup variability is complemented by the inferential analysis. Furthermore, for better data visualization of the inflammatory profiles, we also plotted the descriptive analysis in boxplots (**Figure 74** and **Figure 75**).

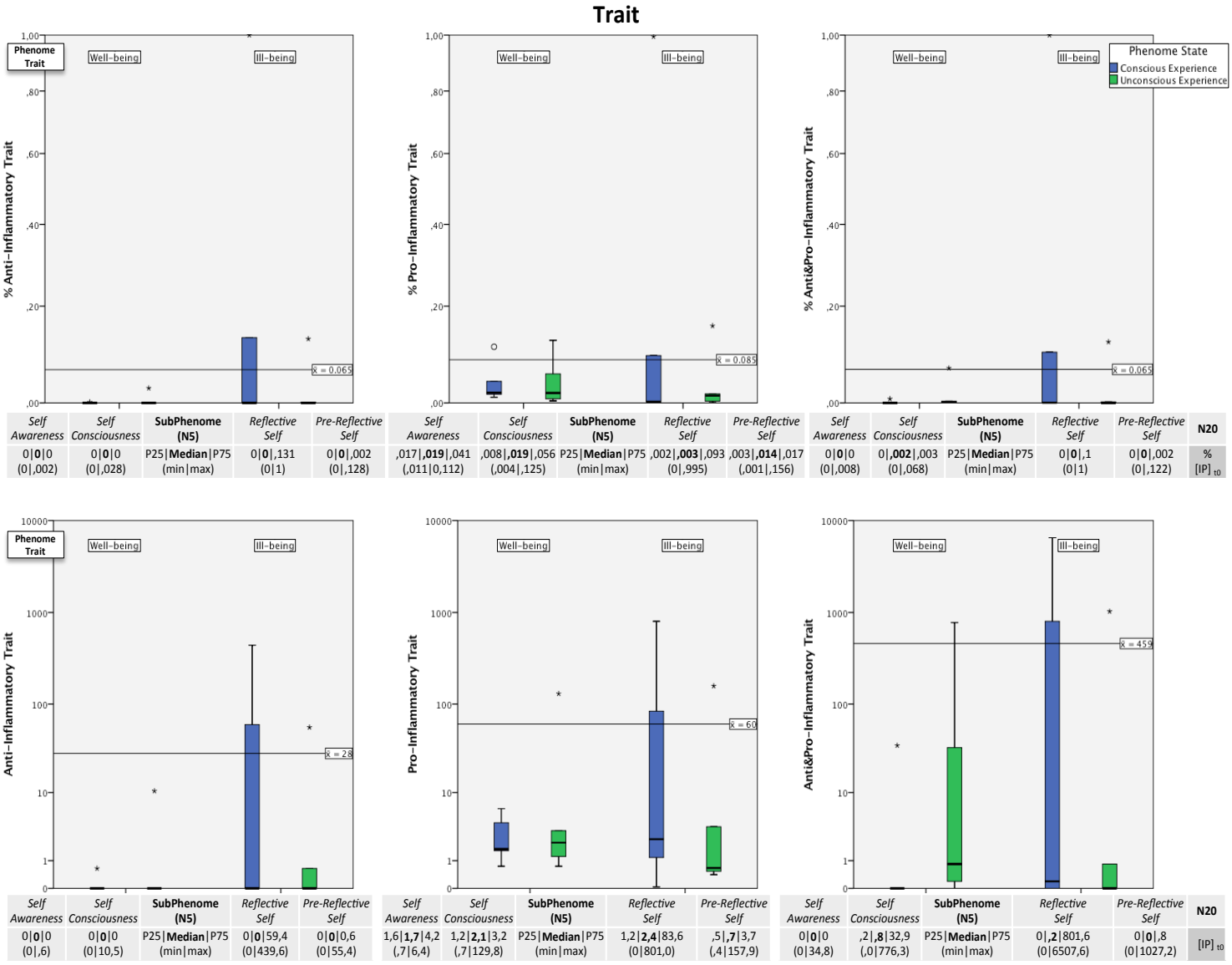
These boxplots summarize, in a simple way, the distribution (Potter et al., 2006) of the Human Th17 Cytokine Assay and provide a more straightforward way to compare the inflammatory profile in the 4 subgroups. They present the P25, the medians (according to the central limit theorem), the P75, the minimum and the maximum for the $\log_{10}$InflammatoryProfile (IP). Like in **Table 9**, we plotted the inflammatory profile in relative % and absolute $\log_{10}$[Obs](pg/mL).

We wanted to understand if there is a difference in the inflammatory profiles, i.e., (i) anti, (ii) pro and anti&pro (iii) in the 4 subphenomes or subgroups: Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self. In this analysis, we only extracted the $\log_{10}$[Obs](pg/mL) by subjects, because if systematized by groups, there would be no graphical representation in the boxplots.

Figure 74 presents the inflammatory profile: (i) anti-, (ii) pro- and (iii) anti&pro-inflammatory profiles of the phenotypic trait (T0) characteristic of each of the 4 distinct subgroups. **Figure 75** presents the inflammatory profile: (i) anti-, (ii) pro- and (iii) anti&pro-inflammatory profiles of the phenotypic state ($\Delta_{T0\text{-}T1}$) characteristic of each of the 4 distinct subgroups.

6.B.4. Strata Characterization

In the second phase of the descriptive analysis, displayed in **Figure 74** and **Figure 75**, we wanted to perform group, or strata, characterization, given by the investigation of each of the 12 variables, systemized by subjects. The objective was to test if there was a constant tendency present in all variables.



Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	N20
0|**0**|0 (0|,002)	0|**0**|0 (0|,028)	P25|**Median**|P75 (min|max)	0|**0**|,131 (0|1)	0|**0**|,002 (0|,128)	,017|**,019**|,041 (,011|0,112)	,008|**,019**|,056 (,004|,125)	P25|**Median**|P75 (min|max)	,002|**,003**|,093 (0|,995)	,003|**,014**|,017 (,001|,156)	0|**0**|0 (0|,008)	0|**,002**|,003 (0|,068)	P25|**Median**|P75 (min|max)	0|**0**|,1 (0|1)	0|**0**|,002 (0|,122)	% $[IP]_{t0}$

Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	N20
0|**0**|0 (0|,6)	0|**0**|0 (0|10,5)	P25|**Median**|P75 (min|max)	0|**0**|59,4 (0|439,6)	0|**0**|0,6 (0|55,4)	1,6|**1,7**|4,2 (,7|6,4)	1,2|**2,1**|3,2 (,7|129,8)	P25|**Median**|P75 (min|max)	1,2|**2,4**|83,6 (0|801,0)	,5|**,7**|3,7 (,4|157,9)	0|**0**|0 (0|34,8)	,2|**,8**|32,9 (,0|776,3)	P25|**Median**|P75 (min|max)	0|**,2**|801,6 (0|6507,6)	0|**0**|,8 (0|1027,2)	$[IP]_{t0}$

Figure 74. Inferential Analysis Of The Neuroimmune Trait Profile

The P25, medians, P75, minimum and maximum, by subjects, for the $\log_{10}$InflammatoryProfile (IP) in % and [analytes] (pg/mL) is presented in 6 boxplots. The Trait profile for the anti- (2 boxplots), pro- (2 boxplots) and anti&pro-inflammatory (2 boxplots) responses, in the 4 subphenomes, is plotted.

In (a) the trait (T0) profile (**Figure 74**), for the variable:

(i) Relative percentage of the anti-inflammatory Trait, all the subgroups had the same median = 0. The total average of the 4 subgroups was 6.6%;

(ii) Relative percentage of the pro-inflammatory Trait, the median scores of the Self Awareness and Self Consciousness subgroups were the highest. However, the subgroup Reflective Self had the highest P75 (9.3%) and maximum (99%) and the subgroup Pre-Reflective Self had the second maximum (15%). The Self Awareness and Self Consciousness subgroups had the lowest maximums (11.2%; 12.5%). The total average of the 4 subgroups was 8.6%;

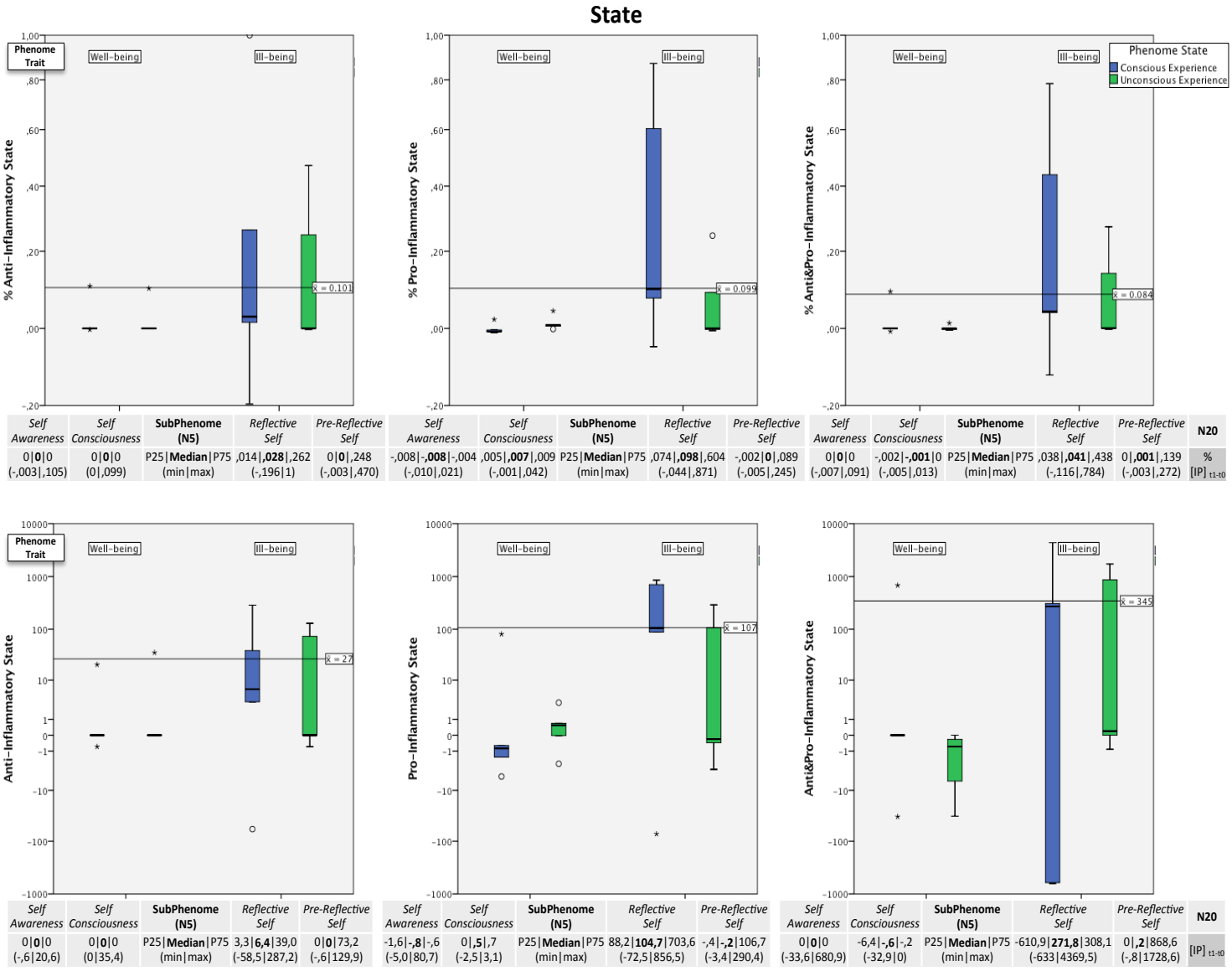
(iii) Relative percentage of the anti&pro-inflammatory Trait, the median score of the subgroup Self Consciousness was the highest, although the highest P75 (10%) and maximum (100%) % anti&pro-inflammatory Trait belong to the subgroup Reflective

Self. The Self Awareness subgroup had the lowest score and maximum. The total average of the 4 subgroups was 6.6%;

(iv) Absolute anti-inflammatory Trait, all the subgroups had the same median = 0. The total average of the 4 subgroups was 28 pg/mL;

(v) Absolute pro-inflammatory Trait, the score of the subgroup Reflective Self was the highest, with a P75 of 83.6 pg/mL and a maximum of 801 pg/mL. The second maximum (157.9 pg/mL) belonged to the Pre-Reflective Self group. The total average of the 4 subgroups was 60 pg/mL;

(vi) Absolute anti&pro-inflammatory Trait, the median score of the subgroup Self Consciousness was the highest, although the highest P75 (801.6 pg/mL) and maximum (6507.6 pg/mL) anti&pro-inflammatory Trait belong to the subgroup Reflective Self. The Self Awareness subgroup had the lowest score and maximum. The total average of the 4 subgroups was 459 pg/mL.



Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	N20
0|**0**|0 (-,003|,105)	0|**0**|0 (0|,099)	P25|**Median**|P75 (min|max)	,014|**,028**|,262 (-,196|1)	0|**0**|,248 (-,003|,470)	-,008|**-,008**|-,004 (-,010|,021)	,005|**,007**|,009 (-,001|,042)	P25|**Median**|P75 (min|max)	,074|**,098**|,604 (-,044|,871)	-,002|**0**|,089 (-,005|,245)	0|**0**|0 (-,007|,091)	-,002|**-,001**|0 (-,005|,013)	P25|**Median**|P75 (min|max)	,038|**,041**|,438 (-,116|,784)	0|**,001**|,139 (-,003|,272)	% [IP] $_{t1-t0}$

Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	Self Awareness	Self Consciousness	SubPhenome (N5)	Reflective Self	Pre-Reflective Self	N20
0|**0**|0 (-,6|20,6)	0|**0**|0 (0|35,4)	P25|**Median**|P75 (min|max)	3,3|**6,4**|39,0 (-58,5|287,2)	0|**0**|73,2 (-,6|129,9)	-1,6|**-,8**|-,6 (-5,0|80,7)	0|**,5**|,7 (-2,5|3,1)	P25|**Median**|P75 (min|max)	88,2|**104,7**|703,6 (-72,5|856,5)	-,4|**-,2**|106,7 (-3,4|290,4)	0|**0**|0 (-33,6|680,9)	-6,4|**-,6**|-,2 (-32,9|0)	P25|**Median**|P75 (min|max)	-610,9|**271,8**|308,1 (-633|4369,5)	0|**,2**|868,6 (-,8|1728,6)	[IP] $_{t1-t0}$

Figure 75. Inferential Analysis Of The Neuroimmune State Profile

The P25, medians, P75, minimum and maximum, by subjects, for the $\log_{10}$InflammatoryProfile (IP) in % and [analytes] (pg/mL) is presented in 6 boxplots. The State profile for the anti- (2 boxplots), pro- (2 boxplots) and anti&pro-inflammatory (2 boxplots) responses, in the 4 subphenomes, is plotted.

In (b) the state $\Delta_{T0\text{-}T1}$ profile (**Figure 75**), for the variable:

(i) Relative percentage of the anti-inflammatory State, the Self Awareness, Self-Consciousness and Pre-Reflective Self subgroups had the superior minimums (≃0 pg/mL), while the Reflective Self subgroup had the lowest minimum (-19.6%). The total average of the 4 subgroups was 10%;

(ii) Relative percentage of the pro-inflammatory State, the median score of the subgroup Reflective Self was the highest (9.8%), while the Self Awareness subgroup had the lowest median (-0.8%) and minimum (-1%). Moreover, the subgroup Reflective Self had the highest P75 (60%) and maximum (87%) and the subgroup Pre-Reflective Self had the second-highest P75 (9%) maximum (24.5%). On the other hand, the Self Awareness and Self Consciousness subgroups had the lowest P,75 (-4%; 9%) and maximum (2.1%; 4.2%). The total average of the 4 subgroups was 9.9%;

(iii) Relative percentage of the anti&pro-inflammatory State, the median score of the subgroup Reflective Self was the highest, with a P75 equal to 43.8%. The second was the median anti&pro-inflammatory score of the Pre-Reflective group, with a P75 equal to 13.9%. The Self Awareness and Self Consciousness subgroups had almost no variation, with the lowest median scores and maximums. The total average of the 4 subgroups was 8.4%;

(iv) Absolute percentage of the anti-inflammatory State, the Self Awareness, Self-Consciousness and Pre-Reflective Self subgroups had the superior minimums (≃0%), while the Reflective Self subgroup had the lowest minimum (-58.6 pg/mL). The Pre-Reflective Self subgroup had the highest P75 (73.2 pg/mL). The total average of the 4 subgroups was 27 pg/mL;

(v) Absolute pro-inflammatory State, the median score of the subgroup Reflective Self was the highest (104.7 pg/mL), while the Self Awareness subgroup had the lowest median (-0.8 pg/mL) and minimum (-5 pg/mL). Moreover, the subgroup Reflective Self had the highest P75 (703.6 pg/mL) and maximum (856.6 pg/mL) and the subgroup Pre-Reflective Self had the second-highest P75 (106.7 pg/mL) maximum (290.4 pg/mL). On the other hand, the Self Awareness and Self-Consciousness subgroups had the lowest P75 (-0.6; 0.7) pg/mL and maximum (80.7; 3.1) pg/mL. The total average of the 4 subgroups was 107 pg/mL;

(vi) Absolute anti&pro-inflammatory State, the median score of the subgroup Reflective Self was the highest (271.8 pg/mL), with a maximum of 4369 pg/mL. The second was the median anti&pro-inflammatory score of the Pre-Reflective group, with the highest P75 equal to 868.6 pg/mL and a maximum of 1728 pg/mL. The Self Awareness and Self-Consciousness subgroups had almost no variation, with the lowest median scores and maximums. The total average of the 4 subgroups was 345 pg/mL.

Henceforth, **Figure 74** and **Figure 75** offered an immune strata characterization. This stratification showed the presence of a statistical tendency. This tendency reflects our trait and state scores explored in **Figure 68**. The tendency of the anti&pro-inflammatory variables, with high relative and absolute scores in intragroup and intergroup inferences, may be justified by the heterogenous and ubiquitous role of IL-21, IL-22 and IL-33 in our neurotypical sample.

However, to better understand this immune statistical tendency in the 4 subgroups, we performed the following inference analysis. This inferential analysis was used for hypothesis testing of the multiple comparisons we wanted to make between [all the analytes; all the samples] of our Human Th17 Cytokine Assay.

The software Bio-Plex Data Pro™ has reserved capabilities to comply to this analysis. Consequently, we did the statistical inference (**Table 10**) with SPSS Statistics 22.

Table 10. Inferential Analysis Of The Neuroimmune Response
Table summarizing the pre-testing, parametric, non-parametric and hypothesis testing for the inflammatory profiles in relative % and absolute values, by subjects and by groups.

Profile (*Self Awareness; Self Consciousness; Reflective Self and Pre-Reflective Self*)	Type	Dependent Variable	Pre-testing: Test of Homogeneity of Variances (Levene Test sig.)	Pre-testing: Robust Tests of Equality of Means (Brown-Forsythe Test sig.)	Paramentric: ANOVA (Between All Groups sig.)	Paramentric: Multiple Comparisons (95% Confidence Interval) with Bonferroni correction (*I* - Phenome State and Trait)	(*J* - Phenome State and Trait)	(I-J) sig.	Non Parametric: Independent Samples* (Kruskal-Walis Test Significance) Between All Groups and Multiple Comparisons sig.	Reject H0
TRAIT for Subphenomes (Groups)	% By Subject	Anti-Inflammatory	,006	,411	,338	*Self Awareness*	*Reflective Self*	,746		Y
		Pro-Inflammatory	,011	,550						N
		Anti&Pro-Inflammatory	,006	,444	,379					Y
	% By Group	Anti-Inflammatory							0	Y
		Pro-Inflammatory							0	Y
		Anti&Pro-Inflammatory							0	Y
	By Subject	Anti-Inflammatory	,006	,406	,332	*Self Awareness*	*Reflective Self*	,735		Y
		Pro-Inflammatory	,011	,484						N
		Anti&Pro-Inflammatory	,009	,447	,385					Y
	By Group	Anti-Inflammatory							0	Y
		Pro-Inflammatory							0	Y
		Anti&Pro-Inflammatory							0	Y
STATE for Subphenomes (Groups)	% By Subject	Anti-Inflammatory	,020	,567						N
		Pro-Inflammatory	,000	,166	,082	*Reflective Self*	*Self Awareness*	,148	0,129	Y
							Pre-Reflective Self	,402		
							Self Consciousness	,184		
		Anti&Pro-Inflammatory	,000	,317	,247	*Reflective Self*	*Self Awareness*	,560	0,283	Y
							Self Consciousness	,444		
	% By Group	Anti-Inflammatory							0	Y
		Pro-Inflammatory							0	Y
		Anti&Pro-Inflammatory							0	Y
	By Subject	Anti-Inflammatory	,026	,648						N
		Pro-Inflammatory	,000	,171	,090	*Reflective Self*	*Self Awareness*	,197	0,263	Y
							Pre-Reflective Self	,479		
							Self Consciousness	,161		
		Anti&Pro-Inflammatory	,019	,712					0,364	Y
	By Group	Anti-Inflammatory							0	Y
		Pro-Inflammatory							0	Y
		Anti&Pro-Inflammatory							0	Y

Not showing inferences/multiple comparisons with a significance level > 0,746. and the mean difference is significant at the level of 0,385. * asymtoptic significances for sig.=0.05

6.B.5. Analysis Of Inferences And Multivariate Factor Analysis

The Inferential analysis examined the same 24 variables of the descriptive analysis. The results of the inferential analysis were obtained by parametric and/or non-parametric hypothesis testing and are summarized in **Table 10**.

The mean difference was considered significant when $p < \alpha=0.385$ and only inferences, or multiple comparisons, with a significance < 0.746 are presented.

The normality, homogeneity of variances, asymmetries in the distribution, sample dimension and scale of the variables determined the type of testing.

Subsequently, the *rationale* of the inferential analysis is explained, and the results described for each of the 24 variables extracted from the Human Th17 Cytokine Assay.

Primarily (Pre-testing) comprehended (i) the rejection ($\alpha=0.05$) of homoscedasticity (Levene Test) and (ii) the confirmation Brown-Forsythe equality of means.

Consecutively, a) One-way ANOVA (Parametric) or b) Independent-Samples Kruskal-Wallis (Non-Parametric) tests were performed, to reject ($\alpha=0.05$ mean difference) intergroup variable score equality (in the (i) Self Awareness group; in the (ii) Self Consciousness group; in the (iii) Reflective Self group; and in the (iv) Pre-Reflective Self group). Afterwards, if rejected, a) ANOVA or b) non-parametric multiple comparisons were also applied, accordingly, to understand which subgroups were responsible for the score mean difference.

In (a) the trait (T0) profile, for the subject variables:

(i) Relative percentage of the anti-inflammatory Trait – it was rejected, with a medium-low significance, that the score was equal between groups (One-Way ANOVA α=0.338) and in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Specifically, the Self-Awareness & Reflective Self subgroups were considered distinct, but with a very low significance ($p<0.746$ (ANOVA));

(ii) Relative percentage of the pro-inflammatory Trait – the equality ($p>0.05$) of means was not rejected between groups or in multiple comparisons;

(iii) Relative percentage of the anti&pro-inflammatory Trait – it was rejected, with a medium-low significance, that the score was equal between groups (One-Way ANOVA α=0.379);

(iv) Absolute anti-inflammatory Trait – it was rejected, with a medium-low significance, that the score was equal between groups (One-Way ANOVA α=0.332) and in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Specifically, the Self-Awareness & Reflective Self subgroups were considered distinct, with a very low significance ($p<0.735$ (ANOVA));

(v) Absolute pro-inflammatory Trait – the equality ($p>0.05$) of means was not rejected between groups or in multiple comparisons;

(vi) Absolute anti&pro-inflammatory Trait – it was rejected, with a medium-low significance, that the score was equal between groups (One-Way ANOVA α=0.385).

In (a) the trait (T0) profile, for the group variables:

(i) Relative percentage of the anti-inflammatory Trait, (ii) Relative percentage of the pro-inflammatory Trait, (iii) Relative percentage of the anti&pro-inflammatory Trait, (iv) Absolute anti-inflammatory Trait, (v) Absolute pro-inflammatory Trait, and (vi) Absolute anti&pro-inflammatory Trait – it was entirely rejected, with a very high significance, that the score was equal between groups (Independent-Samples Kruskal-Wallis α=0) and in multiple comparisons (Kruskal-Wallis Test with asymptotic significances with $p<0.05$). Specifically, the Self-Awareness & Self Consciousness & Reflective Self & Pre-Reflective Self subgroups, in all variables, were considered distinct and the distribution was the same across all multiple comparisons.

In (b) the state $\Delta_{T0\text{-}T1}$ profile, for the subject variables:

(i) Relative percentage of the anti-inflammatory State – the equality ($p>0.05$) of means was not rejected between groups or in multiple comparisons;

(ii) Relative percentage of the pro-inflammatory State – it was rejected, with a high significance, that the score was equal between groups (One-Way ANOVA α=0.082) and in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Specifically, the Reflective Self & Self Awareness subgroups ($p<0.148$ (ANOVA)), the Reflective Self & Pre-Reflective Self ($p<0.402$ (ANOVA)), and the Reflective Self & Self Consciousness subgroups ($p<0.184$ (ANOVA) were considered distinct with a medium-high significance. Alternatively, with non-parametric testing, the Self-Awareness & Self-Consciousness & Reflective Self & Pre-Reflective Self subgroups were considered distinct with a medium-high significance, i.e., $p<0.129$ in independent-samples Kruskal-Wallis test. The distribution was the same across all multiple comparisons

(iii) Relative percentage of the anti&pro-inflammatory State – it was rejected, with a medium significance, that the score was equal between groups (One-Way ANOVA α=0.247) and in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Specifically, the Reflective Self & Self-Awareness subgroups ($p<0.560$ (ANOVA), and the Reflective Self & Self-Consciousness subgroups ($p<0.444$ (ANOVA) were considered distinct with a medium-low significance. Alternatively, with non-

parametric testing, the Self-Awareness & Self-Consciousness & Reflective Self & Pre-Reflective Self subgroups were considered distinct with a medium significance, i.e., $p<0.283$ in independent-samples Kruskal-Wallis test. The distribution was the same across all multiple comparisons;

(iv) Absolute anti-inflammatory State – the equality ($p>0.05$) of means was not rejected between groups or in multiple comparisons;

(v) Absolute pro-inflammatory State – it was rejected, with a high significance, that the score was equal between groups (One-Way ANOVA $\alpha=0.090$) and in multiple comparisons (95% Confidence Interval) with Bonferroni correction. Specifically, the Reflective Self & Self Awareness subgroups ($p<0.197$ (ANOVA), the Reflective Self & Pre-Reflective Self subgroups ($p<0.479$ (ANOVA), and the Reflective Self & Self Consciousness subgroups ($p<0.161$ (ANOVA) were considered distinct with a medium-high significance. Alternatively, with non-parametric testing, the Self-Awareness & Self Consciousness & Reflective Self & Pre-Reflective Self subgroups were considered distinct with a medium significance, i.e., $p<0.263$ in independent-samples Kruskal-Wallis test. The distribution was the same across all multiple comparisons;

(vi) Absolute anti&pro-inflammatory State – it was rejected, with a medium-low significance, that the score was equal between groups (Independent-Samples Kruskal-Wallis $\alpha=0.364$) and in multiple comparisons (Kruskal-Wallis Test with asymptotic significances with $p<0.05$). Specifically, the Self-Awareness & Self Consciousness & Reflective Self & Pre-Reflective Self subgroups were considered distinct and the distribution was the same across all multiple comparisons.

In (b) the state $\Delta_{T0\text{-}T1}$ profile, for the group variable:

(i) Relative percentage of the anti-inflammatory State, (ii) Relative percentage of the pro-inflammatory State, (iii) Relative percentage of the anti&pro-inflammatory State, (iv) Absolute anti-inflammatory State, (v) Absolute pro-inflammatory State, and (vi) Absolute anti&pro-inflammatory State – it was entirely rejected, with a very high significance, that the score was equal between groups (Independent-Samples Kruskal-Wallis $\alpha=0$) and in multiple comparisons (Kruskal-Wallis Test with asymptotic significances with $p<0.05$). Specifically, the Self-Awareness & Self Consciousness & Reflective Self & Pre-Reflective Self subgroups, in all variables, were considered distinct and the distribution was the same across all multiple comparisons.

Finally, a multivariate factor analysis was executed to explore if the descriptive and inferential analysis had the capacity (i) to represent and (ii) to discriminate the differences in the 15 analytes of the Human Th17 Cytokine Assay.

For this analysis, N (number of samples) was equal to 40, as we used duplicate sampling for each of the 20 subjects. Initially, both (i) a factor and (ii) a reliability analysis were conducted in the 20 subjects for the 15 analytes in the Human Th17 Cytokine Assay.

The (i) factor analysis showed in the initial extraction communalities an adequate minimum of 0,962, except for the IL-1β (0.375) and IL-23 (0.773). The communalities in our variables are high, which indicates that the inferential analysis represented the 15 analytes well, apart for IL-1β, that had a low communality (0.375).

Hence, the total variance in our Human Th17 Cytokine Assay was explained by one Eigenvalue component (93%), with a single and final PC1 (0.981) contribution in the component matrix analysis. This analysis demonstrated that the 15 cytokines assay was adequate to be applied in our sample of neurotypical subjects, apart from IL-1β.

Afterwards, a (ii) reliability test of all the 15 analytes was performed to assess their internal consistency in our sample. The reliability analysis showed an initial medium-low Cronbach's $\alpha=0.569$; but if IL-21 was discarded, it would increase to 0.907 and if IFN-γ to 0.933.

Therefore, IL-21 and IFN-γ are analytes that, in our neurotypical sample, do not have a good discriminative power. The analytical difference between all the analytes, in our sample, given by ANOVA Cochran Q test, has a high significance with a $p<0,012$, if IL-21 is discarded.

6.B.6. Neuro-Immune Network: Functional Analysis Of The Immune Response

To conclude the analysis of the immune response in our neurotypical sample, we made a molecular function GO-term analysis (**Figure 76** and **Figure 77**).

Figure 76 shows the PANTHER GO functional classification viewed in a pie chart. It extracts the Slim biological processes of the Human Th17 Cytokine Assay.

Figure 77 shows the Cytoscape v. 3.6.0 functional clustering of the immune interaction networks.

This functional analysis is better plotted in: https://tinyurl.com/Neuro-Immune-Network (retrieved July 25, 2020), an infographic with graphical zooming tools.

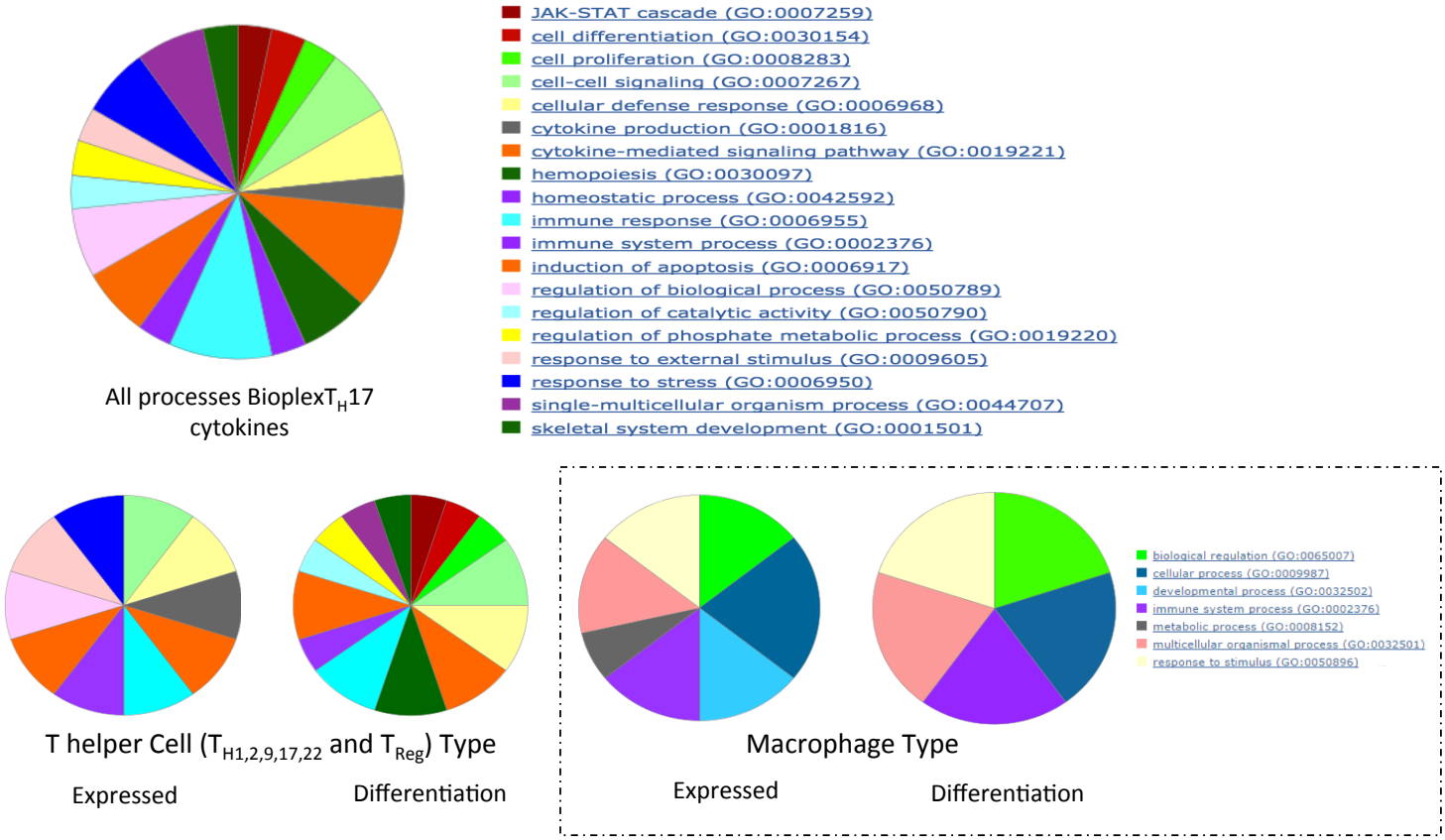


Figure 76. PANTHER GO Functional Classification Viewed In Pie Chart
A Slim biological processes analysis of the Human Th17 Cytokine Assay is presented. The 15 cytokines, detected by our Bio-Plex ProTM assay, are responsible for 19 biological processes. For further analysis, consult the Human Th17 Cytokine DB: https://www.limmit.org/uploads/2/6/8/4/26841837/humanth17cytokinedb.xlsx (retrieved August 09, 2020).

In **Figure 76**, we can observe that the 15 cytokines, detected by our *Bio-Plex Pro*^{TM} assay, are responsible for 19 biological processes.

The Th17 cytokines, in the *Homo sapiens* (Human - taxon 9606) and characterized by Gene Ontology Term (GO), execute the following biological processes: JAK-STAT

cascade (GO:0007259); cell differentiation (GO:0030154); cell proliferation (GO:0008283); cell-cell signaling (GO:0007267); cellular defense response (GO:0006968); cytokine production (GO:0001816); cytokine-mediated signaling pathway (GO:0019221); hemopoiesis (GO:0030097); homeostatic process (GO:0042592); immune response (GO:0006955); immune system process (GO:0002376); apoptotic process (GO:0006915); regulation of biological processes (GO:0050789); regulation of catalytic activity (GO:0050790); regulation of phosphate metabolic process (GO:0019220); response to external stimulus (GO:0009605); response to stress (GO:0006950); single-multicellular organism process (GO:0044707); and skeletal system development (GO:0001501).

The biological processes that are more expressed by Th17 cytokines are not only the cytokine-mediated signaling and the immune response, but also neurologic biological processes like cell-cell signaling and response to stress. The biological processes that are less expressed by Th17 cytokines are the regulation of the catalytic activity and the regulation of phosphate metabolic processes.

To better understand those biological processes, detected by our Human Th17 Cytokine Assay in our neurotypical subjects, we created an embedded type database. This database offers an extensive analysis of the cytokines interaction network. We named this database, the Human Th17 Cytokine DB.

The database is accessible online, in a non-restricted access server, in the LIMMIT Lab, Faculty of Medicine, University of Lisbon, and accessible via the link: https://www.limmit.org/uploads/2/6/8/4/26841837/humanth17cytokinedb.xlsx(retrieved August 09, 2020; *in* Annex K: External Support).

The Human Th17 Cytokine DB reviews the 15 cytokines expression and interaction with specific T helper cell type and macrophage cell type.

Firstly, a brief *in silico* protein characterization was given (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017). The following variables were considered: Uniprot code, entry name, protein name, gene name and mass (in Uniprot database).

Secondly, an assay of protein histophysiology variables was inputted, based on the literature revision in the Introduction. The literature revision detailed the histophysiology of the cytokines and the molecular networks they integrate. This assay included the cell sources and cell sources type, the anti/pro-inflammatory profile, the molecular weight (given in later chapters) and the cell targets.

Thirdly, 2 sets of variables were generated: (a) Expressed and (b) Cytokine for differentiation. Those 2 sets of variables were used both for the T helper cell type and myeloid monocytes. Those were the main immune cell classes (a) expressing and (b) being differentiated by the Th17 cytokines.

The T helper cell types considered were Th1, Th2, Th9, Th17, Th22 and Treg cells. The myeloid monocytes considered were the macrophages M1 (interacting with Th1), M2a (interacting with Th2), M2b (interacting with B cells), M2c (interacting with Treg) and M4. As an example, P05112, IL4_HUMAN, or Interleukin-4, has a mass of 17,492 kDa (Uniprot database). One of its cell sources is Th2 cell, a lymphoid double positive T helper cell.

In the revision of literature, we assessed IL-4 with an anti-inflammatory action, detected in proteomics studies, with 15 kDa, and targeting T and B cells.

IL-4 is expressed by Th2 cells and used for the differentiation of Th2 and Th9 T cell types. Although it is an interleukin not expressed by macrophages, it is used to differentiate M2a macrophages in the interaction with Th2 types of cells.

The analysis of Human Th17 Cytokine DB helped in the understanding of (i) the specific Th17 cytokines that are expressed ubiquitously in the immune system, and (ii)

which cytokines are used for the differentiation of the specific immune cell lineages. With this analysis, accessible in the Human Th17 Cytokine DB and represented in **Figure 76**, 2 main cell source types were distinguished: T helper cell type and myeloid monocytes.

T helper cell types: Th1, Th2, Th9, Th17 and Th22, and Treg cells express Th17 cytokines. However, the most expressed cytokines are IL-4, IL-10, IL-17A, IL-17F, IL-21, IL-22, IFN-γ and TNF-α.

When expressed by T helper type of cells, those 8 cytokines execute all the previously mentioned biological functions (and Gene Ontology Terms), but they do not have a role in the JAK-STAT cascade; in cell differentiation; in cell proliferation, in hemopoiesis, in the homeostatic process, in the regulation of the catalytic activity, in the regulation of phosphate metabolic processes, in single-multicellular organism process, and in the development of the skeletal system (**Figure 76**, bottom left).

However, for the differentiation of T helper cell type lineages, IL-4, IL-6, IL-10, IL-21, IL-23, sCD40L and TNF-α are the cytokines used. When so, those 7 cytokines induce, in the T helper cell type, nearly all the previously mentioned biological functions, but, more significantly, cell-cell signaling, the cytokine-mediated signaling pathway and apoptotic processes (**Figure 76**, bottom left).

Macrophage cell type expresses Th17 cytokines, but not all. Only IL-1b, IL-6, IL-10, IL-23 and IFN-γ are widely expressed in myeloid monocytes. When expressed by macrophages (*in* Human Th17 Cytokine DB), those 5 cytokines have specific functions in biological regulation; in the response to stimulus; in cellular, developmental and metabolic processes; in immune system processes; and in multicellular organismal processes (**Figure 76**, bottom right).

However, only IL-1b, IL-6 and IFN-γ are used for the differentiation of macrophage cell lineages (*in* Human Th17 Cytokine DB). When so, those 3 cytokines induce cellular processes and a biological regulation of macrophages, in response to stimulus (**Figure 76** , bottom right).

Specifically, in our neurotypical sample, we wanted to test the previous functional classification and the biological processing for our 4 distinct mental health subgroups.

Therefore, we advanced with a Cytoscape GO-term analysis of the immune interaction networks (**Figure 77**). This functional clustering follows the bioinformatics methodology being published by the authors (chapter 4), but for the 15 Human Th17 Cytokine Assay.

This functional clustering permitted to better understand (i) how Th17 cytokines interact with each other in our neurotypical subgroups, and (ii) how T helper cell types and myeloid monocytes mediate that interaction.

In **Figure 77**, we can observe two GO-term analysis which are (i) GO Biological Process: Immune System (Diehl et al., 2007), and (ii) KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (Kanehisa and Goto, 2000).

GO Biological Process (on the top of **Figure 77**) is the best known and most used database of biological processes. The Immune System is a specific subset of the GO collection of immunity compounds.

KEGG pathways (on the bottom of **Figure 77**) is a database resource for understanding high-level functions and utilities of a biological system, in our case the Th17 expression in neurotypical subjects, from molecular-level information.

All the 15 analytes are represented. Based on PCA extraction (**Figure 73**), the analysis was performed independently for each of the four subgroups. PCA extraction selected the most expressed and significative analytes of each subgroup.

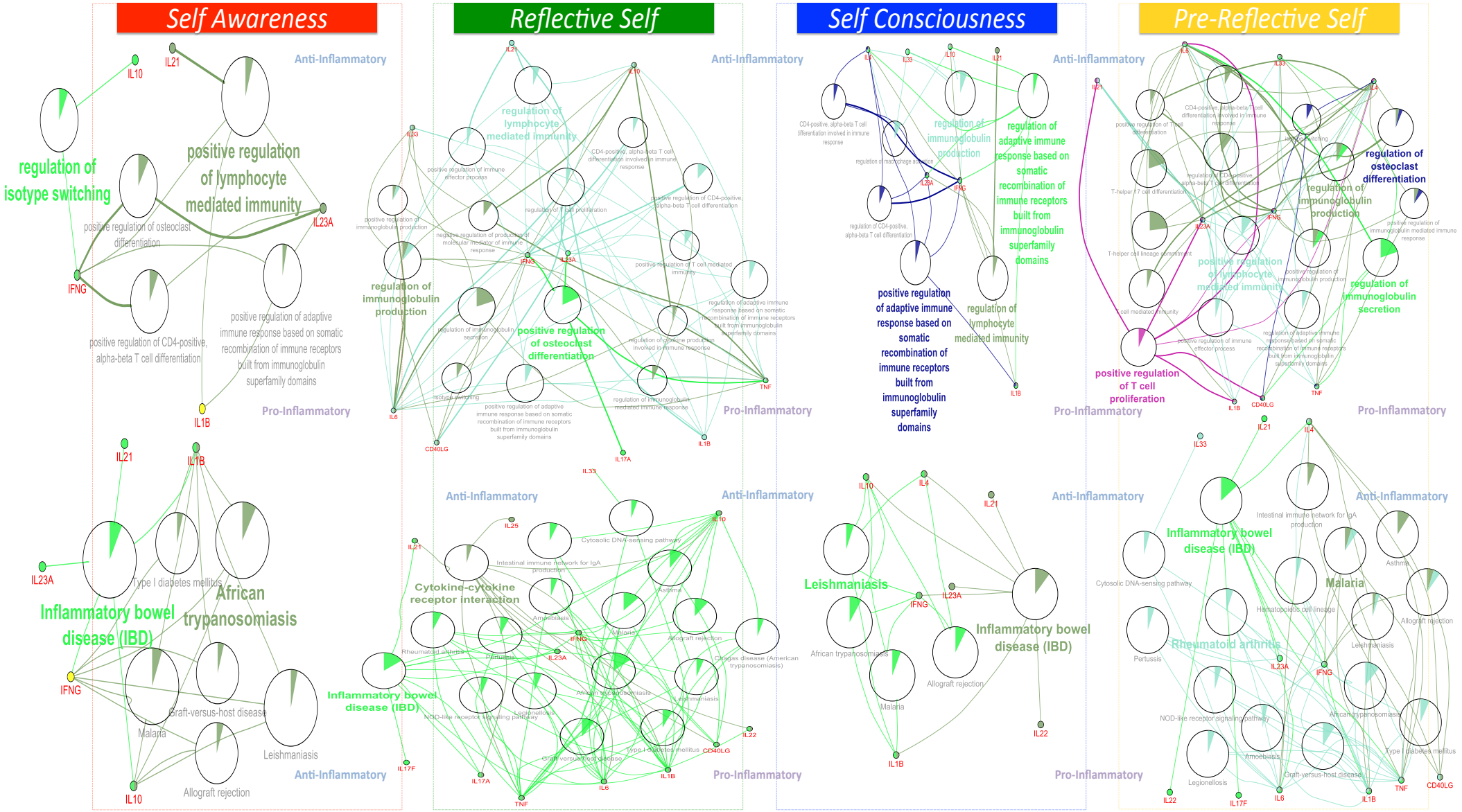


Figure 77. Functional Analysis Of The Immune Response In Neurotypical Subjects

The Immune Interaction Networks are extracted by Cytoscape v. 3.6.0, permitting the clustering of the Th17 cytokines interaction in our neurotypical subgroups.

The Self-Awareness subgroup is characterized by the immune interaction networks of (i) IL-10, IL-21, IL-25; and (ii) IL-33 and IL-1β.

The Reflective Self subgroup is characterized by the immune interaction networks of (i) IL-10, IL-21, IL-25 and; (ii) IL-33 and IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L and TNF-α.

The Self-Consciousness subgroup is characterized by the immune interaction networks of (i) IL-4, IL-10, IL-21, IL-25; and (ii) IL-33; and IL-1β, IL-22, IL-31.

The Pre-Reflective Self subgroup is characterized by the immune interaction networks of (i) IL-4, IL-21 and IL-33; and (ii) IL-1β, IL-6, IL-17F, IL-22, IL-31, sCD40L and TNF-α.

The cystoscope GO-term analysis clustered the analytes in their specific molecular functions, according to GO Biological Process: Immune System and KEGG pathways. Those molecular functions integrate a functional network of the corresponding analytes.

In those interaction networks, we can also observe that the analytes are clustered according to their inflammatory profile: (i) anti-inflammatory or (ii) pro-inflammatory.

(i) IL-4, IL-10, IL-21, IL-25 and IL-33;

(ii) IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L and TNF-α;

IL-23 and INF-γ are analytes that we could cluster neither in (i) anti-inflammatory, nor in (ii) pro-inflammatory profiles, based on the PCA extraction of our Human Th17 Cytokine Assay dataset (**Figure 73**). Therefore, they appear in between (i) an anti-inflammatory and (ii) a pro-inflammatory response.

Specific pathways with $pV \leq 0.05$ were extracted, by Cytoscape v. 3.6.0, for the 4 subgroups.

In the Self-Awareness subgroup (in red), the most significative GO Biological Processes of the Immune System were: the regulation of isotype switching (GO:0045191) and the positive regulation of lymphocyte-mediated immunity (GO:0002708). Those are GO associated with (i) the anti-inflammatory profile.

The most significative KEGG pathway was the regulation of inflammatory bowel disease (H01227) by IL-21, (i) an anti-inflammatory cytokine.

In the Reflective Self subgroup (in green), the most significative GO Biological Processes of the Immune System were: the regulation of immunoglobulin production (GO:0002637) and secretion (GO:0051023) and the positive regulation of osteoclast differentiation (GO:0045672). Those are GO associated with (ii) the pro-inflammatory profile.

The most significative KEGG pathway was the cytokine-cytokine receptor interaction (hsa04060), by IL-1β, IL-6, IL-17A and TNF-α, all (ii) pro-inflammatory cytokines.

In the Self Consciousness subgroup (in blue), the most significative GO Biological Processes of the Immune System were: the adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (GO:0002460) and the regulation of immunoglobulin production (GO:0002637). Those are GO associated with (i) the anti-inflammatory profile. Likewise, less significative but present, is the co-regulation of lymphocyte-mediated immunity (GO:0002706) by IL-1β, (ii) a pro-inflammatory interleukin, and IL-10 and IL-21, (i) anti-inflammatory interleukins.

The most significative KEGG pathway was the regulation of inflammatory bowel disease (H01227), like in the Self Awareness group. However, in this case co-regulated by IL-4, IL-10 and IL-21, (i) anti-inflammatory interleukins, and IL-1β and IL-22, (ii) pro-inflammatory interleukins.

In the Pre-Reflective Self subgroup (in yellow), the most significative GO Biological Processes of the Immune System were: the positive regulation of T cell proliferation (GO:0042102) and the regulation of immunoglobulin production (GO:0002637). Like in the Reflective Self group, those are GO associated with (ii) the pro-inflammatory profile, but, in this case, co-regulated by IL-4 and IL-21, (i) anti-inflammatory interleukins. Moreover, less significative but present is the regulation of osteoclast differentiation (GO:0045670) by pro-inflammatory interleukins but mediated by IL-4, (i) an anti-inflammatory interleukin.

The most significative KEGG pathway was the regulation of rheumatoid arthritis (H00630) by IL-1β and IL-6, (ii) pro-inflammatory cytokines.

From this functional analysis of the immune response in neurotypical subjects, we observe that there is an inflammatory equilibrium. This equilibrium is different in the 4 subgroups. We postulate that this equilibrium may be a physiological mirror of mental health and is synchronized by a stable system configuration.

On the one hand, anti-inflammatory cytokines, e.g., IL-10, portray effective anti-inflammatory properties by inhibiting the expression of inflammatory cytokines, such as TNF-α and IL-6 by activated macrophages. On the other hand, interleukins like IL-1β, IL-6, IL-17A, IL-17F, IL-23, IL-31 or chemokines like INF-γ, sCD40 or TNF-α, are pro-inflammatory, partaking association with autoimmune and inflammatory diseases.

Additionally, IL-21, IL-22 and IL-33 both have roles in this equilibrium and are linked to the regulation of chronic diseases, e.g., cancer, as they are involved in the regulation of proliferation, differentiation and apoptosis, wound healing and tissue reorganization (Akdis, 2011).

To summarize the two-factor analysis and the two reliability tests executed in our neurotypical sample, analytes IL-1β, IL-21, IL-23 and IFN-γ should be revised, when applied in an equivalent population. IL-1β is not representative, IL-21 is not discriminative, and Il-23 and IFN-γ are outliers to our mental health subgroups and inflammatory equilibrium.

In fact, this equilibrium may be an attribute of cognitive faculties (Varela, 1994). Hereafter, to understand this cognitive-immune coupling, it is necessary to analyze more than the individual components of the system, but rather the stereochemically specific interactions with each other, e.g., cytokine communication.

Those individual components are elementary to the whole dynamic functioning of the system, because at this low level of interaction there is absolutely nothing cognitive *per se*. And above all, if molecular interactions between those individual components, as such, are not cognitive in the sense used here, neither is the activation of a cell by the interaction between its membrane-bound receptor molecules and ligands.

Therefore, and ultimately, it is only genuine to define such interactions as instances of recognition if they are studied in the context of a global system, which is indeed, cognitive (Varela, 1994).

Concluding, the equilibrium of anti/pro-inflammatory cytokines levels is critical for the maintenance of inflammatory homeostasis (Yao, 2013; Vassileva and Piquette-Miller, 2014). However, still central and essential is the role that they might have in cognitive homeostasis and well-being (chapter 3).

The excerpt of the research outline (**Figure η**) systemizes the Section II: Molecular Stratification of the experimental study and helps the reader understanding the sequence and integration of the chapter 6 in the overall experiment.

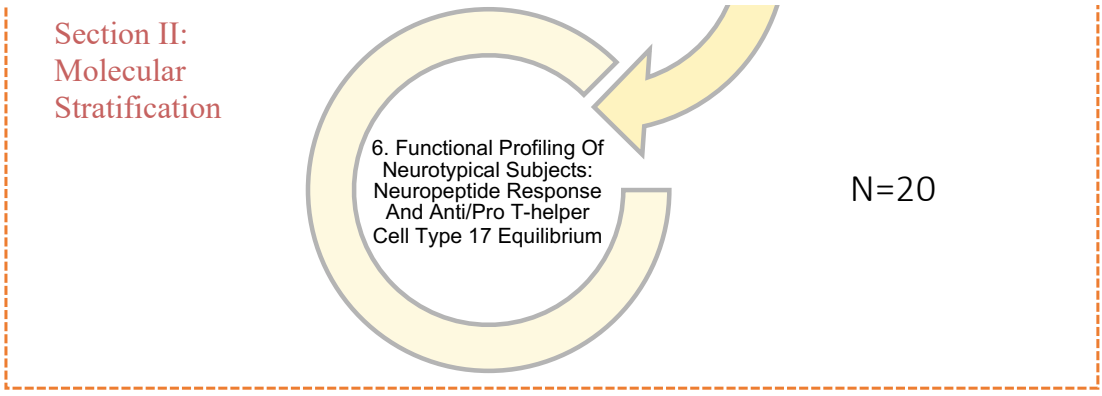


Figure η. Excerpt Of The Master Scheme Revising The Work Developed In The Phd Thesis: Molecular Profiling In Neurotypical Young Adults, Section II: Molecular Stratification
The chapter 6 (Functional Profiling Of Neurotypical Subjects: Neuropeptide Response And Anti/Pro T-helper Cell Type 17 Equilibrium) is displayed, which comprehended the methodology that led to quantification of the neuropeptide and neuroimmune response in the 20 stratified subjects.

Conclusion

Section I: Mental Health Stratification

1. Neurotypical Expression: Cognition And Proteins

The aim of this chapter was to put forward an empirical method that is also consistent with a non-brain-centred account of cognition. We bring forth a conceptual analysis model that, in our opinion, contributes for new non-invasive online research of mental health. Enactive theory offers a plausible theoretical framework for the molecular model that studies subjective experience by standardized salivary protein profiles (Saavedra Silva, Sousa, Silva, Martins, Esteves, Fernandes, Rosa, Correia, Barros, 2017). Only recently did this become possible through the technological development of proteomic analysis. Contemporary research has examined, to some degree, the subjective qualities of neurotypical subjects; however, these issues are yet far from understood. The premise of this thesis is that embodied cognitive features of the human experience are shaped by combined aspects of the brain and the body (Varela, Thompson and Rosch, 1991; Re, Palma, Martins and Simões, 2016).

It is expected that this work will lead to new and interesting questions, which might justify a prospective study where top-down and bottom-up phenomic validation of possible biomarkers is addressed. We hope we were able to show that the concepts of phenome and proteome may be put to practice in order to explore the proposed model.

On the one hand, a phenome allows a categorical stratification and characterization of the embodied consciousness, and, on the other hand, the proteome permits a possible molecular approach to measure human experience and behavior.

Indeed, an observable tendency to neglect reductionist theories on the mind-brain interactions could be raised, but it was not the objective of this chapter. Ultimately, it enabled only to show that this empirical method is a plausible alternative and complement to the gold standard computational and neurocentred research of cognition.

The postulation that was put forward in this chapter was then applied as the experimental design in the empirical investigation of the subsequent chapters to this thesis. Concerning the relation between subjective experience and molecular networks of neurotypical young adults, “omics” science, specifically proteomics, is thus as a feasible and valid perspective in the research of cognition.

2. Cognitive Behavioral Intervention And Mental Status Examination Of Neurotypical Young Adults

It is a standard of neurophysiology to quantify and measure the correlation between behavior and brain function. On the model purposed by this thesis, however, a focus was given rather to the qualitative experience of neurotypical subjects, viz. without any neuropsychiatric condition. What made this research somewhat innovative was the starting point in the Psychological Assessment, which directed subsequently the interpretation to quantitative physiology.

Substantial differences between the experimental and the control group were found. For instance, it was concluded that in the experimental group it was possible to identify, describe and report a subjective experience intrinsic to the Intervention Protocol, i.e., the single cognitive behavioral intervention. The Intervention Protocol (method) and the Psychological Assessment (materials), implementing the four psychological scales, allowed to establish two main outcomes:

(1) to distinguish experiential profiles based on the subjects' evaluations of their own conscious experience;

(2) to define a consequential clinical subtyping strategy.

In what concerns the outcome (1), it was identified, in the experimental group, two subgroups: the higher and the lower tercile. In the control group, it was also found two subgroups: the higher and the lower tercile. These four tercile subgroups were further analyzed in chapter 3, and compared with their protein variations in chapters 5 and 6. Thus, for outcome (1), the chapter 2 focused on the first-person experience associated with cognitive and mental events. In the experiment, cognitive processes appeared relevant and manifested as the subject provided an account. It was explored the first-person account because the familiarity one has with subjective life must give way in favour of a more careful examination.

This first-person methodology was thus crucial because it promoted an effective and sustained examination of phenomenal descriptions, equally compared to third-person accounts or quantitative measurements (Shear and Varela, 1999). For what is recently published, the mental status of neurotypical subjects had not yet been studied on a first-person account. Hence, this first-person account characterized and stratified a neurotypical sample into 4 distinct subgroups, by multiple comparisons of the psychological scales variables, with a good internal consistency and reliability.

In what concerns the outcome (2), "enaction", as conceived by Thompson and Stapleton (2009), grounds subjective experience in macro-micro network interactions. Therefore, and to include adequate molecular descriptions to complement this psychological analysis, the acquisition of proteomics data was foreknown to section II of this thesis. With the data obtained in chapter 2 proving to be a promising framework and research design, a physiological approach was thus prepared to fully assess changes in protein networks.

Henceforth, for the chapter 4, brain protein information by an *in silico* analysis was forecasted (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017). For the chapter 5, the total protein profile characterized by capillary electrophoresis was foreseen. For the chapter 6, it was projected the assessment of neurobiological processes in subjects without any neuropsychiatric condition. After accomplishing these tasks, and to systematize the neurophysiological response, a graphical user interface toolbox was planned.

As primary conclusion, in chapter 2, a methodology that can provide an open link to an empirically based and objective research was set. The overall results of the qualitative, first-person, study should move towards an integrated perspective on mind (Shear and Varela, 1999).

3. Mental Health Profiling In Neurotypical Young Adults

A clinical, or pre-molecular screening, necessary for an efficient molecular biomarker research of neurotypical subjects was proposed. With this study, we primarily aimed to find distinct mental health profiles of the individual without a neuropsychiatric condition.

These mental health subgroups or profiles were the outcome of a clinical stratification that comprehended (i) the generation of a neurotypical knowledge base of the 128 healthy volunteers, (ii) a statistical strategy to stratify them into subgroups with adequate and reliable instruments, and (iii) a descriptive and inferential analysis of those subgroups.

For the correct stratification of mental health phenomes (Bilder et al., 2009) or profiles, we used a neurophenomenological method (Olivares et al., 2015). This method was an eidetic reduction, which made it possible to recognize the essential components of a given memorized phenomenon, and to focus on how subjects pre-reflectively perceive and reflectively described such experiences.

The eidetic reduction was executed by self-report measures of episodic and semantic memory recalls. Validated and structured assessing scales were used to access these phenomena. We suggest that these mental health phenomena, or subphenomes, may be important for a better understanding of mental health (Pacheco, Martins and Simões, 2019).

Henceforth, 4 subgroups or profiles of cognitive functioning, in neurotypical young adults, were stratified, with an average reliability. The identification of those subphenomes, i.e., the sum of their phenotypic states and traits, could then be used to a sequential molecular screening and the establishment of molecular research questions of those discrete subphenomes.

Despite all the limitations in generalizing such a descriptive study, we would like to emphasize, as the main contribution of this chapter, the importance of the identification, the stratification and the characterization of mental states and traits as data features (Hipólito and Martins, 2018).

It is known that in neurobiological functional response studies (Buckner et al., 2005), i.e., in explanatory research, profiles of cognitive functioning, like those found in this study, may show a homogeneous molecular response. This functional response has been particularly documented in the neurotypical individual (Fredrickson et al., 2013). Therefore, with this chapter, we attempted to put forward the possible inference between cognitive and biological substrates, which was then tested in Section II.

Section II: Molecular Stratification

4. Neuronal-Salivary Proteome: *In Silico* Characterization And Bioinformatics Analysis

In this chapter, we assume the research hypothesis that an *in silico* biobank can be generated for the study of the nervous system. We proposed that this biobank may consist of an embedded type database, specifically a literature review of neuroproteomics studies database. As a practical and technical background, we used top-down and bottom-up proteomics studies to acquire the data for the neuronal proteome. Then, we correlated the neuronal proteome with the salivary proteome published by SalivaTec (Arrais et al., 2013; Rosa et al., 2016).

We intend that this *in silico* biobank would eventually be used as a salivary database system for neuronal knowledge-based discovery (Jagarlapudi and Kishan, 2009). To conduct the research hypothesis, an analysis of the 2000-2017 neuroproteomics studies was performed, with samples obtained from different brain structures, both from neuropsychiatric and non-pathological conditions.

Moreover, an annotation of information (via UniProtKB coding) was performed, as it is considered gold standard for the interpretation of the catalogued data: the neuronal proteome. This research and annotation will enable the update of the OralCard database (Arrais et al., 2013), a salivary proteome database, compiling innovative information

regarding the nervous system. A correct annotation is essential for biomarker research studies of neuronal conditions involving salivary collection, sampling and analysis.

The analysis of neuroproteomics studies allowed the identification of a total of 2416 neuronal proteins, of which 765 are present in saliva. 85% of the neuronal proteins were initially considered correlated with a neuropathologic condition, whilst 33% were considered as a possible biomarker of a mental health state or of neurotypical subjects. From those, only 13% were in fact identified, quantified and proposed as disease biomarkers and almost none as a biomarker of a mental health state or of neurotypical subjects (Pacheco, Martins and Simões, 2019).

When compared to the number of proteins already identified in the brain (2416), it emphasizes the need for new proteomics studies with the purpose of identifying a greater number of proteins as a purposed biomarker for specific neuropsychophysiological states and traits. Only with this increase of molecular information, will it be possible, in the future, to identify and validate biomarkers of susceptibility in neuropsychiatric diseases, and more interestingly, to identify them in saliva, a non-invasive biological sample.

The fact that we are talking about biomarkers of susceptibility is that the molecular specificity may depend on a set of two contingencies, which were respected in this chapter. Firstly, proteins or protein networks should, on the one hand, be found exclusively in individuals with neuropsychiatric diseases or, on the other hand, cease to be identifiable when the condition is not present. Secondly, those proteins or protein networks should present changes in their quantification, derived from the deregulated mechanisms, i.e., the biological processes and molecular functions.

Nevertheless, it is, then, necessary to emphasize that many variables could influence the comparison between the biomarker research studies and the reported results themselves. Examples of this were: (i) the lack of standardization in *vivo/in vitro* brain collection protocols and methodologies used in their analysis and (ii) the type of origin of the samples (brain regions and cellular structures).

Hereafter, for the use of saliva as a biological sample for studying neuronal molecules, there was also a normal biological variation of the molecules in saliva (daytime variation, intra-subject variation, age and sex) and an interindividual variation (diet, lifestyles, medications, tobacco, alcohol). Thus, these types of studies, which compiled and integrated proteomics information, were interpreted considering those parameters of variability. Those parameters were also crucial for the development of molecular knowledge and the identification of biomarkers of health and disease.

In order to better respond to that problematic, i.e., the variation of the measurements collected in saliva over time, publications were evidenced and discussed in the introduction and methods. Explanations were presented about the daily variability of the sialometric and sialochemical parameters. In parallel, several publications were also made by SalivaTec in order to study this variability, as well as the study of other parameters of collection and processing. Those publications were put together to the definition of the Standard Operating Procedures (SOP) in salivary samples, and were effectively addressed in this thesis.

As an example of this line of research, throughout the dissertation, the reader is referred to the publication *Protein quality assessment on saliva samples for biobanking purposes* (Rosa et al., 2016) made in a collaboration between SalivaTec and Biobanco-iMM. This publication preceded and validated the protocols of this doctoral study. In experimental terms and to avoid these variations, the Intervention Protocol and Salivary Collection Protocol took place during the mornings of Tuesdays and Thursdays between 09:30 (a.m.) and 10:45 (a.m.), and its processing was executed in the Biobanco-iMM at

12:15. Furthermore, it should be noted that it was not part of the objectives of this work to study the daily variability of sialometric and sialochemical parameters in neurotypical young adults.

Considering the complexity of the correlation between the data entries of our database, our expectations for this research were mainly to propose a methodological tool for analyzing the diverse functions of the neuroproteome. Therefore, we generated an embedded type database, i.e., the Neuro.OralCard, and proposed a bioinformatics strategy for its analysis. This strategy comprehended the use of 3 bioinformatics tools: clustering analysis (with the support of VENNY), PANTHER and Cytoscape.

Regarding the functional characterization of the neuronal proteome, this strategy allowed to verify a high number of proteins involved in metabolic functions, immune system processes and neuropeptide transmission in brain circuits. Contrastingly, a low molecular load and weight was given to almost non-expressed biological processes involved in gamete generation and reproduction. Likewise, RNA metabolic processes were underexpressed, despite the recent neuropathological interest in amyotrophic lateral sclerosis, frontotemporal dementia and spinal muscular atrophy (Alaqeel et al., 2015).

As a result, it was presented by this analysis that 79 specific biological processes, 60 precise pathways, 32 particular cellular components, and 41 distinctive molecular functions were maintained by a full neuronal proteome composed of 55 main protein classes.

All those 267 GOs generate thus complex molecular networks in brain circuits. To analyze those functional networks, we developed a molecular biology atlas for the better understanding of the neuronal proteome, the MolecularCog(G) Atlas. To that matter, twenty-eight molecular network maps were published online, as an open access atlas.

Those results should be better understood, specifically in how they directly contribute for the development of neuropsychiatric diseases and particularly in the recruitment immune system cells and in the neuropeptide response.

Therefore, it was intended that this chapter would provide a better comprehension of how single molecules and molecular system mechanisms might lead to the development of neuropsychiatric conditions. However, further characterization studies were needed to better define and quantify their physiological relationships. This characterization was subsequently provided by the experimental chapter 4 and 5.

Henceforward and that sequential phase of this study, we proposed not an *in silico* (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017), but an *in vivo* characterization, of a selected sample of subjects, via full protein profiling and multiplexed simultaneous immune detection and quantification. This database offered us then an accurate molecular revision of the nervous system for an adequate hypothesis making and testing.

Likewise, the Neuro.OralCard opens a possible reference for further integration of mental health profiles and their molecular response. We expect that this database and the bioinformatics tools used to analyze it may then be used to better characterize a sample of neurotypical young adults (Hipólito and Martins, 2017).

Concluding, we successfully gathered information considered relevant for the analysis and interpretation of neuroproteomic data. Regarding neuronal and mental diseases, although the salivary levels of several proteins reflect the same as *in serum*, additional studies have to be carried out to make suggestions as biomarkers. Given the incidence and prevalence of neuropsychiatric diseases worldwide, associated with the difficulty inherent in an early diagnosis, there is an urgent need to discover biomarkers that can assess individual susceptibility to those conditions.

The identification, quantification and application of diagnostic biomarkers, which allow the early detection of the susceptibility of a given individual to neurodevelopmental diseases, could represent a fundamental contribution for neurologic and mental health (Pacheco, Martins and Simões, 2019). However, for a protein to be considered a reliable molecular biomarker, it is mandatory that it corresponds to strict discovery guidelines (Mayeux, 2004; Al-Tarawneh et al., 2011).

We should thenceforth address the question of how we should propose biomarkers of a neurologic and mental state or trait, i.e., the biomarkers of susceptibility. Hence, we expect that the bioinformatics strategy proposed by this study offers a conceivable answer.

5. Protein Profiling Of Cognition And GUI Toolbox Development In Neurotypical Young Adults

In this chapter, we placed the hypothesis that cognition is embodied and situated in rich molecular environments (Hipólito, 2015a; Hipólito and Martins, 2018). As theoretical background, embodied cognition theory drives the study of the mind within central and non-central nervous system structures. The technique used for the acquisition of molecular profiles, capillary electrophoresis, is an affordable technique for top-down proteomics. This molecular biology technique, usually used with large samples, offers a sufficient sensitivity to characterize molecular states.

However, the total protein profile acquired usually lacks adequate resolution for analyte quantification, when compared to high-throughput techniques, such as nano liquid chromatography-tandem mass spectrometry (chapter 1). Indeed, the acquisition of data with the Experion^{TM} automated electrophoresis system (Biorad®) offers low capacity to discriminate individual proteins and specific molecular weight bands. This low capacity influences the electrophoretic patterns, characterized by a conjunction of proteins that migrate to the same MW and not to a specific and single protein migration. Moreover, the electrophoretic bands usually have an associated error correspondent to the lack of accuracy offered by Experion^{TM} analysis; and what is seen in a molecular weight band should account for this inaccuracy related to the instrument and measure.

Consequently, for the reasons presented above and to ensure a precise quantification and discriminative power, a multiplexed simultaneous immune detection was proposed and used in a sequential phase of this study (chapter 6).

The main objective, in chapter 5, was thus and only to do a primary assessment of the total saliva samples of the participants in this study. This primary assessment had the objective of stratifying the samples and doing a preceding selection of the participants. Thus, it aimed to select a small amount, but the most representative study participants, that advanced to multiplexed simultaneous immune detection.

This objective was fully attained and permitted that the total number of salivary samples were reduced from 256 to the 40 most representative samples. Moreover, it guaranteed full protein stability and quality and no protein degradation. This decrease of sampling in multiplexed simultaneous immune detection (chapter 6), decreased significatively the cost of the overall study. Please note that this analysis is very expensive and that the PhD study ran with minimal funds, allowing thus to conclude the overall objectives of the study.

Henceforward, and taking into account the limitations of this electrophoresis-based technique, we proposed using a MATLAB GUI toolbox to set viable hypothesis and to design possible conclusions. The objective was not to compare an electrophoresis-

based technique with high-resolution methods such as mass spectrometry (real proteomics data), which would not be reliable, but risky and unfounded.

Taking this concern with a lot of care, we combined together, Experion™ and MATLAB, offering a more significant methodological strategy. This strategy was only used as an initial qualitative top-down approach for the molecular stratification of 4 molecular profiles in neurotypical subjects.

Those profiles had already been obtained by previous mental health stratification in chapters 2 and 3 of this thesis. That mental health stratification permitted the choice of the subjects that better represented each subgroup and, therefore, were potentially better candidates for a specific protein profile.

Combined Experion™ and MATLAB analysis advanced with the possibility of further characterizing cognition with a preliminary low-end molecular technique. Moreover, this analysis also offered the consequent hypothesis of quantifying those 4 mental health-molecular profiles with better discriminative power (further developed in chapter 6).

We stress that as a descriptive research, the main hypothesis of this study was centred mainly in its methodology strategies, which were limited by the low financial funds accorded to the PhD and the restraints of using an electrophoresis-based technique versus high-resolution methods such as mass spectrometry. Having this chief limitation in mind, the primary outcomes attained were: i) the pipeline identification of neuronal-saliva protein profiles, ii) the protein stratification of neurotypical young adults, (iii) the GUI toolbox development for data visualization of those strata, and (iv) the selection of subjects advancing for quantification.

Firstly, the identification of the total protein profile, through capillary electrophoresis profile, in 22 control subjects (in T-1), permitted a relative preliminary clustering of proteins (**Figure 41**) in specific MW ranges in the oral protein profile.

The determination of this total protein profile (in T-1 [before and after the experimental procedure]), with the pipeline sample of 5 individuals (**Figure 42**), also showed that in those MW ranges, there were protein expression differences (**Figure 44**). These differences were somewhat consistent with the mental health profiles already found in chapters 2 and 3.

Hence, this translational phenomic approach led us to conceivably propose that a forthcoming analysis could be designed to evaluate the salivary total protein profile as a molecular strategy for categorizing mental health profiles.

Secondly, the analysis of the full sample of 92 neurotypical young adults plotted their expected protein profile. Indeed, to our understanding, there are few studies addressing how to probe mental health states and traits with simple electrophoresis-based techniques. Our current knowledge of the salivary protein profile (Rosa et al., 2012) allowed the design of this larger study, in which proteins may be used as phenome biomarkers.

As referred in Methods, a previous mental health stratification was executed in chapters 2 and 3. Furthermore, this total protein profiling offered a complementary tool to identify the molecular mechanisms altered in each of those strata, i.e., sub-phenomes:

(1) the top phenome of the Experimental group;

(2) the top phenome of the Control group;

(3) the bottom phenome of the Experimental group;

(4) the bottom phenome of the Control group.

The subjects that were most characteristic of each sub-phenome (higher score in the clinical assessment) were the ones whose molecular signature was more significant and relevant. Hence, those subjects had a higher intergroup variability in their protein

profile and better represented the protein distribution of that subgroup. In the comparative analysis between the subgroups (comparative electrophoretic runs), this variability was pertinently displayed with a moderate significance.

Indeed, the Expected Protein Profile of each subgroup was already different before the Intervention Protocol (T0). This alteration confirmed that the phenotypic traits of each subgroup were already suggestively different; a difference which had been assessed by the previous psychological evaluation (Hipólito and Martins, 2017). These *a priori* profiles explore analytically Ansermet and Magistretti's postulates and theory (2004) that states that every individual has its own synaptic traits.

Those synaptic traits can be altered/increased with a specific environmental situation, i.e., a synaptic state. It was thus a state modification, which we observed after the Intervention Protocol Δ (T1-T0). This synaptic state showed an intensification of an already present phenotypic trait. Actually, the first set of results of the Expected Protein Profile served mainly to demonstrate the heterogeneity of a general protein profile and how it becomes more homogeneous, if belonging to subjects previously clinically or cognitively stratified. Specifically, this investigation was intended (i) to generate physiological correlates of cognitive state and traits, and (ii) to analyze the extension to which cognition has an internalization effect. As an example, the identification of a potential protein correlated to cognitive stimuli, S100A8, shown in **Figure 43**, which regulates the pathway of NF-kB Transcription Factors, could mean that a gene transcription cascade could be stimulated by a mental state.

All results obtained in T-1, T0 and T1 supported, to some extent, the relevance of performing a total protein profile on a sample of neurotypical young adults. On the one hand, those results demonstrated that it was possible to use a simple total protein profile analysis as a strategy to assess ordinary changes in protein expression, and as a low-end approach for averagely mental health profiling. On the other hand, they showed relative capacity to demonstrate that different subjects of separate subphenomes may express functional changes in different protein clusters.

Thirdly, MODeLING.Vis permitted an adequate data mining of limited neuroproteomic datasets. This data mining consisted both of an unsupervised/unlabelled and a supervised/labelled machine learning. That is, initially, the subjects were imported, and no labels were given to the learning algorithm, leaving it on its own to find structure in its input. Later, in a posterior phase of the data mining, the subjects were labelled in order to compare the protein concentration (ng/µl) of individual subjects in specific MW intervals.

Initially, all protein profiles, comprising all electrophoretic runs, of all the subgroups, were systematically and randomly uploaded to the algorithms. The algorithms then performed an exploratory data analysis, discovering hidden patterns in protein profile data. A first dataset, i.e., total protein profiles, was inputted for feature learning of functional protein network profiles. Until now, as a limitation, only capillary electrophoresis data had been explored, but this GUI toolbox is also designed for other datasets, for instance, multiplex simultaneous immunodetection.

Likewise, this GUI toolbox opens the possibility for further integration of the acquired psychological data, the intersubjective mental health profile (Hipólito and Martins, 2017). Only relying on standard methods for analyzing those databases (e.g., assessment of the covariance between variables) might provide sub-optimal results due to the high number of measured quantities and possible non-linear relationships (D'Alimonte, 2005) between them. The datasets generated by this full cognitive-molecular study should optimally be intercorrelated in a phenomic multi-data approach.

Hence, our data mining solution was then planned to discover and model patterns, for instance, the EM algorithm used for finding the MW intervals shown in **Figure 78**.

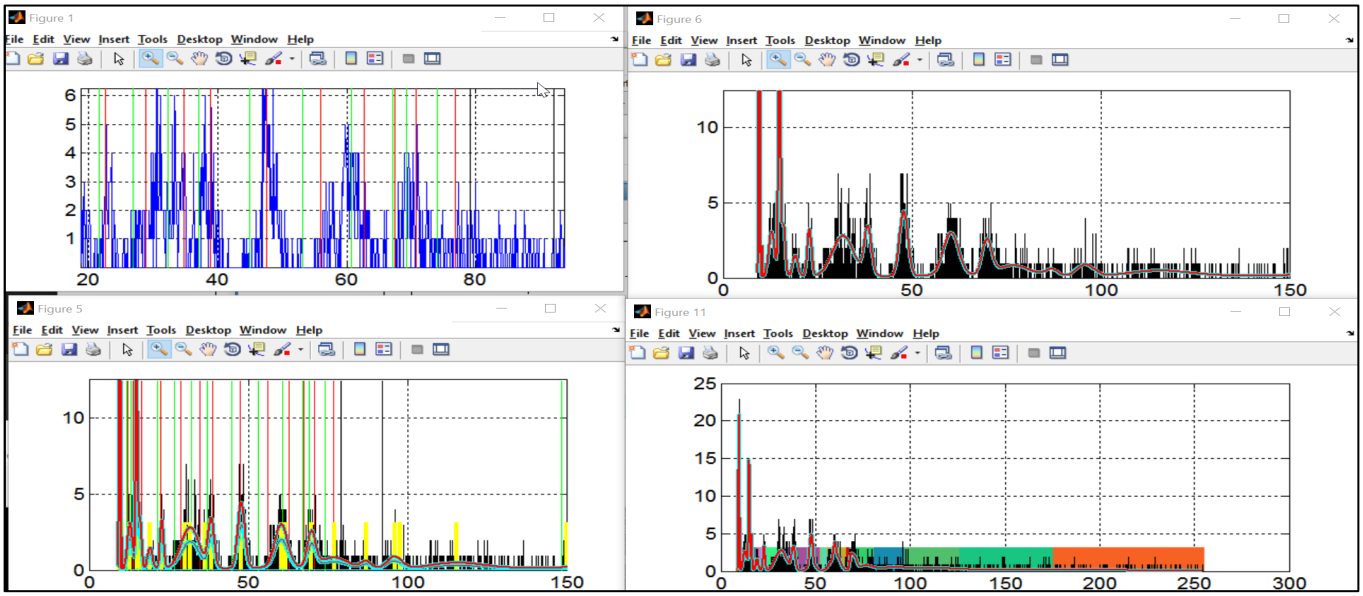


Figure 78. Protein Profile Experion™ Data Acquisition And Algorithm Development
Data mining solution planned to discover and model patterns used to find the MW intervals in the neurotypical sample.

The development of a user-friendly software environment (**Figure 79**) was implemented to enable a throughout exploration of the information embedded in the capillary electrophoresis database.

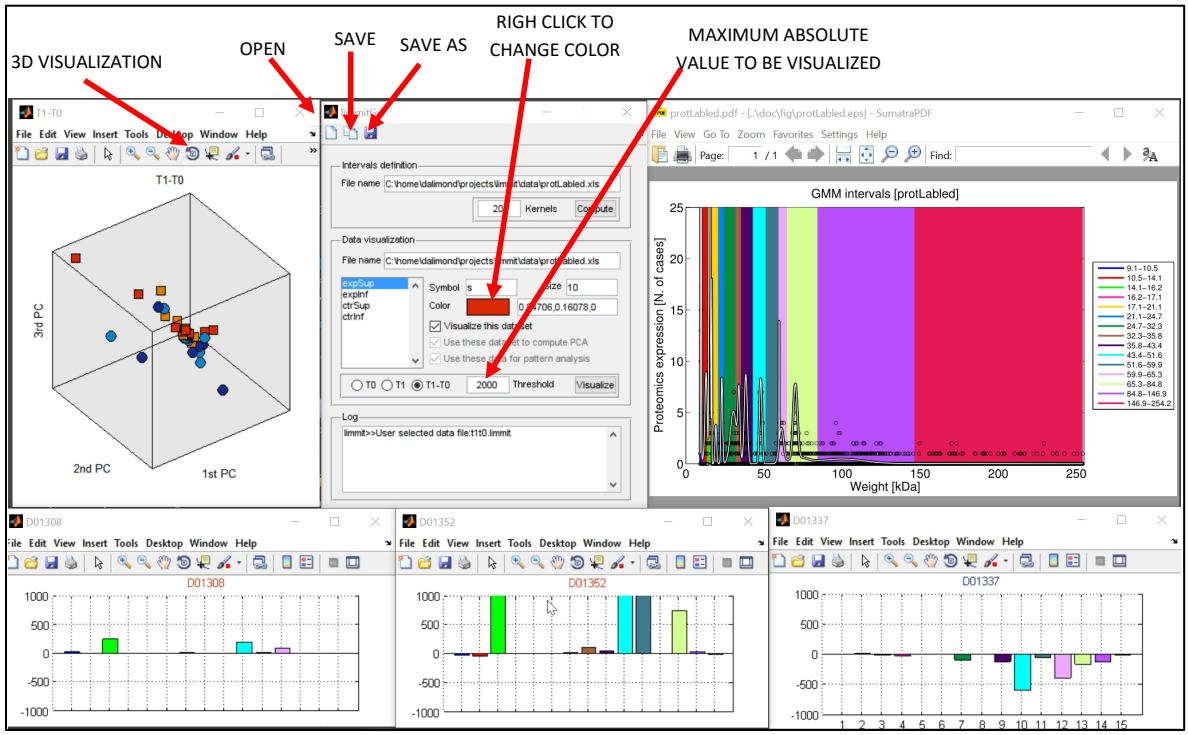


Figure 79. GUI Toolbox
The MODeLING.Vis is used for data mining of different neuroproteomic datasets.
This data mining consisted both of an unsupervised/unlabelled and a supervised/labelled machine learning.

Data visualization techniques were adopted for the interactive query (**Figure 79**) of the molecular profiles. This interactive query, of the acquired Experion™ Protein Profile data, offered the chance to check for functional network profiles. Moreover, it also showed the whole effort of the author to find differences in the electrophoretic separation by Experion™ in the different samples, facing the limitations of a system which prevent the extraction of direct and solid conclusions.

Finally, and for that matter, data classification and regression algorithms are being devised for operational applications, such as recognizing a molecular state, or profile, from the analysis of salivary samples. Expectantly, the same regression algorithms will also be used, in the future, to recognize a mental health state or trait from the analysis of the molecular profiles.

Summarizing, three main components were defined: data visualization, data exploration and development of algorithms. These components encompassed a data mining toolbox, which aimed:

i) to implement and test visualization software that allowed the interactive exploratory of information content embedded in multilayered and multi-source data;

ii) to service the scientific community by distributing, instructing and providing support for software users, i.e., researchers and clinicians.

Fourthly, the results fairly emphasized the molecular strategy we developed for the identification of functional networks, as a complementary and alternative method in neurobiology.

Hence, a follow-up molecular study (chapter 6) of a selected sample is proposed for further proteomic explorations and quantification. This follow-up study can better characterize the molecular substrates of the neurotypical development of young adults, as it probes into the neuropeptide and neuroinflammatory response with a high-resolution method.

Concluding, a descriptive research hypothesis was conducted: the stratification of the neurotypical phenome of young adults and the sum of its phenotypic states and traits. This phenome stratification aims to contribute for a proteomic biobank in development.

Considering the complexity of the correlation between the obtained molecular dataset and the mental health constructs, our expectations for this research were mostly to bring biological theories and insights, and not to perform an explanatory quantification of those molecular/mental health constructs.

For now, and with the limited internal validity offered by low-end techniques, we can only conclude that the neurotypical phenome is a complex result of the intercorrelation of mental health (Hipólito and Martins, 2017, chapters 2 and 3) and the consequent expression of protein networks. Those protein networks, generated in the brain, may be detected in saliva and usually correspond to small neuroimmune molecules or neuropeptides crossing the blood-brain barrier.

Finally, the [9.1;30]kDa molecular weight range should be better quantified, when studying a neurotypical sample, because it offers a possible solution for probing into the neurocognitive response.

Please note that an error variable should be considered and correspondent to the lack of accuracy offered by Experion™ analysis and the identified MW ranges.

These considerations should take this inaccuracy in account but also the process of protein degradation observed and well documented in saliva and fully detailed in chapter 4.

6. Functional Profiling Of Neurotypical Subjects: Neuropeptide Response And Anti/Pro T-Helper Cell Type 17 Equilibrium

It is extensively recognized as self-evident that neurons are an essential core for cognitive phenomena (Varela, 1994). However, we aimed at giving a conceivable counterexample: cognitive-immune coupling. Actually, for a long-time, immunologists naturally attribute "cognitive" faculties to the immune system: recognition, learning, memory and self-/non-self-discrimination (Klein, 1982).

The cognitive immune-coupling mediates (i) analgesia, reward, food intake, metabolism, reproduction, social behaviors, learning and memory host; but also (ii) adaptive immune responses and signaling via transcription factors and cytokine production (Mosmann and Sad, 1996).

In the cognitive immune-coupling, (i) neuropeptides are the essential mediators, while (ii) CD4+ Th cells are the main effectors. This cognitive immune-coupling comprehends small molecules, with a small molecular weight range around [9.1;30]kDa (chapter 5). (i) Neuropeptides act at metabotropic or G-protein-coupled receptors influencing gene expression, synaptogenesis and glial cell morphology (van den Pol, A. N. (2012). (ii) CD4+ Th cells comprise three main classes of helper cells: Th1, Th2 and Th17 (Cua et al., 2003).

On the one hand, (i) the neuropeptides quantified in this study, Orexin, β-Endorphin, Oxytocin and Substance P, have an intricate function in both the nervous and the immune system. Not only do they generate and respond to cognitive life, but also modulate cellular and molecular aspects of the immune response. In neurotypical subjects (Picchioni, et al., 2014), Orexin, β-Endorphin, Oxytocin and Substance P are important for neurodevelopment, and are actors of neurodevelopmental disorders. It is known that they modulate the neuroimmune response in the synaptic transmission (Picchioni et al., 2014) but also in neurogenesis (Bakos et al., 2016).

Likewise, the neuropeptide system may not only be proficient in neurotransmission and modulation of the neuro-immune interface, but also implicated in the cognitive self (Altabe and Thompson, 1996), in developmental self-schemas (McArthur et al., 2017) and in self-concepts, like self-awareness (Fonagy, 2018).

On the other hand, (ii) the cytokines quantified in this study are generated and expressed by Th1, Th2 and Th17 cell types. Th1s regulate intracellular defense, Th2s the antibody or humoral responses, and Th17s are vital in extracellular pathogen defense and in environmental adaptations (Varela, 1994). Th17s have also been described as maintaining both an anti- and pro-inflammatory effect (Annunziato, 2008).

Furthermore, the entire immune system may be, in itself, described as "cognitive", when viewed in interaction with the environment. From that point of view, the minimal level of cognitive organization should comprise not only perceptually high-level, but also low-level, or molecular, intentional actions in an ecological framework.

The incorporation of self-antigens into the dynamic Th17 configurations of lymphocyte clones is a well-studied low-level perspective of the cognitive-immune coupling, i.e., the standard of the immunological self-identity. In fact, as suggested and set forth in this chapter, the discrimination between external/internal factors and the stimulation of anti/pro T-helper immune responses, may also be illustrative of that coupling.

Thus, the immune system might be proficient in perception, learning and, unquestionably, in adaptation, since those characteristics, which are brought to existence by the organism’s own cognitive faculties, are essential for its existence.

To explore if these considerations are appropriated and relevant to neuroscience and to neuronal cognition, we must affirm that neurotransmitters, voltage-dependent ionic channels, neurons and synapses are not, in themselves, "cognitive". Neither is the brain alone, because, so as to be "cognitive", the nervous system requires a functional context coupled to the environment, strictly via intentionality.

That to say that neurons appeared first, phylogenetically, with the protagonism of linking sensory inputs to locomotory actions, which gave rise to correspondent adaptations in the whole system's sensory perceptions, and possibly in the whole system's cellular lineages.

This chapter, to some extent, explored the neuro-immune interface beyond the already advanced knowledge of neurophysiology. For enactive cognitive science, brain and neurons may also be studied into other modes of organization, as explained in chapters 1 and 2. In this chapter, we discreetly advanced with the neuropeptide and immune systems, as actors of high-level cognition and sense-making actions.

Cognitive perception, learning and adaptation are anything but arbitrary, for they are strongly constrained by a "reality principle" which, in the case of living organisms, relates to their viability (Aubin, 1991) and "their ability to maintain their own cellular organization" (Varela, 1994, p. 39).

In conclusion, such viability (Aubin, Bayen and Saint-Pierre, 2011), i.e., optimization, is constantly relative to a particular strategy adopted. Such capacity to maintain its organization (Varela, 1989) follows cognitive faculties which are conveyed into existence by perceptions and actions (Thompson, 2005), and possibly by cognitive-immune coupling interference.

6.1. Limitations Of This Chapter

This study suffices mainly and only for its methodological account, i.e., the combined molecular strategies to evaluate the neuropeptide response and anti/pro Th17 equilibrium. The theoretical account and the hypotheses explored in this descriptive research clearly require further investigation.

Firstly, because the sample size, quantified by multiplexed simultaneous immunodetection, is representative of neither a neurotypical population nor any disease model. Secondly, because an explanatory research would provide a deeper insight into the physiologic mechanisms underlying a variety of combined human neuropeptide and immune-related conditions.

Overall Conclusion And Interpretation

Having presented, discussed and concluded the results of the thesis, this final section summarizes the data and the retained hypothesis of the two sections: Mental Health Stratification and Molecular Stratification.

The Intervention Protocol effectively isolated the mental subphenomes in the sample of 128 neurotypical young adults. On the one hand, the experimental procedure, i.e., the psychotherapeutic technique based on a single cognitive behavioral intervention, was structured to increase awareness and wakefulness by a cognitive exercise of psychophysical training and autobiographical-self memory-recalling, and permitted to isolate mental subphenomes associated with autobiographical-self memory-recalling, positive affectivity and increased self-awareness. On the other hand, the (b) the sham

intervention permitted to isolate mental subphenomes associated to a simple cognitive task of maintaining attention, alertness and concentration during a class.

The Assessment Protocol found four neurotypical subgroups with distinct mental and resulting molecular phenotypes: (i) the Self Awareness subgroup, (ii) the Self Consciousness subgroup, (iii) the Reflective Self subgroup, and (iv) the Pre-Reflective Self subgroup. Being a descriptive study model, preliminary explanatory correlations were made between: (i) the subjective experience, (ii) the total protein profile, (iii) Orexin, β-Endorphin, Oxytocin, and substance P, and (iv) the Th17 cell type response and its biochemical messengers.

The first subgroup characterized by a self awareness phenotype, showed a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a low increase in the [9.8;10.3]kDa and high decrease in the [13.7;17.5]kDa. This reflects the higher molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. More specifically, it was characterized by a most significative increase in Orexin, β-Endorphin and Oxytocin levels, which reflected the elevated neuropeptide response related to the augmenting of specific autonomic and central nervous system functions. In this subgroup and phenotype, this is responsible for the increased reward function and arousal, but also for the elevated associative learning and incentive salience. Furthermore, this neuropeptide response is associated with the increased of the positive emotions and happiness, the reduced fear and anxiety, and the increased predisposition to social interaction. Moreover, there was a complementary effect of the cognitive load on the total inflammatory load. In the interleukin trait and state profiles this subgroup had the lowest pro-inflammatory expression of the 15 analytes studied for the Th17 cell type response, tending rather to an anti-inflammatory response. This is represented by the overexpression of IL-10, IL-21, IL-25 and IL-33. Henceforth, the most significative GO Biological Processes of the immune system were the regulation of isotype switching and the positive regulation of lymphocyte-mediated immunity; while he most significative KEGG pathway was the regulation of inflammatory bowel disease.

The second subgroup characterized by a self consciousness phenotype, showed a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a moderate increase in the [9.8;10.3]kDa and a high increase in the [13.7;17.5]kDa. This reflects the moderate molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. Neurophysiologically, and more specifically the most significative increase in the Oxytocin levels reflected the increased attention and elevated capacity for learning. Furthermore, this neuropeptide response, in this phenotype, is associated with pleasure and with the increased predisposition to social learning. Moreover, there was a complementary effect of the cognitive load on the total inflammatory load. In the interleukin trait and state profiles this subgroup had a low expression of the 15 analytes studied for the Th17 cell type response, tending to no immune response. This is represented by the regulation of IL-4, IL- 10, IL-21, IL-25 and IL-33. Henceforth, the most significative GO Biological Processes of the immune system were the adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains, the regulation of immunoglobulin production, and the co-regulation of lymphocyte-mediated immunity.

The third subgroup characterized by a reflective self phenotype, showed a distinct profile in [9.1;30]kDa molecular weight range, and more specifically a low decrease in the [9.8;10.3]kDa and in the [13.7;17.5]kDa. This reflects the decrease in the molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. More importantly, this was shown by the most significative decrease in Orexin and Oxytocin levels, which reflected the decreased neuropeptide response related to the

diminishing of certain autonomic and central nervous system functions. In this subgroup and phenotype, this is responsible for the decreased activation of the reward system, but also for the lower associative learning and incentive salience. Additionally, this neuropeptide response is associated with the diminished predisposition to positive emotions and tendance to anhedonia, to increased anxiety, and to the decreased tendency to social interaction. Moreover, there was a complementary effect of the cognitive load on the total inflammatory load. In the interleukin trait and state profile, this subgroup had the highest expression of the 15 analytes studied for the Th17 cell type response, showing a relative pro-immune phenotype. This is represented by the overexpression of IL-1β, IL-6, IL-17A, IL-17F, IL-22, IL-31, sCD40L and TNF-α. Henceforth, the most significative GO Biological Processes of the immune system were the positive regulation of (i) immunoglobulin production and secretion, and (ii) osteoclast differentiation; while the most significative KEGG pathway was the cytokine-cytokine receptor interaction.

The forth subgroup characterized by a pre-reflective self phenotype, showed a distinct profile in [9.1;30]kDa molecular weight range and more specifically no increase or decrease in the [9.8;10.3]kDa and very high increase in the [13.7;17.5]kDa. This reflects the marginal molecular expression of the Orexin, β-Endorphin, Oxytocin and Substance P trait and state profiles. Neurophysiologically, in this subgroup and phenotype, this is responsible for a slight decreased attention, arousal and diminished learning. Additionally, this neuropeptide response is associated with certain apathy and the decreased predisposition to social learning. However, there was rather an effect of this demanding cognitive load on the total inflammatory load. In the interleukin trait and state profile, this subgroup had a moderate expression of the 15 analytes studied for the Th17 cell type response, tending to a light pro-immune response. This is represented by the expression of IL-1β, IL-6, IL-17F, IL-22, IL-31, sCD40L and TNF-α. Henceforth, the most significative GO Biological Processes of the immune system were the positive regulation of (i) T cell proliferation, (ii) immunoglobulin production, and (iii) osteoclast differentiation by pro-inflammatory interleukins; while the most significative KEGG pathway was the implication in rheumatoid arthritis.

The combined psychological and neuropeptide/neuroimmune profiles could possibly be justified by modulation of the inflammatory equilibrium to a higher metabolic state. When a task increases stress systems, cognition starts and this equilibrium may be a possible neurophysiological mirror, which is synchronized by a stable system configuration.

Summary

Outcomes And Limitations

In order not to exceed the findings and to be consistent with the objectives, the conclusions of this dissertation are presented exclusively in relation to the sample studied and when applying with the methodology developed in this PhD. To avoid clear errors in the statistical generalization process, the observed results made in this work, point only to a simple categorization of mental health states and traits, as well as a correspondence, or correlation, in their associated molecular states and traits.

As a final remark, I would like to state that the phenomena associated with the patients' subjective experience is hardly evaluated by objective techniques, like molecular biology methods. The two fields of study gathered in this work offer a pragmatic problem, and the solutions presented only avail as an attainable methodological strategy to address this question.

Although translational science in neurology is mainly sustained by molecular and neurophysiological data; in psychiatry, it is utterly and primarily dependent on subjective or observable symptomatology.

Therefore, my first concern, in addressing the problematic raised in this study, was in a detailed first-person data collection. This data collection, explored in Section I: Mental Health Stratification, should be acquired via questions suitable for exploring sensory perceptions, and mental phenomena, such as thoughts, memories, associations, and so on.

But ultimately, my concern in advancing within this problematic was proposing an adequate and sufficient neuro-proteomics reasoning and methodology. This reasoning, explored in Section II: Molecular Stratification, included approaches from (i) full protein characterization to (ii) functional protein networks identification.

Consequently, I submitted a descriptive research that might possibly be used in the research of salivary biomarkers of mental health states, and to be continued in causalistic explanations with a higher sample size and higher internal validity. The cost of such a study, in our neurotypical sample, was an important limitation for its development in a PhD framework.

Considering the limitations mentioned, the resulting work was achieved and the following outcomes were attained along the 6 chapters (articles) of this thesis. Each chapter has been published or is being submitted to peer-reviewed journals in neuroscience or molecular biology.

In chapter I, it is suggested that there are reasons to doubt that cognitive life is only and solely neurocentred. A living system remains alive by perceiving and interacting with its environment, and through interactions that become meaningful in ways that are hardly exclusive of the brain.

It is postulated that systemic protein networks, brain and non-brain located, might play a role in cognitive life. Finally, neuroproteomics, e.g., salivary proteomics, is offered as an empirical method to probe into phenomenal experience.

In chapter II, the two fundamental models to understand mental health are set side by side: the symbolic thinking paradigm and the biological organism model. Likewise, the common difficulty attributed to these paradigms is stated, which is that they do not account for a combined phenomenological and molecular characterization.

Therefore, a conceivable non-reductive solution was purposed to address that problematic. i.e., a descriptive research with a sample of 128 neurotypical young adults. This descriptive research focused both on first-person, via phenomenological assessment, and third-person evaluations, via molecular assessments, of cognitive events.

Finally, and to better separate and isolate cognitive strata in neurotypical subjects, an experimental procedure, consisting of a psychological experiment based on a cognitive behavioral intervention, was created. The result was the screening of 4 distinct mental health profiles.

In chapter III, the 4 mental health strata isolated by the Intervention Protocol were displayed. The 128 neurotypical young adults were stratified by a clinical assessment and statistical method.

The statistical method applied was a profiling strategy based on a proportionate stratified random sampling for neurotypical subject classification. The obtained results led to postulate that 4 subgroups, or subphenomes, with N=23 each, and with different cognitive characteristics, might have distinct molecular expression.

Hence, each of those 4 mental health subphenomes and their molecular fingerprint may have distinct neurophysiology and variate likelihoods to develop a neurological or mental condition.

Chapter I, II and III executed *a priori* mental health stratification for an adequate biomolecular research of our sample of neurotypical subjects.

In chapter IV, the neuronal proteome was revised to synthesize the empirical knowledge for a valid molecular stratification. It is postulated that both neuropsychiatric and neurotypical conditions may result from an altered neuroproteome.

Moreover, it is stated that characteristic neuromolecular signatures can be probed in salivary samples. Subsequently, a database was presented revising 2416 neuronal proteins identified in neuroproteomics, from which 765 are present, detected and identifiable in the saliva proteome.

Furthermore, an *in silico* characterization and a bioinformatics analysis (Correia, Martins, Esteves, Fernandes, Cruz, Rosa, Barros, 2017) was executed by PANTHER and Cytoscape functional clustering. From these analyses, it was concluded that 85% of those neuronal proteins are correlated with a neuropsychophysiological condition, but only 13% are in fact identified, quantified and proposed as biomarkers of susceptibility.

Finally, a functional enrichment determined that predominant proteins in brain circuits are involved in metabolic functions, immune system processes and neuropeptide transmission.

In chapter V, it was hypothesised that neuronal protein networks, identifiable in the salivary protein profile, may be useful to characterize neurotypical cognition.

The previously stratified 92 neurotypical young adults were evaluated according to their total protein profiling. They showed specific and distinct total salivary protein profiles, with a reasonable significance, notwithstanding the low discriminative power of an electrophoresis-based technique versus high-resolution methods such as mass spectrometry. Those molecular profiles were substantially correlated with the previously identified mental health strata.

To better correlate both mental health and total protein profiles, a GUI toolbox was developed – MODeLING.Vis. This toolbox facilitated data mining and exploration of the acquired neuroproteomic data with a low-end method and low-cost technique.

Finally, the outcome of that descriptive analysis was hypothesising the [9.1;30]kDa molecular weight range as an interesting molecular range for adequate quantification. It has been published that this range is characteristic of small neuroimmune molecules and neuropeptides, thus offering a possible solution for probing into the neurocognitive response.

In chapter VI, the [9.1;30]kDa molecular weight range was quantified with multiplexed simultaneous immunodetection. The previously profiled neurotypical subjects were thus evaluated by a combined *HNPMAG-35K | MILLIPLEX MAP Human*

Neuropeptide Magnetic Bead Panel - Neuroscience Multiplex Assay and *Bio-Plex Pro™ Human Th17 Cytokine Assay*. Hence, the 20 neurotypical subjects (5 from each of the 4 subphenomes) with a more distinct and characteristic combined mental health and total protein profile were probed.

The results showed that a neuro-immune interface, or in another denomination the cognitive-immune coupling, is a probable low-level biomolecular structure of high-level neuronal cognition. This structure is regulated, on the one hand, by small peptides such as Orexin, β-Endorphin, Oxytocin and Substance P, and, on the other hand, by Th17 cell type response and its biochemical messengers.

Firstly, the quantification values of Orexin, β-Endorphin, Oxytocin and Substance P in saliva were obtained and published.

Secondly, the quantification values of IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, INF-γ, sCD40L and TNF-α in saliva were obtained and published. Finally, it was concluded that the 20 stratified subjects had a moderately distinct, relevant and significative neuropeptide and neuroimmune response.

As an overall conclusion, I would like to propose that there may be an intricate correlation between (i) the mental health strata and (ii) the neuropeptide and neuroimmune strata, in young neurotypical adults. However, to better explore this intricate correlation, a follow-up causalistic study and analysis should be designed. The results of this thesis led us to consider that the methodology, i.e., the instruments, the techniques and the stratification strategy used in this work should be maintained, but with a higher sample size and the funds to support technical triplicate analysis or the application of high-resolution methods such as mass spectrometry.

"Le Mot De La Fin"

The supervising authors of this PhD thesis, i.e., of the mental health and molecular stratification *rationale*, are (i) Professor Mário Simões, director of the LIMMIT laboratory, Faculty of Medicine, University of Lisbon (FMUL), and it's the senior researchers, where the doctoral student carried out his clinical research work; (ii) Professor Marlene de Barros, Director of the Center for Interdisciplinary Research in Health, Universidade Católica Portuguesa, Portugal, and her senior researchers from the SalivaTec laboratory, where the doctoral student carried out his laboratory research (*wet lab*) work in the collection, processing and treatment of the molecular salivary data; and (iii) the FMUL and the Fundação para a Ciência e a Tecnologia (FCT) researchers who participated in specific tasks of the thesis work, namely Davide D'Alimonte, PhD, principal research scientist (IF-FCT), and his knowledge on developing the MODeLING.Vis Graphical User Interface. All co-authors of this thesis outcomes are properly cited in the results and will be co-authors of the articles that will accompany the publication of this thesis.

The clinical and research staff of LIMMIT laboratory and SalivaTec laboratory reflected with the doctoral student and critically proposed changes included in the final work. The coordination of SalivaTec laboratory in the guidance and full preparation of the thesis manuscript was essential, given its fully recognized work and merit in the area of salivary proteomics. This co-orientation was done in coordination with the principal supervisor in all phases of the project and was fundamental for the execution and completion of the entire research project.

As *"Le Mot de la Fin"*, I would like to uphold that the combined stratification and strategy developed in this work are important not only for producing knowledge in neuropsychophysiology, but also in discovering biomarkers of susceptibility for neuropsychiatric conditions. This descriptive research, and the way it was conducted, feasibly proposed 4 cognitive-molecular strata with distinct phenotypes or phenotypical traits.

In another type of research design, namely in explanatory research, causal relationships between cognitive and molecular life should be further addressed, and by so, better understanding in biomarkers of susceptibility of neuropsychiatric conditions will be developed. Hereafter, the work of this PhD serves only as a *proof of concept* of the methodology applied, showing the technical advances of molecular biology in the study of mental health.

Fundamentally, the work of this dissertation responded to the initially defined objectives. The methodology of the PhD was significantly put to test, and its added value was examined by the established strategy. This publication will be further developed, in a *post-doc*, with a bigger sample and improved clinical and molecular databases, in order to quantify networks of proteins and neurosignaling processes involved in neurotypical subjects or in neuropsychiatric conditions.

Such a study would considerably educate clinicians and researchers in early-diagnosing and screening of nosologies requiring ultra-early interventions. Neurodevelopmental disorders, such as autism, are one of such conditions, benefiting from infant intervention.

Henceforth, and recalling the limitation of this descriptive type of study, I recently published a book chapter (Hipólito and Martins, 2018, *in* Annex A: Original Research)[79], with one of the researchers of MolecularCog(G). This book chapter is an introductory milestone and outcome of the overall motivations for accomplishing this PhD.

"… we ask how to investigate the anomalous experience both from qualitative and quantitative viewpoints. To our knowledge, few studies have focused on a clinical combination of both first-phenomenological assessment and third-person biological methods, especially in autism spectrum disorder therapeutics and diagnosis" (Hipólito and Martins, 2018).

[79] Hipólito, I., Martins, J. E. (2018) A "Second-Person" Model to Anomalous Social Cognition. In: Hipólito, I., Gonçalves J., Pereira J. (eds) *Schizophrenia and Common Sense*. *Studies in Brain and Mind*, vol 12. Springer, Cham. https://link.springer.com/chapter/10.1007/978-3-319-73993-9_4

Review Of The Phd And Publications

Achievements

The work accomplished during this PhD was obtained in the following lab internships and rotations:

Elective Clinical Rotation in Research in Champalimaud Centre for the Unknown, Neuroscience Program, Lisbon, Portugal (2012);

Institute of Pharmacology and Neurosciences, Faculty of Medicine, University of Lisbon, Portugal (2013);

LIMMIT, Faculty of Medicine, University of Lisbon, Portugal (2014);

Biobanco-iMM, Lisbon Academic Medical Center, Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon, Portugal (2015);

Biostatistics IMM (Institute of Molecular Medicine), Faculty of Medicine, University of Lisbon, Portugal (2015);

BIOCANT/CNC (Center for Neuroscience and Cell Biology), University of Coimbra, Portugal (2015);

Data mining and Data visualization, CIMA, University of Algarve, Portugal (2016);

SalivaTec, Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa, Viseu, Portugal (2016, 2017);

Mind & Reasoning (Cognition) Group, Institute for Philosophy of NOVA, NOVA University of Lisbon, Portugal (2016, 2017).

During his PhD program, the student developed the following achievements:

Published **1** original research, **2** book chapter reviews, **1** pilot study **, 1** hypotheses and theory, **2** reviews and **1** conceptual analysis on peer-reviewed journals.

Published (paper, panel, roundtable, talk, workshop and poster communications) on **>50** peer reviewed international congresses and conferences on biomedical sciences.

Started co-supervising MSc thesis and research projects, co-revising scientific publications and having citations of his publications.

Acquired **1** European Union Horizon 2020 (H2020) Grant, **1** University Research Fund, **2** Laboratory (Installations, Materials and Reagents) research infrastructures, **1** preliminary Pharmaceutical Industry Research Coordinator Fund.

Peer-Reviewed Publications [*in PhD*]

Re, T., Ventura, C., Vitiello, G., Simões, M., Martins, J. E. (2015). Transcultural Perspective on Consciousness: a bridge between Anthropology, Medicine and Physics. *Cosmos and History: The Journal of Natural and Social Philosophy*, 11 (2), 228-241. https://cosmosandhistory.org/index.php/journal/article/view/502/841

Martins, J. E., Simões, M., Rosa, N., D'Alimonte, D., Mendes, V. M., Correia, M. J., Barros, M., Manadas, B. (2016). Happiness as a self state and trait of consciousness: saliva molecular biomarkers - a brief revision. *Experimental Pathology and Health Sciences: Research, Clinics, Teaching and Society*, 8 (1): 51-54. http://www.patolex.org/EPHS/revista/201601_files/22%20093.pdf

Martins, J. E., Simões, M., Ferreira, H., Tavares, V., Brito, J., Carvalho, L. X., Carvalho, E. N., Castelo-Branco, M. (2016). Self-reflexive consciousness: a model for the

experimental use of neurofeedback in sensorial immersion in a center for consciousness knowledge. *Experimental Pathology and Health Sciences: Research, Clinics, Teaching and Society*, 8 (1): 55-58. http://www.patolex.org/EPHS/revista/201601_files/23%20093_1.pdf

Re, T., Palma, J., Martins, J. E., Simões, M., (2016). Transcultural Perspective on Consciousness: Traditional Use of Ayahuasca in Psychiatry in the 21st Century in the Western World. *Cosmos and History: The Journal of Natural and Social Philosophy*, 12 (2), 237-249. http://cosmosandhistory.org/index.php/journal/article/view/561/911

Sousa, S., Martins, J. E., Rosa, N., Barros, M., & Correia, M. J. (2016). # 097. Presença de genes de resistência a antibióticos na cavidade oral: uma revisão sistemática. *Revista Portuguesa de Estomatologia, Medicina Dentária e Cirurgia Maxilofacial*, 57, 39-40. doi: 10.1016/j.rpemd.2016.10.095 https://www.sciencedirect.com/science/article/pii/S1646289016301339

Hipólito, I., & Martins, J. E. (2017). Mind-life continuity: A qualitative study of conscious experience. *Progress in Biophysics and Molecular Biology*, 131:432-444. https://www.sciencedirect.com/science/article/pii/S0079610717301165

Book Chapters [*in PhD*]

Hipólito, I., Martins, J. E. (2018). A "Second-Person" Model to Anomalous Social Cognition. In: Hipólito, I., Gonçalves J., Pereira J. (eds) Schizophrenia and Common Sense. *Studies in Brain and Mind*, vol 12. Springer, Cham. https://link.springer.com/chapter/10.1007/978-3-319-73993-9_4

Pacheco, A., Martins, J. E., Simões, M. (2019). Mental health promotion in the community: conceptual analysis. *Cadernos de Saúde,* 2019, vol. 11, no 2, p. 27-36. https://revistas.ucp.pt/index.php/cadernosdesaude/article/view/5166

Postdoc

In his *post-doc*, the author intends to continue the work of his thesis, with his research team, executing European Union Horizon 2020 CORBEL, research and innovation program grant agreement No. 65,424 - CORBEL PID2277 Molecular insight into Autism Spectrum Disorder (ASD), attributed to SalivaTec, Institute of Health Sciences, Center for Interdisciplinary Research in Health, Universidade Católica Portuguesa, Viseu, Portugal. He will be studying a sample of neuroatypical toddlers (i) to permit the integration of multiple clinical and molecular databases for *a posteriori* analysis of ontological and nosological co-expression networks and (ii) to offer data-mining solutions for early screening, molecular stratification and follow-up. Concomitantly, as a resident in child psychiatry in Fondation Pôle Autisme, Centre de consultation spécialisé en Autisme, Geneva, Switzerland, he will attain the clinical knowledge in neurodevelopmental disorders and developmental disabilities.

In 2018, he published a book chapter (*in* Annex B: Book Chapter Review), in *Studies in Brain and Mind*, vol 12., Springer, that introduces the application of the cognitive and molecular profiling techniques developed in this thesis to neuroatypical subjects.

Annex

Annex A. *Mind-Life Continuity: A Qualitative Study Of Conscious Experience*

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Mind-life continuity: A qualitative study of conscious experience

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ABSTRACT

There are two fundamental models to understanding the phenomenon of natural life. One is the computational model, which is based on the symbolic thinking paradigm. The other is the biological organism model. The common difficulty attributed to these paradigms is that their reductive tools allow the phenomenological aspects of experience to remain hidden behind yes/no responses (behavioral tests), or brain 'pictures' (neuroimaging). Hence, one of the problems regards how to overcome methodological difficulties towards a non-reductive investigation of conscious experience. It is our aim in this paper to show how cooperation between Eastern and Western traditions may shed light for a non-reductive study of mind and life. This study focuses on the first-person experience associated with cognitive and mental events. We studied phenomenal data as a crucial fact for the domain of living beings, which, we expect, can provide the ground for a subsequent third-person study. The intervention with *Jhana* meditation, and its qualitative assessment, provided us with experiential profiles based upon subjects' evaluations of their own conscious experiences. The overall results should move towards an integrated or global perspective on mind where neither experience nor external mechanisms have the final word.

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the individual feels the vanity of human desires and aims, and the nobility and marvellous order which are revealed in nature and in the world of thought.

Albert Einstein on Buddhism (1930)

1. Introduction

Thousands of years ago, long before the dawn of science, perceptive access to the world enabled human beings to conduct their affairs. As Koffka (1935, p. 7) put it: "To primitive man each thing says what it is and what he ought to do with it: a fruit says, "Eat me"; water says "Drink me"; thunder says "Fear me"". Over time, by encountering both veridical and erroneous cases in their original world, human beings learned to distrust what things told them, and henceforth developed a new system founded no longer on particulars, but on universals. While the world of primitive man directed his experience—telling him what was good, bad, dangerous, healthy, or safe—today the world of scientific man guides human experience through the intellect, logic, and verified data.

Throughout the ages, however, a challenging question in science has remained, viz. why does physical sensory stimulation have to be accompanied by subjective experience? Were this not the case a neurobiological explanation would suffice, and Beethoven composing the *Ninth Symphony,* or Gödel working on his theorems[1] could be explained by the stimulus-response schema. Subjective phenomena,[2] which constitute the very texture of our existence, are difficult to describe and have thus far been excluded from scientific investigation. This is a very difficult topic for experimental research, particularly because modern Western science starts by quantification, and assumes that psychological experience can be expressed in purely quantitative terms (Weber's law). Of course scientists recognize that only because somethings escape quantification, it does not mean they are to be dismissed from science. On the contrary, as His Holiness the Dalai Lama remarks,

> "It seems that scientific research reaches deeper and deeper. But it also means that more and more people, at least scientists, are beginning to realize that the spiritual factor is important. I say 'spiritual' without meaning any particular religion or faith, just simple warmhearted compassion, human affection, and gentleness. It is as if such warmhearted people are a bit more humble, a little bit more content… If we do not combine science with these basic human values, then scientific knowledge may sometimes create trouble, even disaster" (His Holiness the Dalai Lama, cited in Varela, 1997, pp 4–5).

How, then, do we explain subjective phenomena in a non-reductive metaphysics of the human mind? Psychology, neuroscience, philosophy, and physics, among other areas, study the nature of the subjective phenomenon. Their progressive specialization has marked scientific progress both in Eastern and Western views, experimental and theoretical. This specialization and separation was necessary, but it has inevitably worked against the aim of an integral science (Simeonov, 2010), i.e., a science that is plurally constituted. Owing to this, we propose to look at a new perspective within the study of the mind and subjective experience. This perspective is based on the principle of interdependence between the constituting disciplines, in a dialogue between Eastern and Western traditions. In this paper, it is our aim to show empirically how theoretical cooperation between these traditions may shed light on a non-reductive understanding of life and mind.

A unified approach can be rigorously formulated to accommodate qualitative analysis and quantitative measurements. In our study, we combined a psychological assessment with a molecular measurement of protein profiles, with the aim of contributing to the phenomenological investigation of subjective experience. This is the first in a series of papers in which we focus on the intervention methodology adopted, viz. Buddhist meditative practice. We begin the paper by introducing the *hard problem of consciousness*, discussing the various answers it offers with regard to the phenomenological investigation of subjective experience. We focus in particular on the Western *neurobiology of consciousness* hypothesis, and the *life-mind continuity* thesis. We expect the results of our qualitative experiment (section 4) to show alignment with the *life-mind continuity* thesis.

1.1. The hard problem of consciousness

It is uncontroversial that consciousness is present when, for example, a student attends a class, in a spectator's excitement at a football game, when Galileo worked on his physics, or when Shakespeare wrote *Hamlet*. These activities can be described molecularly as processes whereby excitation on the sensory surface of an animal is conducted by nerve fibres to nerve centers, switched over to efferent nerves, and resulting in muscle contraction or gland secretion. Ordinary man, however, knows nothing of the molecular processes of conscious experience, but he certainly knows *what it is like* to experience a football game or a sunset by the sea.

The East and West provide distinctive conceptions of this subjective experience. Western medicine, for instance, tends to see the mind and body as "split," comprising two separate entities. Eastern traditions, by contrast, suggest that mind and body derive from the same energy (source). These conceptions of consciousness pose more than philosophical issues, particularly because, in the West, dualism between the mind and body has affected how patients are perceived and treated.

The problem of consciousness has occupied the best human minds for thousands of years, and has generated two most common ways of facing the *hard problem of consciousness* in western science, the *physicalist* and the *dualistic* view.

1. The whole problem is illusory. This response holds that there are not two kinds of substance or modes of existence, matter, and mind, but only one, viz. matter. Matter is composed of blindly whirling atoms that, owing to their great numbers and time

[1] For a phenomenological account of Mathematics, see Hipólito (2015).

[2] Conscious experiences such as: when an idea comes to mind; when a childhood memory suddenly returns; when we enjoy a piece of music, or taste a good wine; when we read a novel, or write a letter; or when we watch the sunset, or visit an art gallery, and so on.

available, form all sorts of combinations, among them those we call animals and human beings. Thus, it is scientific conviction that thinking and feeling are merely movements of atoms, even including the feeling of awe accompanying the moment of remarkable scientific discoveries. This materialistic conception of mind has served science well, building up physical and physiological knowledge. Nevertheless, difficulty resides in the fact that between life and mind this conception of consciousness makes an arbitrary discrimination with regard to scientific dignity. They have accepted matter and rejected mind, while each of them may in fact disclose as much of the truth as the other.

2. The second solution draws a Cartesian line between life and inanimate nature belonging together. This response places mind and life together as directed by a power not found in inorganic nature, and therefore as essentially different from it. However, this response does not solve but rather *re-emphasizes* the problem. Numerous speculative attempts have been made to overcome dualism. But pretending that a new name provides a solution to it may do a great deal of harm to science were it to be widely accepted.

Cartesian dualism is theoretically rejected in contemporary science, although, as previously mentioned, in practice we see too often a gap between the body and the mind in western science. The procedure is either to look for the subjective phenomena in the brain (bottom-up paradigm), or to theoretically reject the subjective phenomena while investigating brain function (top-down paradigm). Science cannot empirically deny subjectivity as an ontological phenomenon, nor can it verify or falsify subjective phenomena.

Subjective phenomena have been identified as the *hard problem of consciousness* (Chalmers, 1995). The *hard problem* concerns why physical stimulation must be presented to consciousness with a *subjective feel*. As David Chalmers frames problem (1995, p. 3).

It is widely agreed that experience arises from a physical basis, but we have no good explanation of why and how it so arises. Why should physical processing give rise to a rich inner life at all? It seems objectively unreasonable that it should, and yet it does.

The subjective aspect of experience, elicited from sensory stimulation, was illustrated by Frank Jackson (1982) in a thought experiment known as "the knowledge argument" against physicalism. It can be summarized as follows. Mary is a neurophysiologist who happens to be color blind from birth, but nevertheless specializes in perception and color. Imagine that she knows all there is to know in physical terms about perception before she in fact perceives red for the first time. Would she know *what it is like* to perceive red? Notwithstanding all the knowledge Mary has about the neurophysiology of sight—however detailed it may be—this knowledge cannot give her the experience of what it feels like to see a red rose. Jackson's thought experiment attempts to establish, first, that there are non-physical properties, and second that knowledge of these properties can only be attained through conscious experience. Thus, Jackson's thought experiment attempts to refute the claim that all knowledge is physical knowledge. To the contrary, conscious experience must involve non-physical properties because someone with complete physical knowledge about some phenomenon might yet lack knowledge about *how it feels* to experience the phenomenon itself. From this it follows that Mary does not know *what if feels like* (Nagel, 1974) to see red until she in fact sees red, sc. until she has had the conscious, phenomenal experience of red.

Easy problems, on the other hand, are those that fall within the scope of neuronal and cognitive function. These are usually studied through statistical and computation measurements. Such measuring techniques include behavioral models of yes/no responses (button-pushes, time-responses, etc.), and neuroimaging. Cognitive functions, such as memory, attention, sensory stimulation, are empirically investigated and explained by the standard methods and tools of cognitive science, viz. by computational or neural mechanisms (Chalmers, 1995, p. 2). On these models, to explain access (to consciousness) and reportability (language), for instance, we need only specify the mechanisms by which information about internal states is retrieved and made available for verbal report. To explain the integration of information, we need only exhibit mechanisms by which information is brought together and exploited by later processes. For an account of sleep and wakefulness, for example, an appropriate neurophysiological account of the process responsible for an organism's contrasting behavior in those states will suffice. As Chalmers (1995, pp. 2–3) explains:

> In each case, an approximate cognitive or neurophysiological model can clearly do the explanatory work […] If this phenomena were all there was to consciousness, then consciousness would not be much of a problem.

In cognitive science the *hard problem of consciousness,* or of subjective phenomena, comes up again and again. Indeed, it is well-known to those who study brain function, such as LeDoux, Antonio Damásio, Gerhard Roth, and Francisco Varela. There are two fundamental models to understanding the phenomenon of mind. The first is the computational model, based on symbolic thinking paradigm; the second is the biological organism model, based on the natural life paradigm. The latter involves the thought that we should understand mind from the perspective of life and evolution. In what follows, we will briefly present and discuss two versions proposed within the biological model.

1.2. Neurobiology of consciousness

In recent years, neuroscientists have intensively studied the cerebral activity correlated with conscious experience. Patterns of cell activity have been detected from PET and fMRI scans, which are commonly seen as showing the mind in action. It is said that we can now see where mental arithmetic occurs, where words are formed, where lies are generated, and even where our religious sentiments are located (albeit what we are in fact witnessing are changes in oxygen levels in the blood during these mental activities).

The embryonic neurobiology of consciousness was introduced by Crick and Clark in their *The Astonishing Hypothesis* (1994), followed by *The Quest for Consciousness* by Koch (2004), which further lays down paths to be explored in the next several decades. Koch is optimistic that careful scientific research will provide the answers, but is less impressed with the theoretical limits imposed by philosophers. Although Koch admits that philosophers have frequently formulated questions that challenge scientists, philosophical constraints are often overlooked (Martinez-Conde, 2004). Their model proposes a neurobiological account of experience. Koch narrows down the neural correlates of consciousness for visual consciousness to coalitions of neurons in and around the inferior temporal cortex, projecting to the frontal lobes and supported by feedback activity from the cingulate and frontal cortices. The function of consciousness, he states, is to provide an "executive summary" of the world's status to the planning stages of the brain (i.e. the frontal lobes). Thus, consciousness may be an intermediate-level process (see Jackendoff's, 1987) limited to intermediate brain areas. This view has been illustrated with the idea of a homunculus, sc. the explanation of (human) vision by assuming there is an internal 'viewer', namely a 'little man' or 'homunculus' inside the brain

'looking at a movie' inside a theatre (this has also been called the Cartesian Theatre). The homunculus argument has, however, been the target of criticism and is today generally seen as a fallacy. Another source of difficulty regards how to metaphysically define, and ontologically conceptualize, "visual consciousness". In empirical investigation, one should be cautious when establishing metaphysical correlations of levels of reality. It seems the object of Koch's study is 'vision awareness', that is, the relation between attentional function and vision stimulation, which would fall naturally within the purview of the *easy problems*. It has, however, nothing to do with how subjects experience a certain phenomenon. In other words, it fails to explain the phenomenological experience accompanying sensory stimulation.

Antonio Damásio proposes a theory about the neural basis of consciousness that contests the idea of a homunculus inside the brain (Damásio, 1994, 2010). On Damásio's view, consciousness is a private, first-personal phenomenon that can be studied by combining cognitive and neurobiological methods in order to describe its cognitive nature, behavioral correlates, possible evolutionary origin, functional role, and finally its neuroanatomical and neurophysiological underpinnings (Damásio, 1998). Phenomenal conscious content is, in Damásio's view, a matter of associations that are processed in different brain areas at the same time. More specifically, what makes a conscious state feel like something rather than nothing is explained as a fusion of mind and body, in which neurons become "extensions of the flesh." Phenomenal consciousness is enabled as the result of a procession of neural maps of inner and outer worlds. Indeed, this is what Damásio names *self-consciousness*. Without a self, the mind would lose its orientation: thoughts would be freewheeling, unclaimed by an owner, and we would almost certainly look unconscious (Damásio, 2010; chap. III).

Damásio makes a distinction between three levels of "self", which build upon each other to construct the kind of full-blown consciousness that humans are privileged to experience. On his view, the most basic form of self—the "protoself"—is a neural map of an organism's internal status, i.e., related to primordial feelings of the body present in the normal, waking brain (p. 191). The "core self" is what further extends the "protoself", namely by incorporating interactions between an organism and its environment. It provides personhood, and firmer senses of being. At the highest level, there is the "autobiographical self", which adds information about an organism's past and its expectations about the future. With language as a useful medium, memory and reasoning are enhanced. With this enhancement, narratives become possible, giving the "core self" the sense of being a protagonist, that is, an "autobiographical self".

Damásio's theory is largely centered on the notion and levels of self as exemplified by the brain. His theory relies on neuroimaging techniques and determines that experience results from internal or external physical stimuli. This view therefore requires understanding experience on its reductive construal. Damásio (1994, 2010) rejects dualism by bringing consciousness back to the body (sc. brain function). The reduction of experience to brain function returns us squarely to a reductionist view of consciousness.

Damásio's theory is in opposition to other biological approaches, such as the *life-mind continuity* thesis. According to the latter, notions of both conscious phenomena and *selflessness* play an essential role.

1.3. The life-mind continuity thesis

A variety of phenomena in human experience reveal that, within different evolutionary levels of living organisms, life and mind cannot be thought of as two distinct phenomena. To the contrary, mind and life are two indispensable categories that, by virtue of their internal relation and identical ontology, form a whole.

According to the mind-life continuity thesis there are three levels (Godfrey-Smith, 1998). On the *weak continuity view,* whatever has mind will have life, although not all things that have life have mind. Cognition is a kind of activity of a living system. On the *strong continuity* view, life and mind have a common abstract model or set of basic organizational properties. The functional properties of mind are usually the indispensable properties of life. On the *methodology continuity* view, the understanding of mind requires further understanding the role that it plays in the whole living system (pp. 72–73). Each of the views within the *life-mind continuity* thesis share the perspective that cognition should be studied in the context of "the whole organism" (Godfrey-Smith, 1998).

1.3.1. The enactive approach and autopoiesis

The *life-mind continuity* thesis is accommodated by enactive cognitive science. This is the biological model that emerges from the theory of *autopoiesis*. *Autopoiesis* aims to correlate the data from brain, mind, and consciousness studies (particularly neuroscience laboratory work), with phenomenological insights in the understanding and explanation of subjective experience, and ongoing reflection on one's own personal consciousness, as conceived by Buddhism (Varela et al., 1993; Varela, 1996, 1999; Hayward and Varela, 1992).

Autopoiesis is a theory concerning the inextricable link between life and cognition, insofar as one cannot exist without the other. As Luisi and Houshmand (2010, pp. 85–86) elaborate:

> [The] starting point is the interaction between the autopoietic unit and the environment. The living unit is characterized by biological autonomy and at the same time is strictly dependent on the external medium for its survival. There appears to be a contradiction here, and life must indeed operate within this apparent contradiction. The interaction with this environment is always a very specific one, in the sense that the interaction a butterfly has with the environment is different from the interaction a worm or a human being has with the environment

In other words, the internal structure of the organism changes and adapts according to its environment. The structure of the living organism, together with its previous history of perturbations, determines the reactions that new perturbations will induce. Changes, mutations, and evolution are here seen as the result of maintaining the internal structure of an autopoietic organism.

The term 'cognition' applies only to living entities, and not to the inanimate world. From unicellular to multicellular organisms, from plants to insects, fish and mammals, each one has its own type of cognition corresponding to a different level of life's complexity. Cognition, as seen here, contrasts with the representational/cognitivist model. This is because it consists in a mutual interaction between the inner structure of the system and the environment, in the sense that the environment is 'created' during interaction itself as, for example, when a spider creates a web. It is in this sense that *enaction* is the process of mutual bringing into existence: the organism with its sensorium 'creates' its own world; the environment allowing the living organism to come into being.

According to this theory, the very notion of consciousness derives from the autopoietic organization of life: there is no organic human life without consciousness, and there is no consciousness that is not embodied in organic life. This perspective culminated in the neurophenomenological method (Varela, 1996), which is, according to Varela (1997, in Rudrauf et al., 2003):

> grounded on a pragmatic will to progressively and systematically reduce the distance between subjective and objective [...] a way of narrowing the gap between the mental and the physical

1.4. The free energy principle

There have been recent attempts to formalize the enactive theory with brain physiology. This is known as the Free Energy Principle (FEP). On some generous articulations of this view, free energy minimization occurs not only in biological systems but also takes place in nonliving systems, ranging from synchronization of clocks, to the primordial soup and social networks. Although there are some variants of this hypothesis, it generally holds that organisms act to maintain themselves in their expected biological and cognitive states, and that they can only do so by minimizing their free energy, provided that the long-term average of free energy is entropy (Kirchhoff and Froese, 2017).

A cognitivist view of the free energy principle, however, conceives of the relationship between life and mind under the predictive mind hypothesis. This is a model associating minds with computational processes requiring semantic (i.e. contentful) properties, or allowing the possibility of the mind to be potentially realized independently of life, were it to be given artificial support.

A non-cognitivist approach to the free energy principle (Friston, 2009, 2013), by contrast, implies that mentality is ubiquitous. This is a strong continuity view on particular concepts of life (viz. *autopoiesis* and adaptivity) and mind (basic and non-semantic). On the non-cognitivist view:

> All systems that maintain their variables within a limited range of values can be understood as having some form of mentality or proto-mentality given that the FEP casts any system that is able to maintain structural integrity in the face of a fluctuating environment as engaged in predicting its own future states. That is, retaining integrity rests upon processes the function of which is to maximize model evidence—i.e., these processes exhibit self-evidencing dynamics (Kirchhoff and Froese, 2017, p. 18).

The enactive theory endorses the non-cognitivist view. Subjective phenomena should not be mistakenly understood as a powerful generative brain model of semantic mental representations (cognitivist free energy principle), nor as an application of processing neural maps used to 'recover' the structure of the external world by conceptual reflection on the state of the external environment (as argued by Damásio). On the enactive view, human experience is conceived of as an internal structure of pre-reflective dynamic interaction. Varela adopts the Buddhist notion of a virtual self—or a selfless self—and reconceives it on biological level. *Enactivism,* as informed by Buddhism, thereby assumes a unique perspective.

1.5. Buddhist practice and meditation

The western idea of self is very different from the eastern because the universe does not play an important or relevant role. The self is seen as the enclosed capacity "to order my own thoughts and my own life, to use reason as an instrument to control and order my own life … what's really important is not the particular content of our feelings or thinking but the power to control it reflexively (Varela, 1997, p. 18).

Over the past two thousand years Buddhists have developed sophisticated philosophical, phenomenological, and epistemological notions of egolessness (or selflessness), which form the core of Buddhist thought.

> The Dalai Lama then asked, "In the modern West, when one thinks 'I' or 'I am,' does this necessarily imply that the 'I' so conceived must be posited as being independent or autonomous?" Charles's answer was very Buddhist in flavor. "If you ask people, they say no. But in the way they actually live it, the answer is yes, very powerfully, and much more so than our ancestors who thought of themselves more as part of a larger cosmos. (Varela, 1997, p. 20).

Buddhists refuted the existence of a permanent, unchanging self. In general, all four philosophical schools within Buddhism agree in denying the existence of a self that has a separate nature from the psychological constituents. According to His Holiness the Dalai Lama,

> "There are four major schools of Buddhist philosophy, among which we consider the Prāsaṅgika Madhyamaka school to be the most profound. One school of thought identifies the self essentially with consciousness, whereas the Prāsaṅgika school regards the self as something imputed on the basis of the collection of the aggregates, or the mind and body. The Svātantrika Madhyamaka and all the lower Buddhist schools regard the statement that phenomena exist merely as imputations, not by their own nature, as an expression of nihilism" (Varela, 1997, p. 117).

The self is a flow of our experience: feelings of sadness and so forth arise in response to certain experiences,

> "If in fact the self does not truly exist, then apprehending the self as not being truly existent is, of course, in accord with reality … For a person who has investigated whether or not the self is truly existent, and through this investigation gains some actual experience of the lack of true existence of a self, when for this person a sense of self starts to arise more strongly it would not arise with the sense of apprehending the self as truly existent. Rather, the self would be apprehended without the qualification of being either truly existent or not truly existent. It could also happen for such a person that, although the self appears as if it were truly existent, one knows that it's not. In this situation, the self is apprehended as being like an illusion. It appears in one fashion but one knows it doesn't exist according to that mode of appearance. Hence it's like an illusion." (His Holiness the Dalai Lama, in Varela, 1997, pp. 117–118).

As a practice, Buddhism requires involvement in the same way that scientists are involved in their work, or as a writer's mind is involved in writing (see Brockman, 1996). To these contemplative practices, the self is neither a thing nor an entity, but a process.

According to Evan Thompson (2014), the sort of focused attention practices—as developed by some Buddhist traditions—can be construed as techniques for attending to features of experience that we ordinarily ignore or neglect. In other words, adept meditators can see and notice things that we rarely ever do. Thus, Buddhist contemplative practices can be understood as a kind of phenomenological training that can serve the scientific study of mind.

In Buddhism, mental training is often described as a systematic process of familiarizing oneself with the present character of mental events. This training cultivates a capacity for sustained, attentive awareness of the moment-to-moment flux of experience (Thompson, 2006). This seems to tell against a reflective account of the self, because concepts do not always do justice to the full range of qualities we experience. In the Western tradition, we tend to think that consciousness is something that is either present or

absent: you have it when you are awake, and lose it when you are knocked out. In the East, however, it is essential to distinguish modes of consciousness within the range of, what we in the West call, unconsciousness. Dreaming, lucid dreaming, deep and dreamless sleep, and so-called 'pure awareness' are examples of such modes. Indian philosophy's (see Kak, 1997) major aim is precisely the development of a taxonomy of modes of human consciousness (Thompson, 2014).

Western science, however, has not yet furnished for itself an adequate phenomenology of conscious experience. As such, it is essential that we open the channels for constructive dialogue with the Eastern tradition and its practitioners, thereby enabling their insights to inform our own investigations, and *vice versa.*

2. The empirical study

Here, we focus on the qualitative study, in which we used a Buddhist meditative practice as our intervention protocol. Buddhist mediation was chosen (instead of other possible kinds) in virtue of the autopoietic paradigm. We hope we were able to show, in the theoretical background, why the autopoietic paradigm, inspired in the Buddhist conception of the mind, is stronger in providing non-reductive explanation of subjective experience. Because subjective experience cannot be reduced to brain pictures and/or yes/no answers, this paradigm takes into consideration the rich first-person experience, in the attempt to combine it with third-person, quantitative, measurements.

In this experiment, we had two different groups: one attending a regular class, and another practicing a demanding attentional task, a guided meditation. Both groups were subsequently asked to qualitatively evaluate their experience through a psychological assessment. This psychological assessment covered: 1) altered states; 2) happiness awareness; 3) open awareness to the present; and 4) private-public consciousness. This allowed us, first, to compare control groups to experimental groups, and, second, to probe the experience with regard to its psychological characteristics.

3. Materials and methods

3.1. Materials

The materials comprised an intervention with a meditative practice, and four qualitative tests using the following psychological scales:

- *Abnorme Psychischer Zustaende States of Consciousness* (APZ) (Dittrich, 1998) was used to assess the qualitative properties of what is usually called an 'altered state'. The items in the scale aim to evidence: modifications of thought; intense emotional response; bodily schema changes; perceptual changes; synaesthesia; and meaning alterations, which are typically evoked during meditative practices.
- *Subjective Happiness Scale* (Lyubomirsky and Lepper, 1999) was used to assess global subjective experience. Two items evaluated absolute and relative personal experience; two others items evaluated absolute and relative external experience. SHS measures the construct of subjective happiness and well-being. This scale aims to assess, therefore, the self- and non-self perception experiences of happiness.
- *Mindful Attention Awareness Scale* (MAAS) (Brown and Ryan, 2003) was used to assess open awareness to the present. MAAS taps a unique quality of consciousness related to, and predictive of, pre-reflective self-experience.
- *Self-consciousness Scale – Revised version* (SCS-R) (Scheier and Carver, 1985) was used to assess private-public self-consciousness, considered as sustained consciousness that is relatively stable over time.

The contents and constructs evaluated by each scale are summarized in Table 2. An example of the questions posed to the participants is given for each self-report measure. For instance, in the APZ scale, the main construct evaluated was self-boundaries alterations, composed of 23 binary items. An example of a variable assessed was: "Sounds and noises seemed different from what they usually are.". For the SHS scale, the main construct evaluated was that of self and non-self experiences of happiness, composed of six items on a [0; 7] scaling. An example of a variable assessed was: "Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?". Moreover, for the MAAS scale, the main construct evaluated was open-awareness experiences, composed of 15 items on a [1; 6] scaling. An example of a variable assessed was: "I find it difficult to stay focused on what's happening in the present.". Finally, for the SCS-R scale, the main construct evaluated was private and public self-consciousness, composed of 23 items on five-point Likert scale. An example of a variable assessed was: "I often daydream about myself.".

3.2. Intervention protocol: meditative practice

It is well-established that a variety of mind-body techniques, including yoga, mental imagery, hypnosis, biofeedback, and meditation, are effective at addressing symptoms such as pain, anxiety, nausea, and insomnia. These techniques also help with a variety of medical, emotional, and behavioral issues (Culbert, 2017).

Meditative practice has been shown to play a physiological role in inducing neuroplastic changes in amygdala activity (Leung et al., 2017), as well as preventing age-related changes in cognitive functioning (Cotieret al., 2017). Meditative practice has been revealed as an effective alternative to pharmacology on blood pressure response (Park and Han, 2017), and as a successful cognitive behavioral therapy for epilepsy (Leeman-Markowski and Schachter, 2016). In addition, meditation intervention appears to promote a change in attentional focus from the internal to the present-moment external world, thereby having important implications for the treatment of anxiety (Xu et al., 2017), and generalized anxiety disorders (Hoge et al., 2017).

3.2.1. Jhana meditation

The Pali word *Jhana* (Sanskrit 'dhyana') is often translated as 'meditation', but more accurately refers to 'absorption' into a focused and stable state of concentration. In the classical tradition there are several stages of *Jhana*, each one more focused than the previous (Khema et al., 2001).

Traditional meditation is nowadays amenable to greater scientific understanding. For example, meditation has the short-term goal of stimulating the reward dopaminergic system. If the reward is greater than expected, dopaminergic neurons in the Ventral Tegmental Area (VTA) in the brain stem are activated. The VTA in turn innervates the nucleus accumbens (NAc) in the ventral striatum, which leads to higher centers in the orbital frontal cortex (OFC). Human studies have shown that activity in the medial OFC at the time of a reward correlates with subjective reports (Hagerty et al., 2013). According to Hagerty et al. (2013), *Jhana* meditation has also been associated with specific brain correlates, which may characterize the subjective experience of classical *Jhana* meditation: (1) external awareness diminishing; (2) internal verbalizations fading; (3) self-boundaries alteration; (4) increase of focused attention; and (5) well-being. The brain correlates, obtained by fMRI (BOLD signal) and qEEG (bands power), consisted in: (1) decreased activation compared to

Table 1
Descriptive analysis.

Descriptive Analysis		Instrument	Biometric Data		Abnormer Psychischer Zustand Scale	Subjectiove Happiness Scale	Mindful Attention Awarness Scale	Self-Consciousness Scale (Total)	Self-Consciousness Scale (Private)	Self-Consciousness Scale (Public)
	N	Variable	Age	BMI						
Total	92	Mean	21,67	21,6627	21,79%	79,43%	66,45%	69,33%	71,38%	77,69%
		Median	21,36	21,0937	17,39%	78,57%	65,56%	69,70%	74,07%	76,19%
		SD	1538	2,68483	25,10%	8,64%	10,12%	25,10%	25,10%	25,10%
		Minimum	19	17,57	0,00%	54,00%	46,00%	26,00%	19,00%	24,00%
		Maximum	26	32,64	70,00%	100,00%	94,00%	89,00%	100,00%	100,00%
		P25	20,46	19,8177	4,35%	75,00%	59,17%	60,98%	62,96%	66,67%
		P50	21,36	21,0937	17,39%	78,57%	65,56%	69,70%	74,07%	76,19%
		P75	22,53%	23,0518%	34,78%	85,71%	73,33%	75,76%	81,48%	90,48%

Table 2
Self-report measurement.

Subjective Experience Assesment				
Scale	Constructs	N. Items	Scaling	Variables (items) example
APZ	Self-boundaries alteration	23	binary	"Sounds and noises seemed different from what they usually are." "Time passed faster than usually." "My body seemed bigger/longer than before."
SHS	Self and non-self experiences of happiness	4	[0; 7]	"Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?" "Some people are generally not very happy. Although they are not depressed, they never seem as happy as they might be. To what extent does this characterization describe you?"
MAAS	Open-awareness experiences	15	[1; 6]	"I find it difficult to stay focused on what's happening in the present." "I get so focused on the goal I want to achieve that I lose touch with what I'm doing right now to get there." "I tend not to notice feelings of physical tension or discomfort until they really grab my attention."
SCS-R	Private & public Self-consciousness	23	five-point Likert scale	"I generally pay attention to my inner feelings." "I often daydream about myself." "I know the way my mind works when I work through a problem."

the rest state in the visual (BA 17–19) and auditory (BA 41–42) processing areas; (2) decreased activation compared to the rest state in Broca's area (BA 44,45) and in Wernicke's area (BA 39,40); (3) decreased activation compared to the rest state in the orientation area (BA5); (4) increased activation compared to the rest state in the Anterior Cingulate Cortex (ACC) (BA 32,33); and (5) increased activation compared to the rest state in the dopamine reward system of the brain (NAc in the ventral striatum and medial OFC). Reciprocal connections between the NAc and the medial OFC were found (D'ardenne et al., 2008). The authors suggest the possibility of activating a feedback loop between the two (Hagerty et al., 2013).

In this study, *Jhana* meditative practice was adopted in the experimental group to promote this possible feedback loop, by switching attentional focus to the conscious experience present in the different stages of this type of meditation.

3.3. Study design

This is a randomized, controlled, multicentered and single blinded study. The study, approved by the ethics committee of the Faculty of Medicine, University of Lisbon, was carried out in LIMMIT Lab at the same university as the centralizing research unit, and in collaboration with SalivaTec (Molecular Cognition(G)), Institute of Health Sciences (ICS), Center for Interdisciplinary Research in Health (CIIS), Universidade Católica Portuguesa, Viseu, Portugal, and the Mind-Brain Doctoral College, University of Lisbon.

The study consisted of two groups: a control group attending a regular class, and the intervention group practicing a guided meditation that aimed to promote conscious experience. After this practice, all the participants (control and intervention) were asked to qualitatively evaluate (posttest design) the experience in relation to: 1) altered states; 2) happiness awareness; 3) open awareness to the present; and 4) private-public self-consciousness. Participants were asked to conduct this qualitative evaluation using the four psychological scales outlined above.

3.3.1. Sampling and population of the study

For this study, students from the University of Lisbon (Faculty of Medicine, Faculty of Economy, Faculty of Sciences, and The School of Social and Political Sciences) were recruited. A convenience sample of 128 healthy volunteers, students at the University of Lisbon, non-meditators, were recruited and screened by the inclusion criteria, in order to achieve a proportion of 50% men. Subjects received information and gave informed consent as they enrolled in the study.

3.3.1.1. Sample screening and group characterization. Screening was conducted through a general questionnaire, concerning the following (a) inclusion criteria: 1) 19-25-year-old young adults; male/female; 2) cultural background: university students; 3) normal body mass index (BMI), and 4) non-medicated excepting birth control pills; and (b) exclusion criteria: 1) serious physical illness or uncontrolled disorders of kidney liver, lung, heart, musculoskeletal, rheumatologic, metabolic, neurological or psychiatric; 2) severe chronic or terminal disease, which might affect the CNS or PNS; 3) pregnant or breastfeeding women; 4) abuse of alcohol or addictive substances, prior to the experience.

The allocation was randomized and parallel assigned, based on the students that were attending different lectures. Both experimental and control arm, with a 1:1 allocation, was conserved. The blinding was maintained in the interventions protocol and partially in the assessment protocol.

3.4. Statistical analysis

We used the following software for (i) statistical and data management: IBM SPSS Statistics 22– significance level = 5%; and (ii) laboratory information management: LabWare™ LIMS.

3.4.1. Descriptive and exploratory analysis

Frequency tables (simple/double entries) were used. Scale variables were summarized as mean, standard deviation, median, quartiles, minimum, maximum and/or others order statistics, when the sample distribution justified it. Categorical variables were summarized using frequency and percentage. When the scale of measure was ordinal, order statistics were considered, e.g. tercile. Boxplots were used as a graphic exploratory tool. Moreover, Spearman's rank correlation coefficient was applied.

3.5. Sample size study

The full sample size study is supported by the previous preliminary results of the experimental group [Martins et al., 2016], and, bearing in mind that the main objective was to test the research hypothesis that led to full study (i.e. the evidence that there is a significant change in the proteome profile in the subphenomes characterized in this paper), the following approach was designed. It was, for that purpose, considered a potency (T-Student) of 80%, a significance level of 5%, and an effect size corresponding to 40% of the estimated value for the standard deviation of the variation of the molecular weights in the preliminary study, executed before proposing this qualitative evaluation. Taking into account this option, that corresponds to a medium Cohen's effect size, an appropriate sample size for each group (experimental and control) of 64 elements was found.

3.6. Results and discussion

Through a guided meditation subjects were led to evoke a conscious experience, and later asked to evaluate the experience. We tested the qualitative relation between this experience and 1) altered states; 2) happiness awareness; 3) open awareness to the present; and 4) private-public self-consciousness.

The sample was separated into independent groups: (a) experimental group (64 subjects), and (b) control group (sham intervention) (64 subjects). Then, these groups were stratified by the subjective assessments protocol into Top and Bottom Phenomes, by a statistical strategy, which will further inform the interpretation of a pre-molecular clinical stratification.[3] This first clinical subtyping permitted a stratification resulting in four groups: (i) the Top Experimental Phenome; (ii) the Top Control Phenome; (iii) the Bottom Experimental Phenome and (iv) the Bottom Control Phenome, as shown in Fig. 1.

The four subphenomes (groups) were tested for comparability statistics and external biometric data, possibly confounding variables, and were considered comparable for age, gender and BMI. The qualitative study allowed us not only to characterize the subjective experience of the whole sample (Table 1), but also to look at the differences between the performances of the experimental group in the meditative practice versus the control group attending a class.

As displayed in Fig. 2, there is a clear distinction between the experimental and control groups. The qualitative performance of the psychological task (four scales) allowed us to identify four groups: (1) top experimental group (the higher tercile) constituting

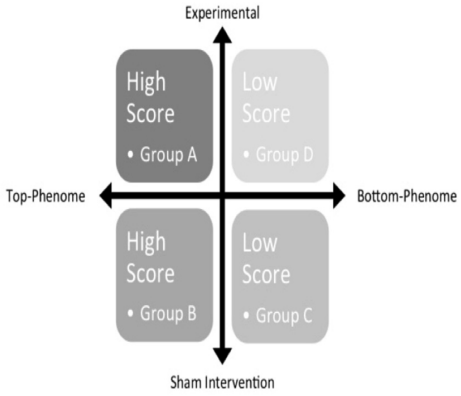


Fig. 1. Stratification of the four groups.

the sample of the best performances during the intervention; (2) the top control group (the higher tercile) indicating the best subjects to focus during the class; (3) the bottom experimental group (the lower tercile) constituting the group of subjects that performed lower (lack of focus) during the intervention; (4) the bottom control group (lower tercile) constituting the group that performed lower (lack of focus) during the class.

Figure 3 presents the comparative results of the phenomenological descriptions of each of the four subgroups. As illustrated in Fig. 2, the four subgroups performed differently on the overall scores of self-report measures. This suggests specific constructions of subjective experience, which resulted in the four subgroups: 1) top experimental group; (2) top control group; (3) bottom experimental group; and (4) bottom control group. These specific psychological characteristics can further be correlated to a specific molecular expression. In Fig. 3, as a proof of concept, we describe the same variables as in Table 2. These variables, or questions posed to the participants in the qualitative assessment, are dissected discretely and ubiquitously for all items of the self-report measures. For instance, in the APZ scale the (1) top experimental group (0.60) reported that “Sounds and noises seemed different from what they usually are”; while (2), the top control group, and (3), the bottom experimental group, did not report this (0.91); while (4), the bottom control group, did not consider the question at all (0).

On the SHS scale, the (1) top experimental group appeared frequently against the statement: “Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?”. This was less true of (2), the top control group; even less so of (3), the bottom experimental group; and only moderately true of (4), the bottom control group.

On the MAAS scale, (1), the top experimental group, somewhat infrequently ” … found it difficult to stay focused on what's happening in the present”, while for (2), the top control group, this was almost never reported; for (3), the bottom experimental group, somewhat frequently reported; and for (4), the bottom control group, this was infrequently reported.

On the SCS-R scale, (1), the top experimental group, report that “daydreaming about themselves” pertains greatly to them; while (2), the top control group, report that the statement pertains only a little to them; (3), the bottom experimental group, report that the statement somewhat pertains to them; and (4), the bottom control group, reported that the statement does not pertain to them at all.

The intervention with a guided meditation and the qualitative assessment with the four psychological scales, allowed us to:

[3] Forthcoming by the authors and collaborators.

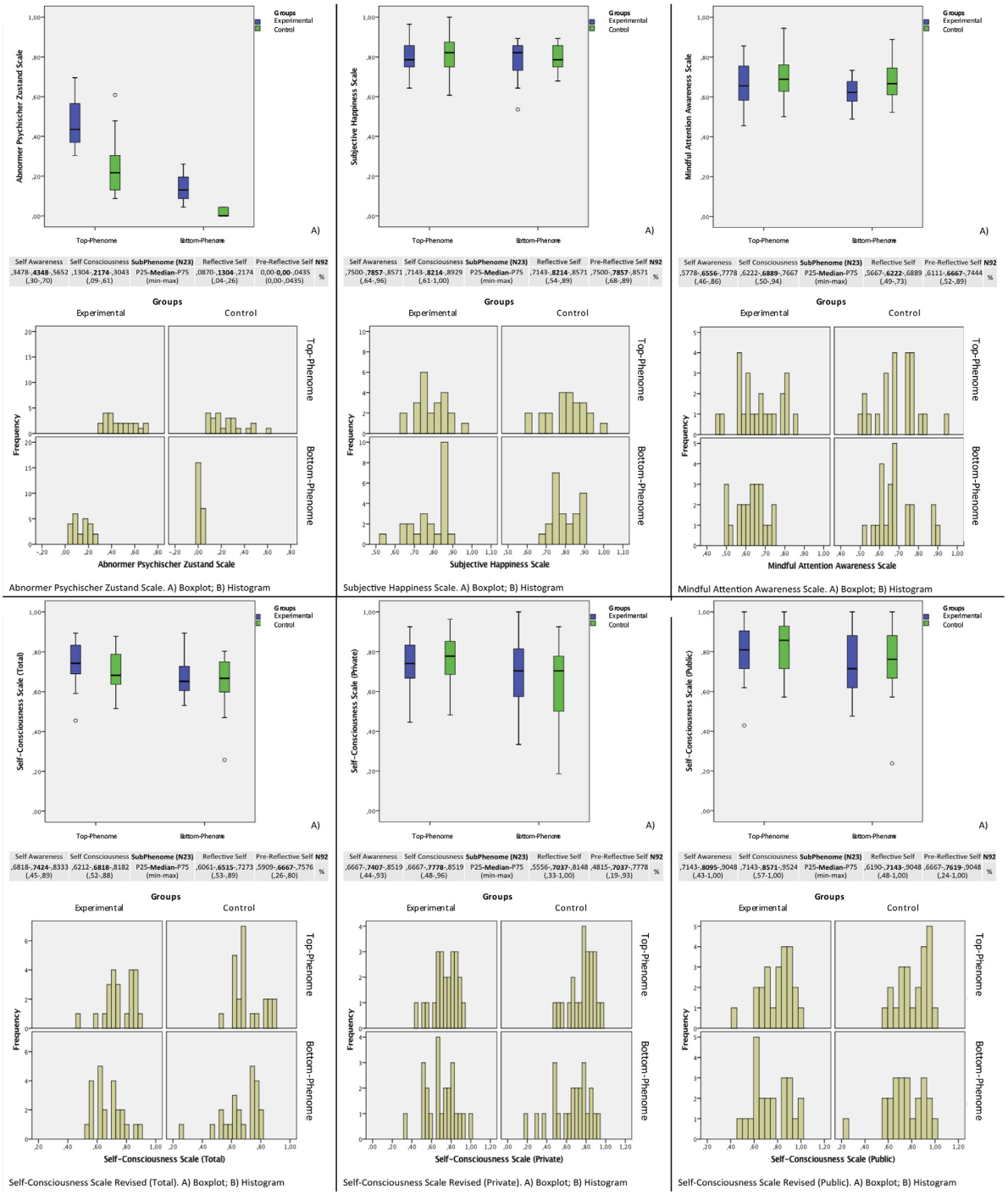


Fig. 2. Qualitative performance control vs. experimental groups.

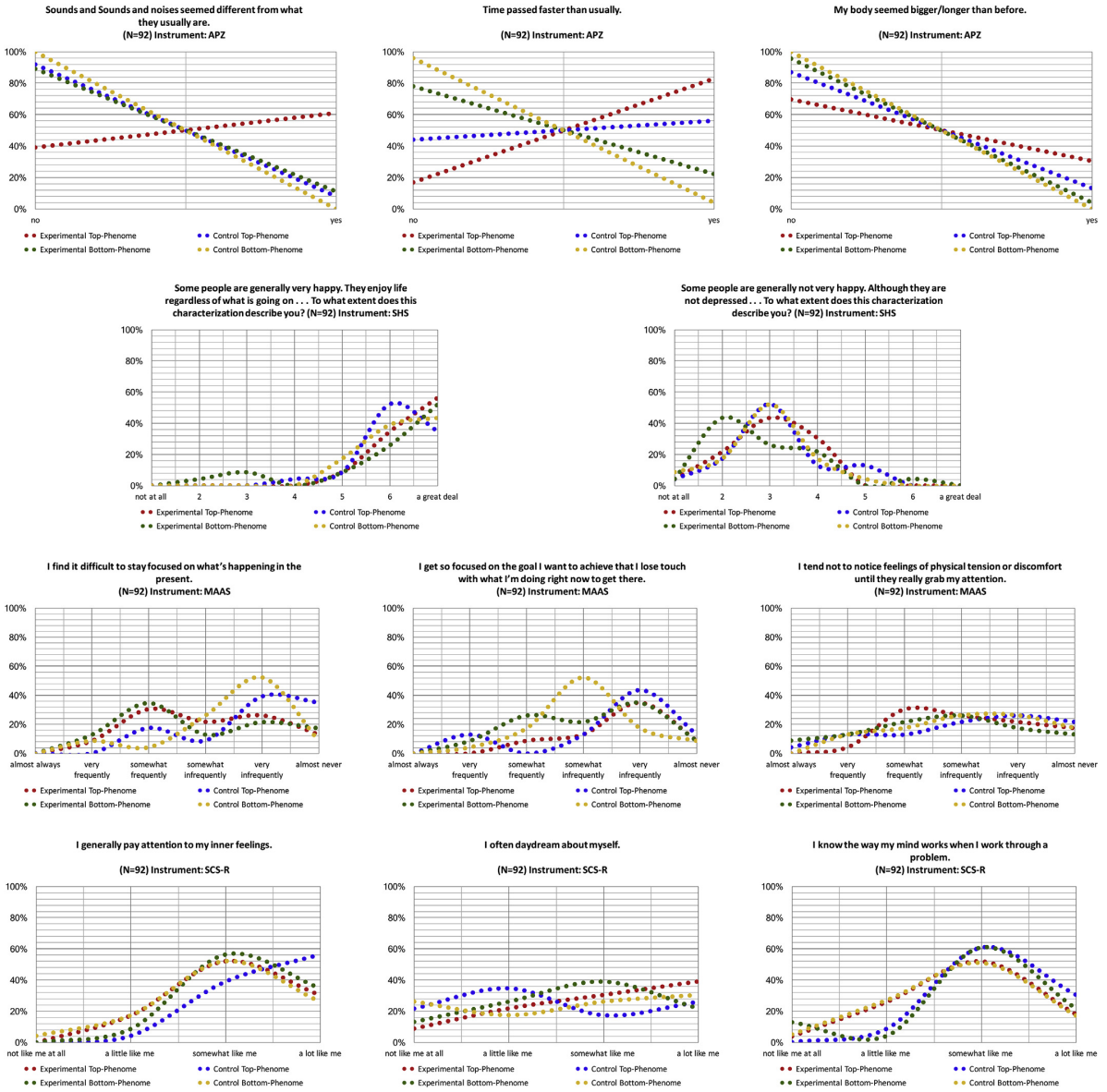


Fig. 3. Comparative results of the phenomenological reports.

(1) Identify experiential profiles based on the subjects' evaluations of their own conscious experience, and
(2) Clinically and phenomenologically understand the groups, and their subtypes, to motivate the investigation of protein correspondence (i.e., quantifying the differences in their proteomic profiles).

The model presented here bears resemblance to the neurophenomenological method, which proposes to introduce into brain research on subjectivity and consciousness first-personal methods of examining experience. Our method, however, constitutes a variation insofar as the quantitative part of the study does not consist in a brain-centered methodology. Another point of difference regards the qualitative evaluation: our participants are not trained in the phenomenological method, that is, they complete the intervention naively. Our quantitative method instead consists in a functional protein profile (proteomics), that is, dynamic protein information that is distributed along the organism itself.

The aim of this study is the understanding of molecular interactions induced by an intervention protocol. We focus on the whole: the proteome, referring to the entire set of proteins produced or modified by an organism or system. Commonly, there are two biochemical methodologies used to study a biological system: a) directed studies (classical biochemical studies), and b) enlarged studies (the omics biochemical studies). This work is not intended as a directed analysis into a specific or exact candidate neural

pathway or phosphorylation process. The sensibility and specificity necessary to conduct such a study is virtually impossible. Rather, we propose a study with a different magnitude, i.e. an enlarged, non-directed perspective, or an omics study. We intend to probe physical interactions between molecules, as an example of "top-down" systems biology, taking an overhead—as well as overall—view of a biosystem (Bruggeman and Westerhoff, 2007; Kiemer and Cesareni, 2007). The work is proposed as a systems biology paradigm, usually defined by contrast to a reductionist biological organization. As Denis (2006) notes, "Systems biology […] is about putting together rather than taking apart". As a method, omics comprises high-throughput analytic techniques (Romualdi et al., 2009). These techniques include, for example, transcriptomics, metabolomics or proteomics, to propose specific testable hypotheses (Alberghina and Westerhoff, 2005); for instance, the result of an experimental induction. Normally, the output of an omics study is a multi-database generation (Baitaluk, 2009). Specifically, in future publications, it will be generated layered results of total proteins profiles, simultaneous immunedetections and quantifications and in-silico characterization, using bioinformatics tools, like cytoscape® and panther®. Top-down systems biology like these could in principle identify molecular interaction networks (Bruggeman et al., 2007). Although there are still today explanatory limitations, the interaction between molecular networks can be correlated to observed behavior of subjective experience, evaluated through self-report measures. Explanatory limitations include, for instance, the incapability of connecting a specific neural pathway to a specific self-reported behavior. Indeed, such an explanatory error resides in the inability to rule out preliminary reasoning, suggesting that such behavioral-molecular correlations were present in the first place.

Subjective experience has been studied as the macroscopic cartography of synaptic connections in a macro-network, but could also be probed on an intra-cellular scale: a micro-network. Those neural networks are regulated upwards or downwards via intracellular regulatory networks of the transcriptome interfaces (Foster, 2015). Insufficient findings describe specific molecular networks that can be elicited through inducting a specific subjective experience. This scientific gap motivates setting up an omics approach. The object of this study is the interplay between mind, body, environment and behavioral characteristics. In specific, this study concerns how subjective experience modulates objective biological measurements (Ulvestad, 2012). Omics methodologies have been recently gathering a complex and extensive quantity of data enabling biomedical sciences to assimilate and define a systems biology framework. Methodologies such as the one proposed by Ritchie and colleagues (2015), assesses biological systems via multi-omics, i.e., from transcriptome to phenome. Omics studies began to characterize different biological substrates. Nevertheless, such studies have only recently looked into neuronal structures (English et al., 2012), giving access to solid neuroproteomics databases (Collins et al., 2006; Behan et al., 2009; Hahn et al., 2009; Ishii et al., 2009; Bayés et al., 2011). We propose that omics studies advance towards insights into the biomolecular underpinnings of subjective experience, for instance, the physiological mechanisms involved in strong emotional states (Chiappelli et al., 2006). Such an advancement in omics studies would offer a neuroscientific approach to the *hard problem of consciousness* (Susan et al., 2005). Nevertheless, no publication yet has used analytical proteomic techniques to evaluate physiological states resulting from the expression of self-conscious experience (Martins et al., 2016). Indeed, the difficulty surrounding such research consists in the fact that self-conscious experience varies both with time, and with the amount of environmental stress that an organism undergoes.

4. Conclusion

It is a standard of modern Western scientific physiology to study natural life by quantifying and measuring the correlation between behavior and brain function. On our model, however, we focused on the *qualitative* experience of a meditative state, centering our method within a fruitful and mutually informative dialogue between Western objective (reproducible) experiments, and Eastern subjective analysis. What makes our research innovative is its starting-point in qualitative assessment, which subsequently directs the interpretation of quantitative measurements of proteomics.

We found substantial differences between the control and the experimental group, allowing us to conclude that the experimental group was able to identify, describe, and report a subjective experience intrinsic to *Jhana* meditation. The intervention with *Jhana* meditation and the qualitative assessment, implementing the four psychological scales, allowed us to: (1) distinguish experiential profiles based on the subjects' evaluations of their own conscious experience; and (2) define a clinical subtyping strategy. We identified, in the experimental group, two sub-groups: the higher and the lower tercile. In the control group, we also found two subgroups: the higher and the lower tercile. These four tercile groups will be further compared with the protein variations.

The study focused on the first-person experience associated with cognitive and mental events. Cognitive processes appear relevant and manifested for the subject that can provide an account. We explored the first-person account because the familiarity we have with subjective life must give way in favour of the careful examination of what it is that we can and cannot have access to, and how this distinction is not rigid but variable. This first-person methodology appears as crucial because without a sustained examination we actually do not produce phenomenal descriptions that are rich and subtly interconnected enough in comparison with third-person accounts, that is, quantitative measurements (Shear and Varela, 1999). To our knowledge, *Jhana* meditation has not yet been studied on a first-person account. We aim in the future to complement this study with synamic protein information (proteomics). This physiological approach, measured by changes in functional protein networks, might prove to be a promising framework for objective, enactive research design. "Enaction", as conceived by Thompson and Stapleton (2009), grounds subjective experience in macro-micro network interactions. A follow-up study, to include molecular descriptions, will supplement the psychological analysis of subjective phenomena as presented here. Namely, the acquisition of multi-omics data of the subjective experience of *jhana* meditation. The first-person account characterizes and stratifies the sample into 4 distinct groups, by multiple comparisons of the psychological scales variables, with a good internal consistency and reliability. Afterwards, the expected total protein profile (t0) of the 4 groups/subphenomes, characterized by capillary electrophoresis, should be discrete. Then, the total protein profile difference (t1-t0) of the 4 groups/subphenomes should show a constant variability in specific proteomic bands, e.g. inflammatory molecular weight range [17 kDa-33 kDa], characterized by capillary electrophoresis. Henceforward, *in silico* analysis, cytoscapeTM and pantherTM, will generate functional protein networks, of the different 4 groups of the precise biological processes, e.g. inflammation, of the neuronal-oral database created with Uniprot® referencing. After accomplishing this tasks, a simultaneous immunedetection multiplexed analysis of the Th17 cytokine and neuropeptides, e.g. substance-p, oxytocin, panel separates and quantifies the neuroinflammatory and neuropeptide molecular response of the 4 distinct groups, and the individual neurophysiological response. Finnaly, a graphical user interface toolbox will

provide exploration, visualization and integration of the different datasets, with several datamining algorithms e.g. expectation-maximization and principal component analysis.

In our study, we adopted a methodology that can provide an open link to objective, empirically based description. The overall results of the qualitative, first-person, study should move towards an integrated or global perspective on mind where neither experience nor external mechanisms have the final word (Shear and Varela, 1999). The mind-life perspective requires a reciprocal influence and determination of first- and third-person accounts. In this paper, we studied phenomenal data as a crucial fact for the domain of living beings, which, we expect, can provide the ground for a subsequent third-person study.

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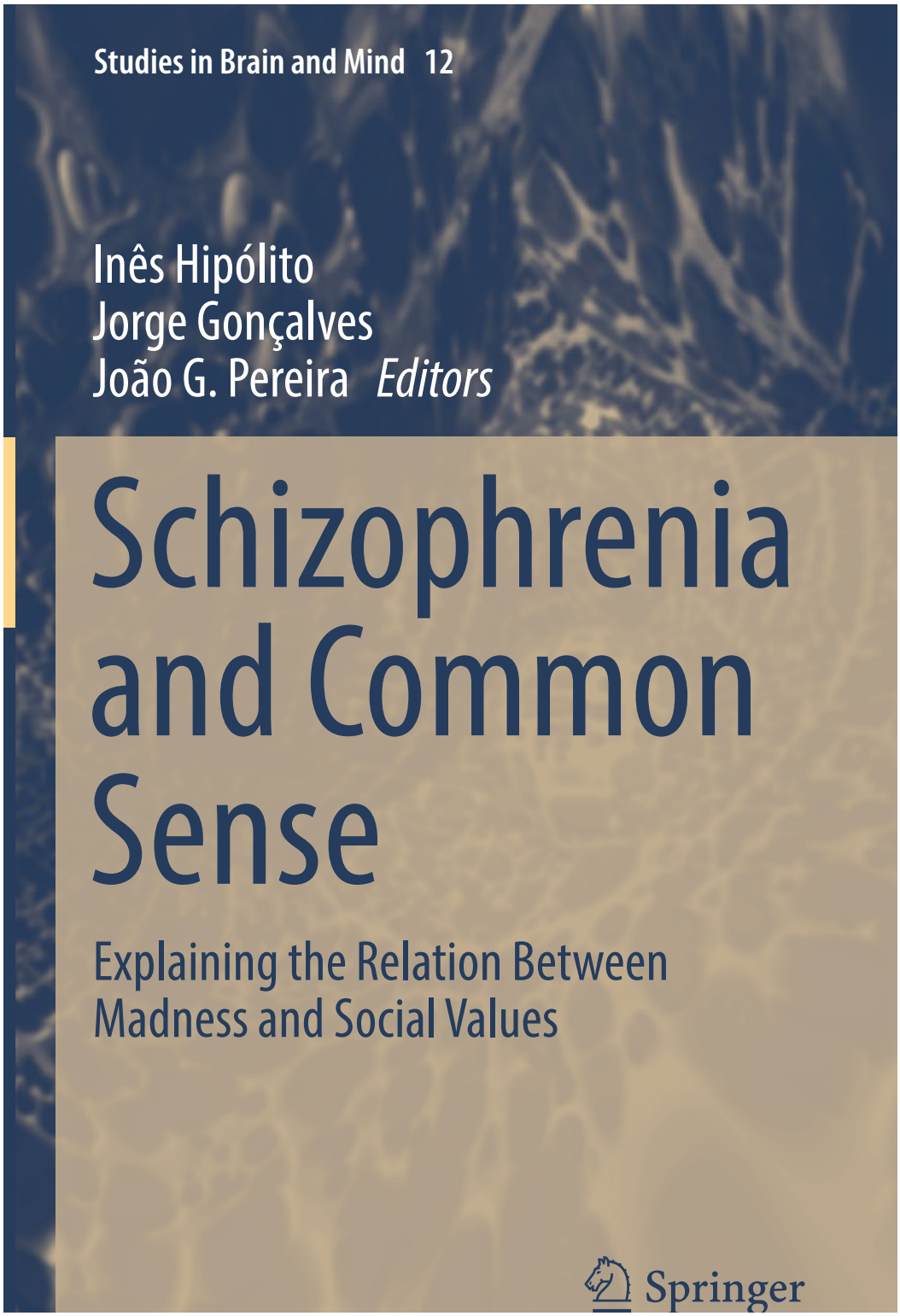
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Annex B. ***A "Second-Person" Model To Anomalous Social Cognition***

Book Chapter Review; *Facsimile;* As co-first author
Published as main doctorate achievement



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Volume 12

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Chapter 4
A "Second-Person" Model to Anomalous Social Cognition



Inês Hipólito and Jorge Martins

Abstract Reports of patients with schizophrenia show a fragmented and anomalous subjective experience. This pathological subjective experience, we suggest, can be related to the fact that disembodiment inhibits the possibility of intersubjective experience, and more importantly of common sense. In this paper, we ask how to investigate the anomalous experience both from qualitative and quantitative viewpoints. To our knowledge, few studies have focused on a clinical combination of both first- phenomenological assessment and third-person biological methods, especially for Schizophrenia, or ASD therapeutics and diagnosis. We will thus attempt to bring forward a second-person scientific design, accounting for both the first-person subjective experiential aspects, and respective third-person neurobiological correlates of embodied aesthetics in anomalous experience. From this proposal, we further explore the consequences to clinical and research practice.

4.1 Introduction

Until recently, schizophrenia and autism spectrum disorder (ASD) were considered distinct nosologic entities, with different objective common underlying mechanisms (Kästner et al., 2015). Emerging studies suggest that there are both clinical and biological links between schizophrenia and ASD. The question regarding whether there is phenotypic overlap or comorbidilty between the two, dates back to 1943, when Kanner first used the term "autism" to describe egocentricism. The distinction

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between the two remained unclear for almost 30 years, until DSM-II included children with ASD under the diagnostic umbrella of schizophrenia. Specifically, under the DSM-5 (APA, 2013), ASD is characterized by persistent and significant deficits in social communication and interaction as well as motor, behavioural and cognitive mannerisms; and diminished or abnormal social interaction abilities (Hipólito, 2014).

Although a clear distinction between the two, schizophrenia and ASD, is nosologically and methodologically important, current emerging studies likewise indicate the genetic similarities between the two disorders (Ellis et al., 2016). Additionally on a cognitive and behavioural level, both disorders show parallels concerning a broad difficulty with social interaction, particularly in the identification of emotion in others (for ASD see Bruggink, Huisman, Vuijk, Kraaij, & Garnefski, 2016; Montgomery et al., 2016. For schizophrenia see Green, Horan, & Lee, 2015; Weisgerber et al., 2015); in the recognition of social cues in general (see for Schizophrenia, Nikolaides et al., 2016; White, Borgan, Ralley, & Shergill, 2016. For ASD see Hipólito, 2014; Hoffmann, Brück, Kreifelts, Ethofer, & Wildgruber, 2016), which has also been correlated with oxytocin receptors (Andari, Richard, Leboyer, & Sirigu, 2016; Guastella & Hickie, 2016; Preckel, Kanske, Singer, Paulus, & Krach, 2016).

On a phenomenological level, literature in this volume noticeably evidences the loss of *common sense*, and disembodiment (see Bizzarri, this volume), as clinical symptoms in schizophrenia (see also, Hipólito, 2016; Nelson, Sass, & Parnas, 2016; Parnas & Henriksen, 2016; Stanghellini, 2000), and in autism (Baron-Cohen, 1997). The just stated genetic, cognitive, behavioural and phenomenological similarities between the two disorders seem, thus, to indicate a shared nosology. If this assumption is correct, we suggest the possible application of a model that aims to understand and to clinically improve the mechanisms underlying *common sense*, both in schizoid and autistic disorders.

4.2 Sense-Making and Molecular Expression

There is a rich literature of first-person schizophrenia reports stating how fragmented perceptual experience is. This may be related to the fact that a disembodiment experience inhibits the *shared intersubjective experience*, and concordantly, *common sense*. So, we propose an experimental model that combines first-person assessments - phenomenological -, and third-person correlates with molecular biology.

On a phenomenological level, the practice of common sense can be related to *sense-making* in the enactive theory. Enactive theory or *enactivism* argues that cognition arises through a dynamic interaction between an acting organism and its environment. It claims that our environment is one which we selectively create through our capacities to interact with the world. In this theoretical framework, the organism

does not passively receive information from its environments, but it participates in the generation of meaning. This theory has been introduced by Francisco Varela, Evan Thompson and Eleanor Rosch, to emphasize the idea that the mind is an enactment of a world.

Accordingly, for Di Paolo and Thompson (2014), sense-making starts with a body, as it is a bodily process of adaptive self-regulation and a constitutive body-cognition link. To be a sense-maker is, among other things, to be engaged in an autonomous on-going social interaction (De Jaegher, Di Paolo, & Adolphs, 2016), which can arise between two or more autonomous agents who are mutually adapting their dynamical coupling. This dynamical coupling usually involves autonomous (De Jaegher & Di Paolo, 2007) and engaged perceptual systems, sensorimotor neuronal structures, and physiological processes in a self-maintaining and self-sustaining organization. In summary, sense-making plays out and happens through the embodiment and situatedness of the cognitive agent: her ways of moving and perceiving, her affect and emotions, and the context in which she finds herself, all determining the intersignificance set by her and the environment. This social significance is of furthermost importance when understanding Schizotypal and autistic disorders, as we shall discuss further on.

The enactive theory has been further developed by Hanne De Jeaegher and Ezequiel Di Paolo, who put forward the hypothesis of *participatory sense-making*. Individuals coordinate their movements and utterances in social encounter, co-generating meaning, while transforming both the on-going interaction process and the individuals engaged in it. Thus, sense-making is a participatory, in the sense that it defined as joint sense-making, which allows social encounter and intersubjective understanding. In social understanding, the body, the interaction processes and the interpersonal experience play a crucial role. Hence, they call for a method that studies the embodied and the interactive aspects of social understanding as the instrument *per se* to yield efficient scientific data (De Jaegher, 2016).

A method to study impaired aspects of intersubjectivity, such as schizophrenia and ASD, should bare in mind that bodily experience of interacting allows a way to address intersubjectivity as a form of not only understanding each other, but also the world together (Reddy, Williams, Costantini, & Lan, 2010). Such a meaningful engagement does not mean the co-existence and mutual necessity of various *first-person perspectives*. In fact, it does mean that these perspectives are, thus, co-created among subjects, in virtue of an autonomy that shapes not only on-going subjective experience, but also their participants (De Jaegher & Di Paolo, 2007; Di Paolo, 2015).

Autonomy plays a key characteristic of the described intersubjective meaningful engagement: the embodied, interactive coordination of sense-making (De Jaegher & Di Paolo, 2007). This concept should, however, be further clarified with the understanding of how it modulates social understanding and cognition (De Jaegher, 2013, 2015; Pfeiffer et al., 2014), specifically in ASD. For that matter, development psychology has illustrated how infants move and participate affectively and intentionally with their mothers, and therefore how they make sense of their interaction

(Bateson, 1979; Bullowa, 1979; Delafield-Butt and Trevarthen, 2015; Hobson, Patrick, Crandell, García Pérez, & Lee, 2004; Malloch & Trevarthen, 2009; Stern, Beebe, Jaffe, & Bennett, 1977; Stern, Hofer, Haft, & Dore, 1985; Trevarthen, 1977). Nevertheless, research still needs to consider a clear, careful phenomenological description of *what it feels like* to connect with others and the great difference it makes to interpersonal understanding, particularly in ASD (De Jaegher, Di Paolo, & Adolphs, 2016; De Jaegher, Pieper, Clénin, & Fuchs, 2017; Reddy et al., 2010).

4.3 Neurophenomenological Method

A better grasp of social understanding, if this theory is correct, would thus yield insights on individual experience, practical bodily engagement, and can be accomplished by a neurophenomenological method (Depraz, Varela, & Vermersch, 2003). The most significant work on this method has been rigorously done by Varela (1996), Varela and Shear (1999), Shusterman (2008), Gendlin (1962), Stern (2004), Van Manen (1990), Ihde (2012), and Petitmengin and Bitbol (2009). These phenomenological approaches preserve the subjective lived experience as crucial for understanding the world, and our social and cultural practices, as sense-making beings (Thompson, 2005). Moreover, *participatory sense-making*, i.e. the intersubjective lived experience, is defined as a mutual circulation between cognitive science and phenomenology (Gallagher, 1997; Varela, 1996; Varela, Maturana, & Uribe, 1991), and naturalized by Roy, Petitot, Pachoud, and Varela (1999), which proves its relevance in ASD and Schizophrenia research.

As outlined in the introduction, Schizotypal and autistic disorders are currently described in literature as involving an impairment of sociocognitive function. We proposed to offer a new perspective on a model that, consonantly with De Jaegher (2016), assesses the phenomenal experience. In these disorders, *common sense* seems to be disrupted. In this section, we suggested that *participatory sense-making*, could provide a suitable background for the way individuals enact intersubjectivity. At this point, the question that still remains to be answered is how, then, to improve *common sense* in patients suffering from schizophrenia or ASD. We consider that this first-person phenomenological background and method could ideally be completed by a third-person biological measurement. In biological terms, there may be a possible relation between protein expression (Hipólito and Martins, 2017), possibly of interleukins - a group of secreted proteins and signal molecules - and the inhibition of common sense. It has been shown by Kolodner and Guzdial (1996) that cognitive load stabilization promotes sense-making. Likewise, for van Bruggen, Kirschner, and Jochems (2002), interactive activity with others reduces extraneous cognitive load, which may be essential when the environment demands heavy cognitive resources, especially when with cognitive impairment like ASD and Schizophrenia. According to these empirical results, it seems reasonable to consider that the practice of *common sense* may have a neuroendocrine-immune expression.

4.4 Second-Person Model[1]

Third-person studies are usually denoted as quantitative methods. They are applied by neuroscience, mostly on the sensorimotor studies, and by cognitive psychology, essentially focusing on the study of cognitive functions, such as language, memory, attention, etc., all of which belong to the object of study which has been called the *easy problem* (Chalmers, 1995). The methodological techniques are usually brain-centred and include imaging, time-response and button-pushes. An alternative *third-person approach* is molecular biological method, which usually tend to look for biomarkers - an indicator of some biological state, usually measured to examine normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention - which are bodily distributed network of proteins. Although, the identification of clinically useful biomarker tests still remains very challenging, there are already tentative studies for ASD, which include genetic, biochemical, neuropsychological, neurophysiological and neuroimaging *third-person measurements*. However, in psychopathological research there are crucial difficulties when advancing with genetic, proteomic and environmental contributions and their association with the cognitive phenotypic heterogeneity (Anderson, 2015), and it was not possible until today to find its possible biomarkers. Omic research, - which involves large number of analytes and observations, with transcritomics, proteomics, and metabolomics (Dudley, Häßler, & Thome, 2011; Maurer, 2012), - is a potential network perspective that potentially brings innovation to the *third-person method,* particularly because of their concern with both neurodevelopmental traits and neuroinflammatory states (Onore, Careaga, & Ashwood, 2012). A transcriptomic-proteomic approach has the unique characteristic of being a non-directed enlarged study, which may shed light on the potential protein networks involved in ASD. Moreover, a salivary sampling method, shown to be better than drawing blood (Lim, Garssen, & Sandalova, 2016), has the characteristics of (i) being an ubiquitous biological substrate (Lim et al., 2016); (ii) being easily collected noninvasively (Lawrence, 2002; Rosa et al., 2016) and, thereafter, not interfering with the cognitive state shared in an aesthetic *second-person* model of autism ASD; and (iii) being an important research tool (Chiappin, Antonelli, Gatti, & Elio, 2007) for clinical screening and/or diagnostic (Malamud, 2011).

Indeed, as an example of this approach, Arrais et al. (2013) already identified and annotated, in silico, i.e. in a bioinformatics tool OralCard, salivary proteins associated with ASD, like the eukaryotic translation initiation factor 4E (Gkogkas et al., 2013), analysed by combined proteomic techniques (Chi et al., 2009).

Quite the reverse are the *first-person methods*, which attempt to assess what is beyond the cognitive function, that is, the phenomenal experience and self-consciousness, and all the phenomenon under the scope of the *hard problem* of

[1] Note our notion is distinct from Varela's *second-person method*, which has merits to its own credit.

consciousness (Chalmers, 1995). These methods are usually applied by phenomenology, which aims to assess the qualities of the experiential phenomena.

In this paper we would like to suggest, as an experimental method, a combination of *first-person assessments* with *third-person method;* what could be called a *second-person model*.

The first-person perspective of subjective experience is a valuable measuring tool particularly in psychiatric investigation and clinical practice. Various perspectives and method have been provided (for an exhaustive account see Piccinini, 2009). In psychology and neuroscience, the subjects issuing first-person reports and other sources of first-person data play the epistemic role of (self-) measuring instruments. Data from measuring instruments are public and can be validated by public methods (Piccinini, 2009, p. 2). In other words, first-person data are as public as any other scientific data. Piccinini also notes most of first-person methods generate data from verbal behaviours, but some don't. First-person data need not be based on first-person reports, or even on verbal reports of any kind. For instance, subjects may be asked to express their level of happiness (or pain) by pointing at images of smiling vs. frowning faces. "For this reason, the term "first-person report"—which is often used in this context—is too restrictive to capture all sources of first-person data. Instead, I will use "first-person behaviour" to denote any behaviour that is a source of first-person data (p. 2).

Daniel Dennett appears to assimilate Piccinini's proposal into his "heterophenomneology" (see Dennett, 2017), which consists in applying the scientific method with anthropological bent. In other words, Dennett suggests combining self-reports with all other available evidence to determine the mental state and discover how the subject sees the world. Although Dennett's view takes first-person data to be public, Dennett claims that (i) scientists should be agnostic about the truth value of first-person reports and (ii) they should interpret all first-person reports as expressions of beliefs (e. g., Dennett 2003). This means his view is that the primary explananda of a science of consciousness are the first-person behaviours, contrary to Piccinini's view, who considers that the primary explananda are the mental phenomena.

On the first-person level, phenomenology has proven to be an important tool to address intersubjective experience (De Jaegher Di Paolo, et al. 2016; De Jaegher, Pieper, et al., 2016). In contemporary research, cognitive scientists increasingly use the term 'phenomenology' to designate a first-person description of 'what it is like' of experience. The phenomenological method offers an indispensable scientific alternative, as a descriptive truly scientific study of the mind (Husserl, 2012, p. 102; Gallagher & Zahavi, 2013). The phenomenological method is not some kind of introspective psychology. Instead, it is concerned with the conditions of possibility of phenomena and appearances, that is, with the description and analysis of the details of the experiential dimension in order to clarify the cognitive contribution of the knowing subject (Gallagher & Zahavi, 2013), and the engagement between social beings and their actions.

Furthermore, Franz Brentano's neo-Aristotelian theory is understood as the science of phenomena, that is, the study of their qualitative properties, and elicited subjective experiences. His method is called experimental phenomenology,[2] and was developed mostly thanks to Carl Stumpf (1873, 1883), who stimulated the experimental investigation culminating in Gestalt psychology, and further developed by Koffka (1935), and Wertheimer (1923). This is known as The Berlin School of experimental psychology, which today is seen with a renewed interest in neuroscience of perception (see; Bagdasaryan & Le Van Quyen, 2013; Jäkel, Singh, Wichmann, & Herzog, 2016; Kovács & Julesz, 1993; Koenderink, 2010; Todorovic, 2011; Wagemans, 2015). In the model here we suggest that on the first-person account, experimental phenomenology (descriptive psychology) might be applied in the schizotypal and autistic disorders, focusing on the qualitative aspects of the phenomenal anomalous experience. From this perspective, the qualities of perception and kinaesthetic phenomena should be a central feature when considering human perceptive experience in general, and Schizophrenia and ASD in particular.

Perception, under experimental phenomenology lens, is not a matter of colour or smells; rather, it is about the relationship between sensations happening in consciousness and how we inhabit the world. The perspective we put forward is very much inspired by the pragmatic view of Wittgenstein (1963, 1984, 1993) and by the phenomenological view of Husserl (1970) and Merleau-Ponty (1962), since perception is a matter of the essential link between our embodied nature and our own mode of being in the world, - both as subjects and as members of intersubjective communities. In short, perception is a practice of perceiver's interactions with the world entailing a substantive commitment about what the perceivers are and what the world is.

A good example of the important clinical features within *first-person* assessment has been shown by, for instance, sensory-motor rhythmic abilities (De Jaegher, 2013, 2015), particularly because they allow for the measurement of social skills through timing and coordination (De Jaegher, 2007), either in a switch between different rhythms or in a co-coordination (McGann & De Jaegher, 2009). Accordingly, the interaction process and its dynamics can be empirically measured by qualitative performance, assessing how subjects (i) make sense of the world by moving around in it and with it; (ii) synchronize movements with others when interacting with them; and (iii) coordinate sense-making activities, affecting not only the world sense-making, but also of others and of themselves.

To our understanding, research on Schizophrenia and ASD therapy should then focus on how patients participate in each other's *sense making*, sharing meaning together and through interaction. Recently, there have been studies addressing these concerns, based particularly on art therapies. Interaction between the autistic patient and the parents and the therapists is vital as a *synch* dance (Trevarthen, Aitken, Vandekerckhove, Delafield-Butt, & Nagy, 2006). This may be understood also in a

[2] There are two main "classical" versions of phenomenology: the Husserlian (transcendental) theory and the experimental (empirical) version of Stumpf, Meinong, Michote, among others.

literal sense. Dance as been described as valuable to fostering sensory integration and regulation, enhancing bodily synchrony and *praxis*, that is, the ability to play and engage in meaningful action within the world (Amos, 2013). In fact, Koehne, Behrends, Fairhurst, and Dziobek (2015) showed that fostering social cognition through an imitation and synchronization-bases dance-movement in adults with ASD improved emotion's inference.

Moreover, Hildebrandt, Koch, & Fuchs (2016) tested dance movement therapy intervention, through mirroring in movement, in adults with ASD and Asperger's syndrome, with the aim of increasing body awareness, social skills, self-other distinction, empathy and well-being. This study showed that participants, after the intervention, improved well-being, body awareness, self-other distinction and social skills.

Likewise, Gavron and Mayseless (2015) explored joint-painting procedure as self-report measures of the quality of mother-child relations. Their results show art therapy treatment to be positive in psychotherapy. Other methods, focused on *second-person* perspective framework, include music therapy, which although already focusing on this, might need to acknowledge the interactive *sense*, concerning social and meaning-making aspects. For instance, improvisational musical approaches have linked the deep continuity between cognition and improvisation (van der Schyff, forthcoming/2017). Moreover, interactive improvisation music therapy for children with ASD may be applied as a possible treatment model (Geretsegger et al., 2015), setting the theoretical basis for an interactive or participatory sense-making therapy.

4.5 Discussion

The majority of current studies on ASD take a psychophysical stance with experiments being carried out using classical *third-person* neuroscientific methodologies, which means they mainly consider the *stimuli* of neuronal activity. This method, however, seems insufficient *per se*, since we also need to analyse and describe qualitative perceptive phenomena in order to understand how meaning-making is processed in its interactive and social manner. Phenomenology constitutes a valid method to precisely address this accounts: the characteristics of other person's subjective experience and intersubjectivity, therefore offering to the experimental scientist indirect evidence about that experience (Lappin, 2013). To our knowledge, few studies have focused on a clinical combination of first- and third-person methods: *second-person* model, especially for Schizophrenia, or ASD therapeutics and diagnosis, which are indeed what we propose here.

To ensure a conceptual continuity between sociocognitive functions of Schizophrenia and ASD, subjective experience and intersubjectivity, and to overcome epistemological difficulties we propose a series of premises to guide research designs:

1. the scientific study of the universals of subjective experience is a paradigmatic case of intersubjectivity;
2. individuals coordinate their movements and utterances in social encounter, co-generating meaning and co-transforming the on-going interaction process;
3. the clinical setting should focus on the enhancement of the shareable meanings (intersubjectivity) between the patient and the medical doctor; aesthetic appreciation could be used as a tool;
4. subjective experience should be brought into focus by the methodological combination of first- (qualitative) and third-person (quantitative) methods, that is, a second-person model.

4.6 Conclusions

Anomalous identification of emotion in others have been studied, in ASD, by Bruggink et al. (2016), who come to the conclusion that cognitive emotion regulation may be purposed as a therapy. Montgomery et al. (2016, p. 1931) suggests that adults with ASD "may need more support, particularly in mentalizing and complex emotion recognition". As noted elsewhere (Hipólito, 2014), ASD patients have difficulties in the recognition of social cues in general, because "autistics' mode of empathy is more rule-driven, and their participation in social and moral scene differs from ordinary subjects. As autistics are unable to grasp human emotions intuitively and pre-reflectively through bodily attunement, they, instead, rely on pure logic (p. 273). While many of us are empathically engaged spontaneously and become immediately attuned to the concerns of others, autistics rely more on rules of conduct and general principles in order to navigate the social landscape. This might be described as a "cold" methodology that engages the interest, to a great extent, in order to bring about the sort of affective, bodily attunement that for ordinary subjects is already there. Ordinarily, this affective, bodily attunement, occurs spontaneously and pre-reflectively via affective framing.

Likewise, Green et al. (2015), revised and identified the same social impaired processes in schizophrenia. Indeed, this lack of social attunement is a condition that is also present in schizophrenic patients. Hipólito (2016) investigates the second-person approach pointing out the importance of experiencing and interacting with others as our primarily way well-being. Hipolito identifies the second-person perspective taking can be used to point out specific features of social cognition and acquisition of knowledge, not only about other person's mental states such as their beliefs, desires and intentions, but also insight about the meaning of their utterings (p. 610).

A second-person method seems crucial, when investigating the difficulty ASD and schizophrenic patients show on the lack of intuitively and intersubjective attunement with others. Hipolito (2016, p. 611) offers the following explanation,

Imagine, for instance, you are studying a patient's brain. In this case, the third-person perspective is helpful because the disease might have a define location in

space that can be assessed by a scanner. In contrast, you apply the first-person perspective when you wonder whether or not a brain scan is enough to understand the nature of the disease. If so, you need to find out how your patient feels, what are his beliefs, desires and intentions. Is he delusional? Hallucinating? Is he scared? Motivated? Irritated or depressed? In assessing our patient, the first-person perspective provides no information because his mental state is different from our own. Similarly, the third-person perspective cannot help because there are no objective facts upon which to access the person's thoughts and feelings. Therefore, to infer our patient's feelings is a paradigmatic case of social cognition. For it is not about one's own mental states; it is not just about facts.

From this remark, Hipolito (2016, p. 612) concludes that,

> Because (…) [n]ot all social encounters demand a third-person perspective, in fact, the second-person perspective seems to be the most accurate from a biological point of view, either to the online and offline intersubjectivity . . . empirical research should make a significant distinction, both between the first-person, third-person or the second-person approach.

Hipólito & Martins, 2017 also provide an account of the second-person method as a combination of qualitative analysis of the subjective experience and quantitative measurements of molecular protein networks. The authors focus the study in phenomenological data that will further be correlated with the protein profile for the subjective experience. The overall results, the authors claim, should move towards an integrated or global perspective on mind, where neither do experience (First-person person) nor do external mechanisms (third-person person) have the final word. It seems hence appropriate to use methods that focus on the intersubjective experience, that is, the interpersonal processes involved.The following conclusions can be here drawn: (a) shareable meanings generated within subjective experience should be studied in a *second-person perspective;* (b) qualities of perception and kinaesthetic phenomena are central features when considering human perceptive experience in general, and ASD in particular; (c) phenomenological approaches preserve the subjective lived experience, which is crucial, as sense-making beings.

We claimed a scientific study on impaired social cognition and subjective experience should combine *first-* and *third-person perspectives*. This combination could be achieved by a *second-person* method, accounting for subjective, mental, experiential aspects and biological and neuronal ones. We therefore proposed that the phenomenological *first-person study* could be followed by a *third-person* method that uses proteomics and salivary samples as *third-person* measuring tool.

In this paper our major concern was to address phenomenological experience of non-sense as it is *first-personally* reported in literature by psychotic spectrum patients: schizophrenia and ASD. We discussed and attempted to understand how would it be possible to ease and conduct the therapeutic and epistemological process from *nonsense* to common sense. Thus, we presented a potential experimental design consisting in a *second-person* model. We proposed artwork and experiential aesthetics as an important tool to the diagnosis and therapeutics of ASD, particularly because it focuses on the qualities of phenomenal experience to assess the

shared vehicle of intersubjective meaning. With this methodological procedure, we expect to contribute to the current methodological debate with a model that directly approaches schizophrenia and ASD anomalous qualitative and subjective experiential aspects, and objective biological accounts, as a major concern to its diagnosis and therapeutic.

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Annex C. *Happiness As A Self State And Trait Of Consciousness: Saliva Molecular Biomarkers - A Brief Revision*

Pilot Study; *Facsimile;* as first author
Published with PhD supervisors as main doctorate achievement

Short Communication

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Happiness as a self state and trait of consciousness: clinical hypnosis and saliva molecular biomarkers - a brief revision

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Abstract

The relationship between consciousness and neural activity, i.e., between mental and brain states, has been widely studied. However, the relation between consciousness and its biochemical markers is still undetermined. Altered states of consciousness (ASC), can be a learning process for the achievement of subjective states of happiness. Clinical hypnosis and guided meditation are fast learning techniques, leading to the desired subjective state. This subjective state of happiness has saliva molecular biomarkers and can be correlated with psychological assessments. Therefore, it is proposed a new approach of altered state of consciousness induction, associated with subjective states of happiness, using a specific methodology and analysis, named The Pursuit of Happiness model. This model: (1) Studies the use of a hypnotic induction technique, named The Way of Happiness, to achieve states and traits of consciousness associated happiness. (2) Proposes, in parallel, a biomedical analysis named: States and Traits of Consciousness: Saliva Molecular Biomarkers. (3) Correlates the objective saliva molecular biomarkers with the subjective psychological assessments. (4) Develops a Knowledge Discovery Database with data-mining algorithms and bioinformatics tools to discover patterns that relates ASC, subjective states of happiness, saliva molecular biomarkers, peak experiences and self-consciousness.

Keywords

Consciousness, happiness, neuroproteomics, data mining, bioinformatics

INTRODUCTION

Consciousness can be defined as the immediate perception that the subject has of what is happening inside or outside of him, including the knowledge of his thoughts, feelings and actions. Consciousness is understood as the momentary reality that the individual experiences according to his perception and self-consciousness [1,2] and correlates with the concept of experience described in Nietzsche's philosophy, with triple meaning of immediacy, significance and incommensurability [3].

The concept of ASC, defined as 'a qualitative alteration in the overall pattern of mental functioning, such that the experiencer feels his consciousness is radically different from the way it functions ordinarily' [4], may be understood as common ground among neuroscience, medicine and the science of consciousness.

ASC have been used in medicine as an alternative mechanism for the acquisition and integration of knowledge [5]. Both natural and induced have been described as ways to reach an altered state of consciousness.

The natural one corresponds to meditative practices, in which the learning process for the achievement of that ASC is usually long.

The induction techniques are faster, as they always involve a second individual, in addition to the subject of the practice. That individual leads the induction and facilitates the achievement of a desired ASC.

Meditative practices have been linked to the induction of ASC and to neuroplasticity, possibly leading to new brain correlates [6]. In fact, they induce long-term changes in self-consciousness, observed both in high and low level cortical representations [7].

The neurophysiological changes induced by meditative practices are of two types: (1) occurring during meditative practices - state changes, and (2) accumulating over months or years and persisting even when not on meditative practices - i.e., trait changes [8]. Therefore, also ASC can be seen as state and trait of consciousness.

One way of attaining the proposed state and trait of consciousness, is the mindfulness meditative practice. Mindfulness is described as sustainable awareness aimed at a non-reactive consciousness [8, 9, 10]. This promotes physical and mental well-being with positive emotional traits [11], and modulates the limbic-neocortical emotional systems connectivity [12], therefore the need to integrate their principles in medicine [13].

Meditative practices and induction techniques, e.g. clinical hypnosis, [14, 15], respectively natural and inductive technique, have been linked to the attainment of ASC, leading to neuropsychophysiological and anatomical correlates [16, 17, 18]. These are well studied, and have been recognized as an effective clinical tool [19]. These techniques are also a vehicle for inducing peak experiences, associated with subjective states of happiness [16,

20] and described as "flow state" [21]. Moreover, induction techniques, are used as first-line therapeutic practices of acute and chronic syndromes [22].

Few findings describe specific biomarkers that can be elicited through ASC, showing some relationship with psycho-neuro-immune-endocrine models [19]. Moreover, different ways of reaching, perpetuating and intensifying subjective states of happiness, through induced ASC, should be scientifically approached, because they have been rarely associated with biochemical markers.

In fact, ASC and the induction of subjective states of happiness and peak experiences have been only extensively and specifically associated to endogenous dimethyltryptamine pathway [23, 24]. At this point it is postulated that these ASC, natural or induced, may be associated to the sense of self-consciousness, and used to promote subjective states of happiness, and ultimately peak experiences.

Consciousness Evaluation

Diagnosis using neuroimaging and electrophysiological tools in search for objective markers of consciousness is being employed. However, such tools cannot be considered as diagnostic per se, but as assistants to the clinical evaluation, which, at present, remains the gold standard. As well as the Glasgow Coma Scale and Coma Recovery Scale–Revised remains the gold standard in the behavioral assessment [25].

The fMRI and EEG recordings provide mutually consistent evidence on the neural correlates of ecstatic meditations. The multiplicity of brain states suggests that there may be a vast ASC available through meditation, depending on which brain regions are given awareness and which are inhibited from awareness [16].

Also, different scales have been used to assess consciousness in its different components, (1) being evaluated as an ASC [26], (2) as an induced state and trait of consciousness [27], (3) as a study of self-consciousness [28] or (4) possibly as associated with subjective states of happiness [29]. These different scales may evaluate autobiographic memories of happiness recalled from the extended consciousness (the autobiographic self) of the subject [30].

In this study, the gold standard are the behavioral and psychological assessments, by the application of scales that evaluate the subjective and phenomenological experience, which should contribute to certify that the subjects are in the desired state and trait of consciousness.

The biomedical analysis uses saliva molecular biomarkers, stored in Biobanco-iMM, Lisbon Academic Medical Center, Lisbon, Portugal. The neuroproteomic analysis of the saliva molecular biomarkers is expected to enable the recognition of states and traits of consciousness, if sufficient data is gathered and analyzed with data-mining algorithms and bioinformatics tools. The objective here is to provide an alternative, innovative and non-invasive consciousness assessment in its different components and states, e.g. happiness.

Neuroproteomics of Consciousness and Happiness

The problem is that there is no validated objective "consciousness meter" that can be used as a proof or disproof of consciousness [31]. The real challenge lies in designing tools to allow an objective, scientific measure of subjective report [32].

Indeed, this is already an active area of study [33, 34] and could in principle produce biomarkers of consciousness that allow for reporting the phenomenological experience [35]. Therefore the need to understand thoroughly the neural correlates of consciousness, possibly through salivary molecular biomarkers.

Neuropathophysiologic mechanisms studied with neuroproteomic analysis of Serum S-100, serum NSE, S-100 CSF and NSE CSF [36] are correlated with the prediction of regaining consciousness after global cerebral ischemia [37], and could be seen as biomarker of self-consciousness after cardiopulmonary resuscitation. Other biomarkers obtained through proteomics like MAP-2 can in principle provide insight into the mechanism of neuronal remodeling [38] as well as discriminate between patients with deficits in consciousness [39].

Other example, but linked to physiological mechanism, is the N,N-DMT, obtained with liquid chromatography–tandem mass spectrometry [24]. Biochemical, physiological, and behavioral experiments indicate that N,N-DMT is an endogenous agonist for the sigma-1 receptor [40] or serotonin 5-HT1A/5-HT2A-R receptor [41]. These studies, could uncover the neurologic controversy of ASC and the induction of a state and trait of consciousness associated with happiness and peak experiences [23]. It is already known that optimism and positive emotional states also modify the salivary proteomic biomarker profile [42].

Moreover, using proteomics, it would be possible to monitor the modulatory effects of metabotropic muscarinic receptors, on inhibition of adenylyl cyclase, excitation of phospholipase C, and activation of K+ channels. In turn, such activation would alter the phosphorylation states of intracellular proteins, ion channels, enzymes, and transcription factors, from millisecond to long-term time scales [43].

However, no one yet, seems to have combined proteomic technology with changes in brain states resulting from experience, the environment or how it influences the conscious experience, e.g. happiness or self-consciousness, and therefore the main objective of such study.

In addition, peptides such as encephalins might as well serve as additional parameters for influencing and reflecting baseline states and traits of consciousness, as measured by analytical high-pressure liquid chromatography on peptide-containing fractions of plasma for identification by matrix-assisted laser desorption– ionization–time-of-flight mass spectrometry [43].

Biomarkers that reflect mental state have been identified in saliva. These biomarkers, like amylase, chromogranin A, and cytokines, are known stress markers, but there are few reports demonstrating their relation with a positive mental state [44].

Concluding, the physiological approach through molecular biomarker analysis of saliva might be a genuine and interesting framework for the research of these study internal variables [45].

However, it would be important to bear in mind that this study would show at best an index of a degree of a state and trait and consciousness, not consciousness itself.

Experiments characterizing biological correlates might provide insight into the causal relationship between physical and phenomenological descriptions.

Data Mining and Patterns Recognition

A complete set of neuroproteomic and psychological data is collected in this work to study the (inter subjective) happiness state and trait of consciousness. The resulting amount of information embedded is hence massive.

Only relying on standard methods for analyzing this data base (e.g., assessment of the covariance between variables) might provide sub-optimal results due to the high number of measured quantities and possible non-linear relationships between them.

Data mining solutions are then planned to discover and model patterns that relate ASC, subjective states of happiness, saliva molecular biomarkers, peak experiences and self-consciousness. The development of a user-friendly software environment is also scoped to enable a throughout exploration of the information embedded in the database. Specifically, data visualization techniques shall be adopted for the interactive query of related ASC and physiological relationships. Finally, data classification and regression algorithms based on neural network models are devised for operational applications such as recognizing ASC from the analysis of salivary samples.

CONCLUSION

Summing up there are evidences of innovative and non-invasive techniques of evaluation of states and traits of consciousness, e.g. subjective happiness or self-consciousness. This evaluation combines both phenomenological and subjective data, obtained through psychological assessments and scales, and neuroproteomic and objective data, obtained through salivary sampling.

Data mining solutions are implemented to offer pattern

recognitions to correlate both evaluations: subjective and objective.

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Annex D. *Verbal Protocol Of The Experimental Procedure*

Cognitive behavioral intervention

The script of the single cognitive behavioral intervention is based on the publications of the PhD supervisor (Simões et al., 1998; Simões, 2002; Simões et al., 2013), was initially tested in the pilot study of this dissertation (Martins et al., 2016a) and as the experimental procedure of this work.

The verbal script was kept rigorously in all subjects of the study, (i) to avoid confounding variables specific to the experimental procedure, and (ii) to maintain the repeatability of the intervention and the reliability of the psychological measures.

The 1st phase, the *Arousal Phase* (Britton et al., 2014) is a conditioning procedure (Wager and Atlas, 2015). With the duration of 15 min, it aims to modulate arousal, or the level of consciousness.

The 2nd phase, the *Awareness Phase* (Laureys, Gosseries and Tononi, 2015), is an expectancy procedure (Kirsch et al., 2014). With the duration of 30 min, it aims to modulate awareness, or the content of consciousness.

1. 1st Phase, The *Arousal Phase*

Close your eyes… feel good and quiet with your eyes closed… offer this moment to be with you in full… in silence… In Peace…

Breathe Deeply…

Hold your breath for a few moments…

Let go of the air slowly… and RELAX…

Once again…

Breathe deeply feeling comfortably NICE… and loosen the tensions of your body…

Relax… now Breathe Deeply… and feel comfortably safe…

comfortably Protected… release the air slowly through your nose…

Um!!!

What a pleasurable feeling in becoming aware of the air that enters and nourishes your body.

Your body is all nourished by this clear, comfortably healthy air that oxygenated your body, and emotions.

Feel your body now.

Imagine a LIGHT, your favorite color…

Wrapped by a cloud in the form of a soft cotton filling your body.

Um! Feel this colorful cloud filling your feet.

Become aware of your left foot… relax it.

Become aware of your right foot… relax it.

Now let this soft, colorful cloud fill your legs…

Relax your left leg.

Relax your right leg…

This feeling of relaxation also continues by your belly… Relax your abdomen…

Relax your heart…

Relax your arms… hands… fingers…

Breathe once again deeply…

Feel your body relaxed…

Relax your posterior region… the back… your column… feel filled by this colorful cloud…

Your body is RELAXED, RELAXED.

Take it, take it, light.

How nice to enjoy being LIGHT?

And this colorful cloud continues to increase…

Gently filling your face.

Unwinding all your face.

Your face, your mouth… relax your tongue inside your mouth… relax your eyes… relax your forehead… your nose…

The air comes in with great fluidity now…

Relax your ears…

Um! What a nice feeling of relaxing all your hair…

Gently filled by this little colorful cloud, touching all your head…

Um… that feel comfortably healthy

Comfortably nice…

Comfortably IN PEACE…

IN PEACE.

(PAUSE)

2. 2nd Phase, The *Awareness Phase*

Lightness, delicacy… Well-being is with you now…

You feel so light, so light, so light that you are…

In pleasant situations of your childhood.

It could be a day with your old friends at school… or who knows… your neighbors…

A great joke that amused you so much… and made him smile with joy!

You're in the moment now.

Maybe a day of partying…

A long-awaited party… or maybe a party in your town… or a party at school…

You see nice, fun things going on at this event… it's so good to be there…

Um!

And that person who was always with his family… could be an aunt who liked it so much… who knows a friend of your mother's… or maybe a neighbor who talked about funny things or gave him incredible moments… or some unbelievable gift.

It's a birthday. It may have been yours or a friend's… a birthday party…

Um!

That piece of cake! The sing congratulations… the games with the little friends… the noise of the voices of adults and children as a song…

The birthday table… the sweets… the cake!

Oh! The piece of birthday cake…

What a delight! Was it chocolate? Was it fruit? What was your favorite cake? And present… what's the gift from your childhood you really liked?

What would you like to play with and with whom?

What about the TV? Which cartoons would most like to watch?

And the movies… and the series?

What about school? Do you remember when the day you started reading?

The pleasure of putting the little letters together and understanding what they were saying?

What about the break? What would you like to do at halftime?

What about the school snacks?

What about the teacher you admired the most? What did you think was beautiful at school?

A special poster? A special tree? A room? A corner of the school where he really felt good…

What about your friends at school? What you talked about… I'd be really happy.

And what did you learn? What was the theme of the school you really liked to learn?

What about the study visits? Where was the best? What was interesting there?

And the games… the sing inside the tour bus… so much joy on this journey…

What about your favorite food?

Um! Remember that tasty ice cream that made you grow mouthwatering?

Or that sweet guy who adoptees! He savored every PLEASANT moment of his childhood.

(PAUSE)

What about nature? Have you ever played with the rainwater? Or have you ever showered in the rainwater? And play with the flowers and the insects…

And some pet of yours or a friend… a cat… a dog… a little bird

Oh! And the ladybugs when they appreciate it near us… butterflies…

Anyway… Your favorite toy when you were a kid… and his nickname that made him angry, but that he knew that whoever called him that was because, deep down, he loved him so much.

And when he was sick, and someone took care of him with a lot of pampering… a soap or a special food that day…

And parties like Christmas… Did you believe In Santa Claus?

Did you write him letters? Was there special food on Christmas Day? Family reunion?

What about other important parties in your childhood? Feel all these Positive Memories of your childhood… be there now… let your memories come up…

With images… Sounds… body sensations… Smells…

All! relating his childhood to phases of JOY.

Allow this pleasurable feeling to pack your body and emotions now…

Breathe for a moment and visualize a colorful, beautiful, crisp picture, and represent your childhood! Your HAPPY phase! Keep this happy childhood phase inside you…

And…

Whenever you remember a scene from your childhood… in the HAPPY Phase… will have a sense of well-being… and happiness.

(REPEAT PAUSE 3 TIMES THIS SENTENCE, AND WHISPERING LAST TIME)

And now breathe deeply.

Release the air slowly…

You're a teenager now.

A teenager…

Look at your teenage body…

What was the style of that time? The kind of clothes I'd like to wear…

If you're a man, your favorite pants, the clothes you're cutest with, your sneakers… did you use anything else special?

What you did with your physical appearance to feel better… more beautiful?

And if you're a woman. What did you wear that you liked very much? The necklaces, the earrings, the haircut, and the make-up… the lipstick… the shadow… the special way of feeling like a teenager.

What about the changes in the body? Stop being a girl or a boy and feel like A Man or A Woman?

How did you feel about growing up? And the compliments… He's already a boy. a girl…

What about your friends? What about your class?

When did you start going out alone? Where were you going?

Hanging out with your colleagues? With friends?

Go to the movies? Watch movies on the net?

Games on the net?

And the messages sent?…

And the first kiss or kiss that really missed…

And that special boy… or that special girl in your life.

And the pleasant memories of this phase…

And the school? What was good?

New interests?

And your projects… your desires…

And the parties with the friends of the class… special pranks that just remembering makes you want to laugh… what you had that caught another people's attention… your smile… your look… the way he spoke… their way of dialoguing… what was his strength?… Yes, it had a very special way of being… feel that moment… this moment…

And the songs I spent hours singing… the clips of your songs…

And your dreams awake… thought about being admired by a lot of people? Conquer the love of your life/ have the car of your dreams? Have a wonderful future with your way of being… magical solutions to all problems…

Savor and intensify your imagination in everything that relates to your youth in Happy Moments… generally, they are significant situations for you… and that is nice to remember at this time… get involved in your happy youth phase… see the colors of this phase… the smell… the special people for themselves, the sounds of this era… Experience.

Remember this phase of happy youth now…

And know that every time you remember a positive memory of your Happy Youth… IMMEDIATELY, a sense of well-being, will occur… at any time in his life.

(REPEAT PAUSE 3 TIMES THIS SENTENCE, AND WHISPERING LAST TIME)

Breathe deeply and allow this Phase of your Happy Youth to be within you.

(PAUSE)

Breathe deeply again… feel that you are in a new phase of your life.

It's an adult being.

Go to positive memories of this phase of your life…

Look at the dreams you've managed to fulfill…

Study to a certain level… the effort this has caused you…

Take responsibility… yes, because being an adult is to walk with its objectives with independence and determination.

See your skills and skills… what's really right to do… a drawing, a painting, a dissertation, a poetry, writing…

Your significant achievements in your life…

The conclusion of the bachelor's degree…

What most positively marked this stage of completion of studies…

Your marriage or marriage with someone who believed it could make you happier… Your professional side… his accomplishments in the work that made him happy…

A compliment received… An adult achievement…

A walk… a movie… a book… a scene… The birth of a future son… The acquisition of an object after much idealize so… a car or set up a house… a project carried out.

Real friends… Which makes you feel happy…

The encounter with the family…

A day of well-being with friends…

A party… a diversion…

A wake up after a deeply well-slept night…

The savor of life… a sunset… a walk… a dance… listen to a certain song… feel in PEACE!

See a great friend… The motivation of a special meeting with someone who is affectionately special to him…

Being entirely with you at any given time… the flow with you…

Or if you love… to be pleasantly with your own company… to learn something new… the curiosity of life…

Being in a really relaxing place… can be on a beach, or in a field, on the mountain, or perhaps in a place created only by you.

Savor this place…

Feel… the smell of clean clothes… wet earth smell… the smell of a perfume that is special to him… the smell of the morning in the spring… the smell of your favorite flower…

And colors that make you good… feel your blue… or its yellow… who knows the red… feel your favorite color and well-being that it provides you… or see the objects you admire… a painting, a poem, a photograph, a city… images that provide your well-being…

And your favorite songs… the sound… the melody… the lyrics and musicality…

Your relationship with nature… the time well lived in contact with nature… a beach, a lake, a mountain… a garden…

A moment when he felt that his duty was fulfilled… managed to finish a task or project that wished very much… something simple, like riding a mobile with the instructions or making a recipe for a cake that worked… or even something more elaborate like organizing a meeting, learning a language… computer science… the welfare of overcoming obstacles.

Anyway, rescue your positive memories from being an adult.

Give yourself at this moment with all the visual, synesthetic, auditory, taste… entirely to the pleasure of being an adult…

And every time a reminder of your PHASE OF BEING HAPPY ADULT, IMMEDIATELY a Sense of pleasure and well-being will come to you

(REPEAT PAUSE 3 TIMES THIS SENTENCE, AND WHISPERING LAST TIME)

Breathe deeply…

Now he's a person who already has a good Life Trajectory.

You've experienced a lot of things in life.

What have you learned positively in the latter?

What did you accomplish in the life that really gave you Pleasure?

How can you enjoy your time with greater creativity?

Have you learned it?

What positive challenges do you still want to achieve?

Determine that now on you will be a more positive person about Life.

What do you want to leave as a landmark here on earth with your experience?

Which people really let your heart smile?

What really makes you feel good?

See your life project for everyday life.

Let it be simple, but that it means meaning to you.

Feel really HAPPY that life gives you the happiness of being alive.

Feel light and happy to have continued your trajectory so far… That will continue.

Breathe deeply and experience fully and healthily this phase of maturity in which you are.

And… know that every time you remember this current phase of yours, your Positive Memories of your wisdom will provide you WITH SERENITY and well-being.

Live it deeply…

Breathe deeply… let this well-being inside you.

And… once again, breathe deeply…

Create a picture where you can be represented with Positive Memories of your childhood, adolescence, and adulthood…

This painting is your production… he's very valuable… is a unique example in the world … is your Positive Trajectory of Life.

There is in this picture a pleasant smell, sounds that reach your ears well, images that delight your vision, and a texture that makes you good to the body.

Place this painting in a place that is very special to you.

Now give your painting a name. And remember… every time you visit this picture of your Positive Memories, a pleasant sense of well-being, peace, serenity and stillness will involve your whole being.

View your frame again.

From your Positive Memories… and feel the lightness of being, in peace and serene…

Breathe deeply

(PAUSE)

Once again visit your Positive Memories framework… and feel comfortably HAPPY.

Breathe deeply…

(PAUSE)

The time has come to return to the here and now…

I'm going to count from 1 to 3…

And by reaching 3 you will open your eyes with a sense of Peace, Harmony and HAPPINESS.

And every time the Positive Memories of your life trajectory appear, you will immediately feel comfortably Happy in Peace and healthy.

1. Become aware of your breath.
2. Tome awareness of your body. Move your feet, your hands, your head. Take an ambiguous awareness of my voice… of the noises around…
3. Soft and slowly OPEN your eyes, squirt the whole body… stretch… feeling HERE and now, in this room, in the city… Here and now! Having in itself the feeling of well-being and HAPPINESS!

(PAUSE)

Portuguese translation for the Portuguese sample of neurotypical young adults

1st phase, the *Arousal Phase*

Feche os olhos... sinta-se bem e tranquilo com os olhos fechados... ofereça este momento para estar consigo por inteiro... em silêncio... em PAZ…

Respire. Profundamente…

Retenha a respiração por alguns instantes...

Solte o ar devagarzinho... e RELAXE…

Mais uma vez...

Respire profundamente sentindo-se confortavelmente AGRADÁVEL... e solte as tensões de seu corpo...

Relaxe-se... agora RESPIRE Profundamente… e sinta-se CONFORTAVELMENTE Seguro...

confortavelmente protegido... solte o ar devagarzinho pelo nariz...

Hum!!!

Que sensação PRAZEROSA em tomar consciência do ar que entra e nutre o seu corpo.

O seu corpo está todo nutrido por este ar límpido, confortavelmente saudável que oxigenou seu corpo, e emoções.

Sinta o seu corpo agora.

Imagine uma LUZ, da sua cor preferida…

Evolvida por uma nuvem em forma de um algodão macio a preencher o seu corpo.

Hum! Sinta esta nuvem colorida a preencher os seus pés.

Tome consciência de seu pé esquerdo... relaxe-o.

Tome consciência de seu pé direito... relaxe-o.

Agora deixe esta nuvem macia e colorida preencher as suas pernas...

Relaxe sua perna esquerda.

Relaxe sua perna direita...

Esta sensação de relaxamento também continua pela sua barriga... Relaxe seu abdómen...

Descontraia o seu coração...

Relaxe os seus braços… as mãos… os dedos...

Respire mais uma vez profundamente...

Sinta o seu corpo descontraído...

Relaxe a sua região posterior... as costas... a sua coluna... sinta-se preenchido por esta nuvem colorida...

O seu corpo encontra -se RELAXADO, DESCONTRAÍDO.

Leve, leve, muito LEVE.

Como é bom desfrutar de ser LEVE.

E esta nuvem colorida continua a aumentar...

Preenchendo suavemente o seu rosto.

Descontraindo todo o seu rosto.

A Sua face, a sua boca... descontraia a sua língua dentro da boca... relaxe os seus olhos... descontraia a sua testa... o seu nariz...

O ar entra com muita fluidez pelas suas narinas agora...

Relaxe as suas orelhas...

Hum! Que sensação agradável de relaxar todo o cabelo...

Gentilmente preenchido por esta pequena nuvem colorida, a tocar toda a sua cabeça...

Hum... que sensação confortavelmente saudável

Confortavelmente agradável...

Confortavelmente em PAZ...

Em PAZ.

(PAUSA)

2nd phase, the *Awareness Phase*

A Leveza, a delicadeza... o bem-estar está consigo agora...

Sente-se tão leve, tão leve, tão leve que está…

Em situações Agradáveis da sua infância.

Pode ser um dia com seus antigos amigos da escola... ou quem sabe… os seus vizinhos...

Uma grande brincadeira que o divertiu muito... e o fez sorrir de alegria!

Está neste momento agora.

Quem sabe um dia de festa...

Uma festa tão aguardada… ou quem sabe uma festa na sua cidade... ou uma festa na escola…

Vê coisas agradáveis, divertidas acontecendo neste evento... é muito bom estar lá...

Hum!

E aquela pessoa que estava sempre com a sua família... pode ser uma tia que gostava muito... quem sabe uma amiga de sua mãe... ou quem sabe um vizinho que falava de coisas engraçadas ou lhe dava incríveis momentos... ou algum inacreditável presente.

É um aniversário... pode ter sido o seu ou de um amigo... uma Festa de aniversário...

HUM!

Aquele pedaço de bolo! O cantar os parabéns... as brincadeiras com os amiguinhos... o barulho das vozes dos adultos e das crianças como uma canção...

A mesa do aniversário… os doces… o Bolo!

Ah! O pedaço de bolo de aniversário...

Que delícia! Era de chocolate? Era de frutas? Qual era seu bolo preferido? E presente... qual o presente da sua infância que realmente gostou?

O que gostava de brincar e com quem?

E a televisão? Quais os desenhos animados que mais gostava de assistir?

E os filmes... e as séries?

E na escola? Lembra-se de quando foi o dia que começou a ler?

O prazer em juntar as pequenas letras e entender o que diziam?

E o intervalo? O que gostava de fazer no intervalo?

E os lanches da escola?

E a professora que mais admirava? O que achava bonito na escola?

Um cartaz especial? Uma árvore especial? Uma sala? Um cantinho da escola em que realmente se sentia bem...

E os seus amigos da escola? O que vocês conversavam... ficava mesmo alegre…

E o que aprendia? Qual era o tema da escola que gostava mesmo de aprender?

E as visitas de estudo? Onde foi a melhor? O que havia lá de interessante?

E as brincadeiras… o cantar dentro do autocarro da excursão… tanta alegria nesta viagem...

E a sua comida preferida?

Hum! Lembra-se daquele saboroso gelado que lhe fez crescer água na boca?

Ou aquele doce que adora! Saboreou cada momento AGRADÁVEL de sua infância.

(PAUSA)

E a natureza? Já brincou com a água da chuva? Ou já tomou banho na água da chuva? E brincar com as flores e os insetos...

E algum animal de estimação seu ou de um amigo... um gato... um cão... um passarinho.

Ah! E as Joaninhas quando apreciam perto de nós... as borboletas...

Enfim... O seu brinquedo preferido quando era criança… e o seu apelido que o deixava zangado, mas que sabia que quem o chamava assim era porque, no FUNDO, o amava muito.

E quando estava doente e alguém lhe cuidava com muitos mimos... uma sopinha ou comida especial naquele dia…

E as festas como o natal… acreditou no Pai Natal?

Escreveu-lhe cartas? Havia comida especial no dia de natal? Reunião em família?

E outras festas importantes na sua infância? Sinta por inteiro estas Memórias Positivas da sua infância... esteja lá agora...deixe as suas lembranças surgirem…

Com imagens… sons… sensações corporais… cheiros...

Tudo! relacionando a sua infância com fases de ALEGRIA.

Permita que esta sensação prazerosa embale o seu corpo e emoções agora...

Respire por um momento e visualize um quadro colorido, bonito, nítido, e que represente a sua infância! A sua fase FELIZ! Guarde esta fase de infância feliz dentro de si...

E…

Sempre que se lembrar de uma cena da sua infância… na Fase FELIZ… terá uma sensação de bem-estar... e felicidade.

(REPETIR PAUSADAMENTE 3 VEZES ESTA FRASE, E SUSSURRANDO NA ÚLTIMA VEZ)

E agora respire profundamente.

Solte o ar lentamente…

Você agora é um Adolescente.

Um Adolescente…

Veja o seu corpo de adolescente...

Como era a o estilo dessa época? O tipo de roupa que gostava de usar...

Se é homem, as suas calças preferidas, a roupa com que ficava mais bonito, os seus ténis... usava mais algo especial?

O que fazia com sua aparência física para se sentir melhor… mais bonito?

E se é mulher. O que usava que gostava muito? Os colares, os brincos, o corte do cabelo, e a maquilhagem... o batom... a sombra... o jeito especial de se sentir uma adolescente.

E as mudanças no corpo? O deixar de ser menina ou menino e se sentir Homem ou Mulher?

Como se sentia em crescer? E os elogios... Já é um rapaz... uma rapariga...

E os seus amigos? E a sua turma?

Quando começou a sair sozinho? Para onde ia?

Passear com os colegas? Com os amigos?

Ir ao cinema? Ver filmes na net?

Jogos na net?

E as mensagens enviadas?...

E o primeiro beijo ou o beijo que realmente deixou saudades...

E aquele rapaz especial... ou aquela rapariga especial na sua vida.

E as lembranças agradáveis desta fase...

E a escola??? o que houve de bom??

Novos interesses?

E os seus projetos... seus desejos...

E as festas com os amigos da turma... brincadeiras especiais que só de lembrar dá vontade de rir... o que você tinha que chamava atenção dos outros... o seu sorriso... o seu olhar... a maneira como falava... a sua forma de dialogar... qual era o seu ponto forte?... sim, tinha uma maneira muito especial de ser... sinta esse momento... este momento…

E as músicas que passava horas cantando... os clipes das suas músicas...

E os seus sonhos acordados... pensava em ser admirado por muita gente? Conquistar o amor da sua vida/ ter o carro dos seus sonhos? Ter um futuro maravilhoso com a sua maneira de ser... soluções mágicas para todos os problemas...

Saboreie e intensifique a sua imaginação em tudo o que se relaciona com a sua juventude em momentos felizes... geralmente, são situações significativas para si... e que são agradáveis de recordar, neste momento....envolva-se por inteiro na sua fase de Juventude feliz... veja as cores desta fase... o cheiro... as pessoas especiais para si, os sons desta época... vivencie.

Relembre esta fase da juventude feliz agora…

E saiba que todas as vezes que você se lembrar de uma Memória Positiva da sua juventude Feliz… IMEDIATAMENTE, uma sensação de bem-estar, ocorrerá… em qualquer momento da sua vida.

(REPETIR PAUSADAMENTE 3 VEZES ESTA FRASE, E SUSSURRANDO NA ÚLTIMA VEZ)

Respire. Profundamente. E permita que esta Fase da sua juventude feliz esteja dentro de si.

(PAUSA)

Respire profundamente outra vez... sinta que está numa nova fase de sua vida.

É um ser adulto.

Vá para lembranças positivas desta fase da sua vida...

Veja os sonhos que conseguiu concretizar...

Estudar até um determinado nível... o esforço que isto lhe causou...

Assumir responsabilidades... sim, pois ser adulto é caminhar com os seus objetivos com independência e determinação.

Veja as suas habilidades e aptidões... aquilo que tem mesmo jeito para fazer... um desenho, uma pintura, uma dissertação, uma poesia, escrever...

Suas realizações significativas na sua vida...

A conclusão da licenciatura...

O que mais marcou positivamente esta etapa de conclusão dos estudos...

O seu casamento ou união com alguém que acreditou que lhe poderia fazer mais feliz... O seu lado profissional... as suas realizações no trabalho que o deixaram contente...

Um elogio recebido... Uma conquista de adulto...

Um passeio... um filme... um livro... uma cena... O nascimento de um futuro filho... A aquisição de um objeto depois de muito o idealizar... um carro ou montar uma casa... um projeto realizado.

Verdadeiros amigos... O que lhe faz sentir-se feliz...

O encontro com a família...

Um dia de bem-estar com os amigos...

Uma festa... uma diversão...

Um acordar depois de uma noite profundamente bem dormida...

O saborear da vida... um pôr-do-sol... uma caminhada... uma dança... ouvir uma determinada música... o sentir-se em PAZ!

Rever um grande amigo… A motivação de um encontro especial com alguém que lhe é afetivamente especial...

O estar inteiramente consigo em determinado momento... o fluir consigo...

Ou se amar... o estar agradavelmente com a sua própria companhia... o aprender algo novo... a curiosidade da vida...

Estar num local realmente relaxante... pode ser numa praia, ou num campo, na montanha, ou quem sabe num local criado somente por si.

Saboreie este local...

Sinta... o cheiro de roupa limpa... cheiro a terra molhada... o cheiro de um perfume que lhe é especial... o cheiro da manhã na primavera... o cheiro da sua flor preferida...

E cores que lhe fazem bem... sinta o seu Azul… ou o seu amarelo… quem sabe o vermelho... sinta a sua cor preferida e o bem estar que ela lhe proporciona... ou veja os objetos que admira... um quadro, um poema, uma fotografia, uma cidade... imagens que proporcionam o seu bem estar...

E as suas canções preferidas... o som... a melodia… a letra e a musicalidade...

A sua relação com a natureza... o tempo bem vivido em contacto com a natureza... uma praia, um lago, uma montanha… um jardim...

Um momento em que sentiu que seu dever foi cumprido... conseguiu terminar uma tarefa ou projeto que desejava muito... algo simples, como montar um móvel com as instruções ou fazer uma receita de um bolo que deu certo... ou até algo mais elaborado

como organizar uma reunião, aprender um idioma...informática... o bem-estar de vencer os obstáculos.

Enfim, resgate as suas Memórias positivas de ser adulto.

Entregue-se neste momento com todo os sentidos visuais, sinestésicos, auditivos, gustativos... inteiramente ao prazer ser adulto...

E todas as vezes que uma lembrança desta sua FASE DE SER ADULTO FELIZ, IMEDIATAMENTE uma Sensação de prazer e bem-estar virá até si

(REPETIR PAUSADAMENTE 3 VEZES ESTA FRASE, E SUSSURRANDO NA ÚLTIMA VEZ)

Respire profundamente...

Agora é uma pessoa que já tem uma boa trajetória de vida.

Já vivenciou muitas coisas na Vida.

O que aprendeu positivamente nestes últimos?

O que realizou na vida que realmente lhe deu Prazer?

Como consegue usufruir o seu tempo com maior criatividade?

O tem aprendido?

Que desafio positivo ainda quer alcançar?

Determine que agora em diante será uma pessoa mais positiva em relação à vida.

O que deseja deixar como um marco aqui na terra com sua vivência?

Quais as pessoas que realmente lhe deixam o coração a sorrir?

O que realmente lhe faz sentir bem?

Veja o seu projeto de vida para o quotidiano.

Que seja simples, mas que tenha significado para si.

Sinta- se realmente FELIZ por a vida lhe dar a felicidade de estar vivo.

Sinta-se leve e feliz por ter continuado a sua trajetória até aqui... Que continuará.

Respire profundamente e vivencie de forma plena e saudável esta fase de maturidade em que se encontra.

E... saiba que todas as vezes que se lembrar desta sua fase catual, as suas Memórias positivas da sua sabedoria vão lhe proporcionar SERENIDADE e Bem-estar.

Viva-o profundamente…

Respire. Profundamente... deixe este bem-estar dentro de si.

E... mais uma vez, respire profundamente...

Crie um quadro onde possa estar representado com Memórias positivas da sua infância, adolescência, e da sua fase adulta...

Este quadro é uma produção sua... ele é muito valioso... é um exemplar único no mundo… é a sua Trajetória positiva de vida.

Há neste quadro um cheiro agradável, sons que lhe chegam bem aos ouvidos, imagens que encantam a sua visão, e uma textura que lhe faz bem ao corpo.

Coloque este quadro num lugar que lhe é muito especial.

Agora dê um nome ao seu quadro. E lembre-se... todas as vezes que visitar este quadro das suas Memórias positivas, uma sensação agradável de bem-estar, Paz, serenidade e quietude envolverá todo o seu ser.

Visualize novamente o seu quadro.

Das suas Memórias positivas… e sinta a leveza de ser, em paz e serena...

Respire profundamente

(PAUSA)

Mais uma vez visite o seu quadro das Memórias Positivas... e sinta-se confortavelmente FELIZ.

Respire profundamente...

(PAUSA)

Chegou o momento de retornar ao aqui e agora...

Eu vou contar de 1 até 3...

E ao chegar até 3 você abrirá os olhos com uma sensação de Paz, Harmonia e FELICIDADE.

E todas as vezes que as Memórias positivas da sua trajetória de Vida aparecerem, imediatamente sentir-se-á confortavelmente Feliz, em Paz e saudável.

1- Tome consciência da sua respiração.
2- Tome consciência do seu corpo. Movimente os pés, as mãos, a cabeça. Tome também consciência da minha voz...dos barulhos em redor...
3- Suave e lentamente ABRA os olhos, espreguice todo o corpo... alongue-se... sentindo-se AQUI e agora, nesta sala, na cidade... aqui e agora! Tendo em si a sensação de bem-estar e FELICIDADE!

(PAUSA)

Annex E. ***Case Report Form***

MOLECULAR PROFILING IN NEUROTYPICAL YOUNG ADULTS



CASE REPORT FORM

Subject X

Subjective Happiness Scale - Escala de Felicidade Subjectiva (SHS)

Desenvolvida por: Sonja Lyubomirsky (1999).

Instruções: Para cada uma das seguintes frases, por favor, escolha o ponto da escala que o descreve de forma mais apropriada.

1	2	3	4	5	6	7
Uma pessoa não muito feliz						Uma pessoa muito feliz

1	Em geral, considero-me:	
2	Comparado com os meus pares, considero-me:	
3	Algumas pessoas são geralmente muito felizes. Apreciam a vida independentemente do que se passa à sua volta, aproveitando o melhor de tudo. Como considera essa pessoa?	
4	Algumas pessoas não são geralmente felizes. Embora não estejam deprimidas, nunca parecem tão felizes como poderiam ser. Como considera essa pessoa?	

Mindful Attention Awareness Scale - Escala de Conhecimento, Atenção e Consciência (MAAS)

Desenvolvida por: Kirk Warren Brown and Richard M. Ryan. Brown, K.W. & Ryan, R.M. (2003).

Instruções: Abaixo está um conjunto de afirmações sobre a sua experiência quotidiana. Usando a escala 1 a 6, por favor, indique com que frequência tem, ou não, atualmente cada experiência. Sendo que:

1- Quase sempre; **2**- Muito frequentemente; **3**- Algo frequente;

4- Algo infrequente; **5**- Muito infrequente; **6-** Quase nunca.

Por favor, responda de acordo com o que realmente reflecte a sua experiência e não o que você acha que sua experiência deveria ser.

1	Posso estar a sentir alguma emoção e só fico consciente dela algum tempo mais tarde.	
2	Eu parto ou entorno coisas por descuido, por não tomar atenção, ou porque estou a pensar noutra coisa qualquer.	
3	Acho difícil permanecer focado no que acontece no presente.	
4	Tendo a andar depressa para chegar onde quero ir e não dou muita atenção ao que vou experienciando ao longo do caminho.	
5	Tendo a não dar conta de sensações de tensão física ou de desconforto até eles me puxarem a atenção.	
6	Esqueço-me do nome de uma pessoa logo a seguir a terem-mo dito pela primeira vez.	
7	Parece que estou em "modo automático", sem dar muita atenção ao que estou a fazer.	
8	Eu apresso-me de umas atividades para as outras sem lhes dar realmente atenção.	
9	Fico tão focado no objetivo que quero atingir que perco o contacto com o que estou a fazer neste momento para chegar a esse objetivo.	
10	Faço tarefas ou trabalhos automaticamente, sem dar atenção ao que estou a fazer.	
11	Dou por mim a ouvir alguém por um lado, mas a fazer outra coisa ao mesmo tempo.	
12	Conduzo para sítios em "piloto automático" e depois pergunto-me porque é que lá fui parar.	
13	Dou por mim preocupado com o futuro ou com o passado.	
14	Dou por mim a fazer coisas sem lhes prestar atenção.	
15	Eu petisco sem ter consciência de que estou a comer.	

Abnorme Psychischer Zustaende, Secondary scale: Altered States of Consciousness - Questionário de Estados Modificados de Consciência (APZ)

Desenvolvida por: A. Dittrich and Mário Simões (1986).

Instruções: Nas páginas seguintes encontra-se uma série de afirmações, que podem ser respondidas com "**Sim**" ou "**Não**".

Por favor avalie se:

durante o procedimento realizado

fez uma experiência correspondente, assinalando com uma **cruz [X]** o "Sim" ou o "Não".

Por favor, observe os seguintes pontos:

1- Não tenha medo de responder uma afirmação com "Sim", por talvez pensar que a sua experiência não tenha sido "**normal**". Muitas pessoas têm sob certas condições experiências estranhas, sobre as quais não falam no entanto com outras.

2- Por favor, responda **rapidamente** e não deixe afirmações **sem resposta**. Naturalmente que com estas curtas perguntas não podem ser consideradas todas as particularidades de uma experiência. Talvez, por isto, algumas não correspondam exactamente ao que experimentou. Decida-se mesmo assim, no caso de cada questão, da melhor maneira da qual se possa lembrar, se fez ou não uma tal experiência durante o seu estado de então ou, se a sua resposta tende para "Sim" ou para "Não".

Por favor, vire agora a página e comece a responder às questões.

N.	Escala Secundária Estados Modificados de Consciência (APZ)	SIM	NÃO
1	Sons e ruídos soavam de modo diferente do que usualmente.		
2	O tempo passava mais depressa do que habitualmente.		
3	Qualquer pensamento sem importância não me "saía da cabeça".		
4	Tive consciência de que atrás do meu "Eu" habitual se escondia um outro "Eu".		
5	O chão, sobre o qual estava, parecia oscilar.		
6	Tinha um zunido nos ouvidos.		
7	Não me podia lembrar do que havido acontecido duas horas antes.		
8	Tinha o sentimento vago de que comigo ia acontecer uma coisa importante.		
9	Partes do meu corpo pareciam não me pertencer mais.		
10	Tinha a impressão de que os meus membros eram maiores do que habitualmente.		
11	Eu estava convencido de já ter vivido a mesma situação.		
12	As coisas ao meu redor tinham outro odor que normalmente.		
13	Estava cansado e esgotado, porém ao mesmo tempo completamente acordado.		
14	Parecia-me que já tinha sonhado o que estava a viver no momento.		
15	Relações estranhas entre factos em si distantes uns dos outros tornaram-se claras para mim.		
16	Tinha dificuldades em distinguir entre o que eu imaginava e o que eu na realidade vivia.		
17	Não sabia mais onde estava realmente.		
18	Tinha a impressão que podia pensar mais depressa e com mais clareza do que habitualmente.		
19	Vinham-me tantos pensamentos à ideia que já não conseguia ordená-los.		
20	Eu estava extremamente acordado e supersensível.		
21	Tinha a impressão de que tudo o que se passava à minha volta se relacionava comigo.		
22	Tinha a impressão de que já não podia mais controlar os movimentos do meu corpo.		
23	Sentia-me influenciado pela corrente elétrica, raios ou hipnose.		

The Self-Consciousness Scale (Revised version) - Escala de Autoconsciência – Revista (SCS-R)

Desenvolvida por: Scheier, M. F., & Carver, C. S. (1985).

Instruções: Por favor responda às seguintes questões sobre si mesmo. Usando a escala abaixo, refira de que maneira as questões representam a sua vivência pessoal. Sendo que:

3 = Muito semelhante a mim; **2** = Algo semelhante a mim;

1 = Pouco semelhante a mim; **0** = Não semelhante a mim.

Por favor, seja honesto tanto quanto consiga, e tente que uma resposta não influencie outra resposta. Não há respostas certas ou erradas.

1	Eu estou sempre tentando-me entender.	
2	Eu preocupo-me com o meu estilo de fazer as coisas.	
3	Eu levo tempo para vencer a minha timidez em situações novas.	
4	Eu penso muito sobre mim mesmo(a).	
5	Eu preocupo-me com a maneira como me apresento.	
6	Eu frequentemente "sonho acordado(a)" comigo mesmo(a).	
7	É difícil para mim trabalhar quando alguém me observa.	
8	Eu nunca me analiso ou questiono.	
9	Eu fico envergonhado(a) muito facilmente.	
10	Eu tenho consciência do meu aspecto.	
11	Para mim é fácil falar com desconhecidos.	
12	Eu geralmente estou atento aos meus sentimentos.	
13	Eu geralmente preocupo-me em causar uma boa impressão.	
14	Eu constantemente penso sobre as razões das minhas ações.	
15	Eu fico nervoso(a) quando tenho de falar em frente de um grupo de pessoas.	
16	Antes de sair de casa eu verifico como está a minha aparência.	
17	Em algumas situações eu distancio-me (mentalmente), para me poder analisar de forma objetiva.	
18	Eu preocupo-me com o que os outros pensam de mim.	
19	Eu dou-me rapidamente conta das minhas mudanças de humor.	
20	Eu geralmente estou consciente da minha aparência.	
21	Eu consigo dar-me conta do modo como a minha mente trabalha quando estou a resolver um problema.	
22	Grandes grupos de pessoas deixam-me nervoso(a).	

Questionário Geral – Avaliação clínica semiestruturada

Código da amostra:_____Data da amostra:_____

1. O dador concorda ser contactado no futuro, caso seja necessário obter novos dados clínicos?

Sim Não Se sim, contacto telefónico/e-mail: _____
Naturalidade: _____

2. Género

Feminino Masculino

3. Raça/Subgrupo populacional

Caucasiana Negra Asiática Cigana Outra; Qual? _____

4. Mês e Ano de Nascimento _____ de

5. Dados Biométricos

Altura: _____Peso: _____

6. Atividade Física

Como passa a maior parte do seu dia?

Sentado na maioria das vezes Pé a maior parte do dia, sem grandes deslocações
Andar a pé com algum peso Esforço físico intenso

Que tipo de exercício faz no seu tempo livre?

Nenhum Atividade física ou desporto casual
Atividade física regular, várias vezes por mês Tempos de treino diversos por semana

7. Tabaco

Fuma ou fumava? _____

Diariamente Ocasionalmente Nunca

Quantidade diária (cigarros por dia): _____ Em que idade começou a fumar: _____

Em que idade deixou de fumar: _____

8. Álcool

Consome álcool:

Diariamente Ocasionalmente Nunca

Quantidade diária (especificar bebida e número de copos por dia):

Em que idade começou a beber: _____ Em que idade deixou de beber: _____

9. Procedimento efetuado neste estudo

Compreende o significado de Estado Modificado de Consciência? Sim ☐ ou Não ☐

- Por experiencia/vivência pessoal? Sim ☐ ou Não ☐

Com que frequência é sujeito a Estados Modificados da Consciência?

☐ Nunca	☐ Meses	☐ Horas
☐ Anos	☐ Dias	☐ Minutos

Que actividades pratica para induzir estes estados?

☐Meditação	☐ Yôga	☐Actividade Física
☐ Sonho Lúcido	☐Etnomedicinas	☐Artes
☐Outras Actividades	☐ Quais?.....	

Compreende o significado de Auto-Consciência? Sim ☐ ou Não ☐

- Considera o procedimento a que foi sujeito o tornou mais auto-consciente? Sim ☐ ou Não ☐

Compreende o significado de Felicidade? Sim ☐ ou Não ☐

- Considera que vivenciou um estado de Felicidade associado no procedimento que foi sujeito? Sim ☐ ou Não ☐
- Considera o procedimento a que foi sujeito o possibilitou de vivenciar um estado e traço de consciência que lhe pertence intrinsecamente? Sim ☐ ou Não ☐

Compreende o significado de *Peak Experiences*? Sim ☐ ou Não ☐

- Por experiencia/vivência pessoal? Sim☐ ou Não ☐
- Considera que vivenciou uma *Peak Experience* no procedimento que foi sujeito? Sim ☐ ou Não ☐

Compreende o significado de Relação Médico-Doente? Sim☐ ou Não ☐

- Considera o procedimento a que foi sujeito possa ser importante na Relação Médico-Doente? Sim ☐ ou Não ☐

10. Medicamentos que toma habitualmente:

Medicamento	Dose (Facultativo)	Início do tratamento (mês/ano)

11. Indique as doenças que lhe foram diagnosticadas:

Doença	Diagnóstico (mês/ano)

Informação ao dador de amostras biológicas

Título do projecto de investigação

Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa - Banco de amostras biológicas Humanas para fins de investigação biomédica

Objectivo do Estudo

A criação de um banco de amostras biológicas humanas permitirá o desenvolvimento de ferramentas de diagnóstico e investigação em múltiplas áreas da medicina, com especial impacto em doenças crónicas, como as doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Contudo, este objectivo só será cumprido com a colaboração dos doentes e de indivíduos saudáveis, através da doação de amostras biológicas que serão guardadas e preservadas em condições apropriadas de forma a serem utilizadas para futuros estudos. Caso o doente ou indivíduo saudável e/ou o seu representante legal decida participar, terá de fazer apenas os procedimentos habituais de uma consulta.

Procedimentos

No caso de concordar em participar neste projecto, ser-lhe-á colhida uma amostra biológica. A amostra habitualmente solicitada será aproveitada a partir da colheita de sangue e/ou urina que irá efetuar. Para os indivíduos que estejam a realizar exames diagnósticos ou que estejam a ser sujeitos a tratamentos cirúrgicos poderá ser pedida autorização para colheita de uma pequena amostra do material removido durante o procedimento (como por exemplo saliva, liquido cefalo-raquidiano, tecidos removidos para biópsias ou removidos no decurso de cirurgias). Estas colheitas serão efetuadas sem alterar os procedimentos médicos habituais e sem interferir com a rentabilidade diagnóstica do procedimento ou com o sucesso da cirurgia. . Esta amostra será preservada em condições apropriadas e as informações clínicas com ela relacionada serão introduzidas numa base de dados, passando a sua identificação pessoal a estar codificada e não acessível aos utilizadores das amostras.

A doação da amostra é voluntária e revogável, sendo que o dador, ou o seu representante legal, tem o direito de retirar a amostra e/ou interromper a colaboração assim que achar conveniente, sem necessidade de justificação e não podendo ser descriminado por isso. O dador ou o seu representante legal deverá manifestar por escrito a sua vontade em retirar a amostra ou interromper a colaboração e nestas situações a amostra será imediatamente destruída.

O Biobanco do IMM propõe-se armazenar as amostras biológicas e seus possíveis derivados tais como, soro, plasma, DNA, RNA e células. No caso da colheita de sangue ou em qualquer outra circunstância de colheita para a qual seja necessário um acto médico invasivo será adoptada uma técnica de imortalização de células, evitando-se assim nova colheita de amostra.

O biobanco do IMM não divulgará resultados envolvendo o material biológico. No entanto, o dador poderá escolher se quer ser informado dos resultados com potencial relevância para a sua saúde. O pedido de resultados deverá ser feito por escrito para o Biobanco do IMM pelo dador ou representante legal e deve ser expresso no consentimento informado.

Serão cumpridas todas as normas éticas aceites internacionalmente para o uso de matérias biológicos para fins de investigação. Todos os projetos que fizerem uso das amostras depositadas no Biobanco do IMM serão submetidos à Comissão de Ética competente para a sua avaliação.

Identificação das amostras e Confidencialidade

A existência de um biobanco pressupõe a existência de uma base de dados contendo informação clínica referente ao doente ou indivíduo saudável. Após a colheita, as amostras serão identificadas por um código de forma a preservar a privacidade.

Durante o desenvolvimento de um projecto de investigação, a equipa de investigação poderá ter necessidade de recolher informação do processo clínico para a execução do estudo. O anonimato será, contudo mantido, ou seja os dados constantes do seu processo clínico serão fornecidos ao investigador, mas sem qualquer identificação, ou qualquer informação que permita saber a quem pertencem.

A descodificação apenas poderá ser efectuada pelo médico (que será o responsável pela base de dados, de acordo com a informação fornecida à Comissão Nacional de Protecção de Dados - CNPD), em caso de absoluta necessidade, por motivos de saúde do dador e a pedido deste, e sempre de acordo com as disposições legais em vigor.

Os dados serão tratados confidencialmente, de acordo com a Lei, com os regulamentos e de acordo com as normas éticas aprovadas pela Comissão de Ética do CHLN/FMUL e pela CNPD.

Os dados resultantes dos estudos realizados serão alvo de publicação de uma forma anónima e agregada, em termos de percentagens ou de dados numéricos, nunca individualmente.

Tempo de conservação

As amostras serão conservadas por um período de 20 anos no Biobanco do Instituto de Medicina Molecular (IMM) da Faculdade de Medicina da Universidade de Lisboa, sob a responsabilidade da Equipa ligada a este estiver devidamente credenciado pelas entidades competentes. As coleções de amostras serão avaliadas periodicamente, nomeadamente para aferir da sua qualidade, podendo ser destruídas ou, findo o período da conservação, poder-se-á solicitar a prorrogação da conservação. Nestas condições excecionais o Biobanco do IMM poderá recontactar os dadores.

Comunicação e divulgação de dados

Os dados genéticos e as amostras biológicas colhidas para fins de investigação científica podem ser transferidos para outras organizações ou centros de investigação, para fins de pesquisa e somente em projetos desenvolvidos conjuntamente com o IMM, mediante consentimento do participante expresso na declaração de consentimento informado.

Possíveis Benefícios para os Participantes

Esta é uma doação altruísta, não havendo por isso qualquer compensação para o dador. Não se garante que este estudo envolva quaisquer benefícios directos para o participante. Se algum dos estudos puder ser relevante para a saúde do dador, este será informado, se essa for a sua vontade expressa na declaração de consentimento informado.

Contudo, a sua participação proporcionará a aquisição de conhecimentos que poderão vir a beneficiá-lo a si ou a terceiros no futuro.

Riscos físicos previsíveis

Na maioria dos casos, os riscos e o desconforto associados serão mínimos ou inexistentes. Nas colheitas associadas a procedimentos com fins diagnósticos ou terapêuticos, os riscos e o desconforto serão os inerentes ao procedimento em si. Em qualquer dos casos, o dador será sempre antecipadamente informado dos riscos e grau de desconforto associados aos procedimentos.

Participação Voluntária e Direitos de Abandono

O presumível dador terá toda a liberdade para se recusar a participar no estudo ou retirar o seu consentimento, suspendendo a participação em qualquer momento e, consequentemente, as amostras serão destruídas. A participação é voluntária e a sua recusa em participar não envolverá qualquer penalização ou perda de benefícios. A recusa ou abandono não colocarão em risco o direito a receber tratamento ou assistência médica, presentemente ou no futuro.

O dador poderá retirar o seu consentimento nas modalidades **sem contacto futuro** (as amostras poderão ser usadas normalmente até se esgotarem, mas não serão estabelecidos futuros contactos para a obtenção de mais amostras) ou **sem uso futuro** (não serão estabelecidos futuros contactos e as amostras serão imediatamente destruidas e os registos eliminados).

Se tiver qualquer dúvida, em qualquer momento, mesmo após a colheita, sobre este estudo poderá contactar o Director do Biobanco do IMM:

Prof. Doutor Sérgio Dias e Doutor Joaquim Polido Pereira,
dirigindo-se a: Drª Angela Maria Afonso

Biobanco

Instituto de Medicina Molecular

Faculdade de Medicina da Universidade
de Lisboa Telefone: +351 217999437

Ext:47047/92903

Email: immbiobanco@fm.ul.pt

Termo de Consentimento Informado

Declaração de consentimento informado

Conforme alei 67/98 de 26 de Outubro e a "Declaração de Helsínquia" da Associação Médica Mundial (Helsínquia 1964; Tóquio 1975; Veneza 1983; Hong Kong 1989; Somerset West 1996, Edimburgo 2000; Washington 2002,

Tóquio 2004, Seul 2008, Lorenza 2013) – quando se aplicar

Designação do Estudo: Molecular Profiling in Neurotypical Young Adults.

Eu, abaixo-assinado

_____

,

fui informado de que o Estudo de Investigação acima mencionado se destina a medir possíveis alterações de biomarcadores salivares, através de técnicas de proteómica, elicitados pela aplicação de uma intervenção única do tipo cognitivo-comportamental baseada em mindfulness, após sessão única. Sei que neste estudo está prevista a realização de questionários e colheita e análise de saliva, bem como uma tarefa cognitiva, tendo-me sido explicado em que consistem e quais os seus possíveis efeitos.

Também sei que algumas análises, especificamente a análise de proteómica, não podem ser efectuados neste hospital e que, por isso, têm que ser realizadas noutras instituições.

Foi-me garantido que todos os dados relativos à identificação dos Participantes neste estudo são confidenciais e que será mantido o anonimato.

Sei que posso recusar-me a participar ou interromper a qualquer momento a participação no estudo, sem nenhum tipo de penalização por este facto.

Compreendi a informação que me foi dada, tive oportunidade de fazer perguntas e as minhas dúvidas foram esclarecidas.

Aceito participar de livre vontade no estudo acima mencionado.

Concordo que sejam efectuados os exames e a colheita de amostras de saliva para realizar as análises que fazem parte deste estudo.

Também autorizo a divulgação dos resultados obtidos no meio científico, garantindo o anonimato.

Nome do Investigador:

Dr. Jorge E. Martins

Contacto:

jorge.e.martins@campus.ul.pt

Data	Assinatura
___/___/_____	_____

Declaração de Consentimento Informado

Banco de amostras biológicas para fins de investigação biomédica

Investigador:_____

Hospital: _____

Nome do dador: _____

Número de estudo do dador: _____

Eu, , portador do

bilhete de identidade/cartão do cidadão n.º [ ], declaro ter tomado conhecimento e aceitar participar neste projecto, de forma a contribuir para a criação de um banco de amostras biológicas com informação clínica associada, para fins de investigação biomédica.

Aceito que a minha amostra biológica seja utilizada em projectos de investigação de mecanismos das doenças, diagnóstico precoce, fatores de prognóstico e novos alvos terapêuticos em múltiplas áreas da medicina, nomeadamente nas doenças oncológicas, cardiovasculares, neurológicas, ósseas e imunológicas. Poderei revogar a autorização para utilização da minha amostra biológica e informação clínica em qualquer altura.

O objectivo do banco de amostras biológicas foi-me claramente explicado e foi-me dada a oportunidade de colocar questões sobre o seu funcionamento, bem como os procedimentos relativos à colheita e utilização da minha amostra biológica e dados a ela associados.

Declaro que aceito participar, voluntariamente, neste estudo. Especificamente concordo com os seguintes pontos:

- Consinto a colheita de material biológico (sangue / ..... / .....) e autorizo a conservação de amostras no Biobanco, de modo a que possam ser usados para pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, sem fins lucrativos;

Sim Não

- **Esta opção é para ser respondida apenas por participantes que já cederam amostras biológicas colhidas no âmbito de outros projetos.** Nestas circunstâncias, autorizo a transferência para o Biobanco as minhas amostras biológicas, préviamente colhidas no âmbito de outros projectos, de modo que elas possam ser utilizadas em pesquisas futuras, incluindo estudos genéticos e cultura de linhas celulares por investigadores portugueses e estrangeiros, mas sem fins lucrativos;

Sim Não

- Estou consciente de que minha participação é voluntária e que posso em qualquer altura solicitar a destruição das minhas amostras biológicas, invalidando assim o consentimento informado prévio, sem justificar, tendo recebido a garantia de que o meu pedido não desenvolverá discriminação;

Sim Não

- Declaro que quero conhecer resultados que possam ser relevantes para a minha saúde.

Sim Não

- zo ser contactado novamente pelo Biobanco do IMM para pedido de atualização sobre a minha situação clínica;

Sim Não

- Autorizo o contacto do Biobanco do IMM a familiares meus para pedido autorização de colheita de amostras biológicas e/ou informação clínica;

Sim Não

_____ _____

Data Assinatura do Dador/Representante Legal

Em caso de representante legal, este actua na qualidade de:

□ Titular do poder paternal, quando o dador é menor
□ Tutor, quando o dador foi declarado interdito
□ Herdeiro, quando o dador faleceu

Discuti este estudo de investigação com o participante e/ou o seu representante legal, utilizando uma linguagem compreensível e apropriada. Informei adequadamente o participante sobre a natureza deste estudo e sobre os seus possíveis benefícios e riscos, considerando que o participante compreendeu a minha explicação.

_____	_____	_____
Data	Nome do Médico	Assinatura

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Annex F. ***Mental Health Promotion In The Community: Conceptual Analysis***

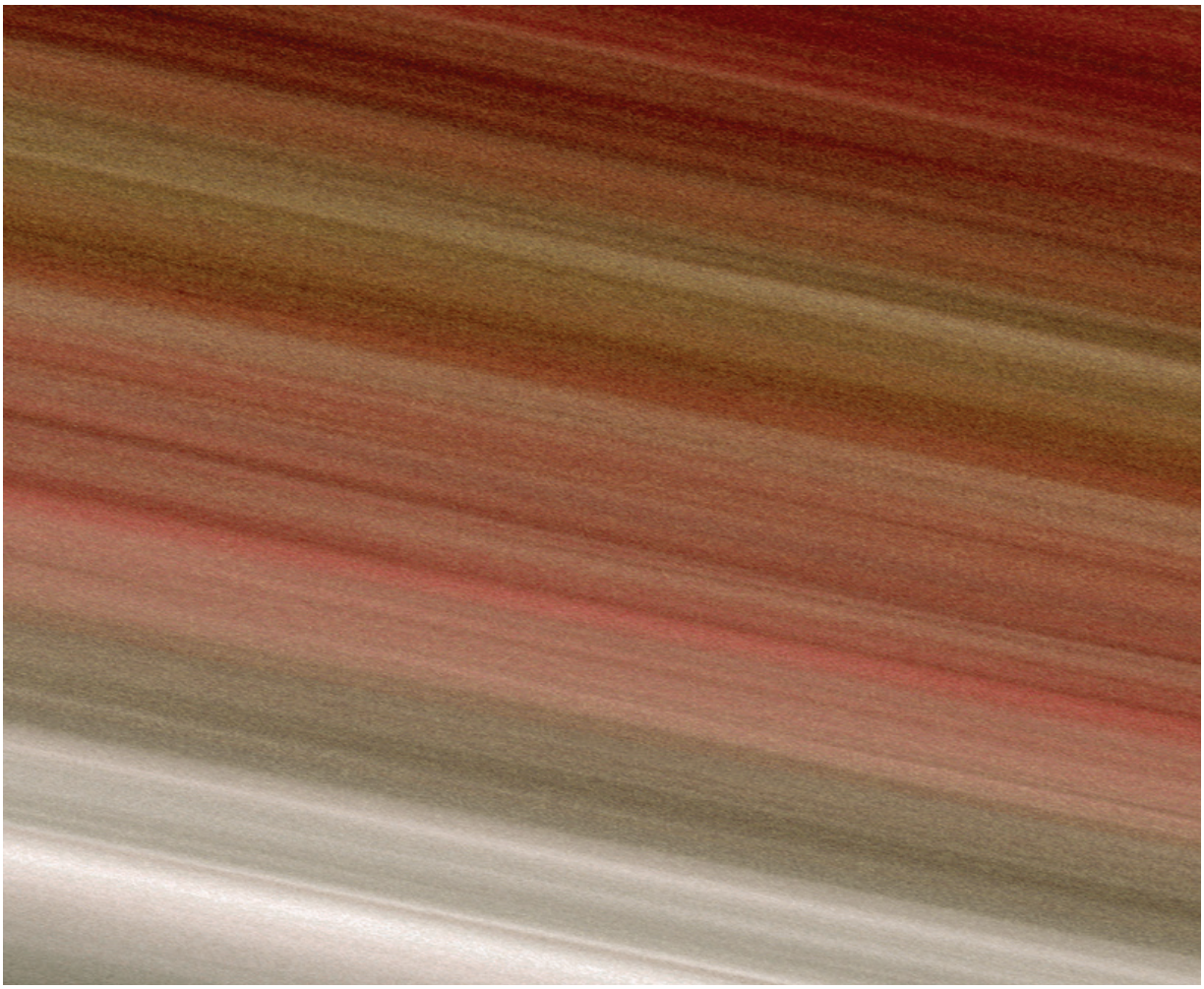
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Promoção de saúde mental na comunidade: análise conceptual

Mental health promotion in the community: conceptual analysis

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Palavras-chave
Consciência do eu; consciência de morbidade; medicina geral e familiar; meditação; neurociência Afetiva e Cognitiva; psiconeuroimunologia; psicoterapia.

Resumo

Introdução: Recentemente, a neuropsiquiatria tem-se centrado, quase que exclusivamente, na correção de alterações fisiopatológicas, menosprezando inevitavelmente certos fatores psicossomáticos de doenças crónicas da comunidade. Assim sendo, torna-se necessário o desenvolvimento e aplicação de saúde mental que utilize uma abordagem mais compreensiva no tratamento de doenças incapacitantes e nos cuidados de saúde primários.
Objetivo: Este estudo pretende rever o efeito da consciência do eu e consciência de morbidade, como descrito subjetivamente por pacientes durante intervenções psicoterapêuticas ou por adultos saudáveis em práticas meditativas.
Material e métodos: Para avaliar o efeito descrito, uma análise conceptual é proposta como método. Revendo a literatura atual sobre o "state of the art" de dinâmicas cerebrais e eixo psiconeuroimunológico, testou-se a correlação entre a experiência subjetiva do paciente e a neurociência afetiva e cognitiva. Como hipótese de investigação, pretendeu-se evidenciar os paradigmas científicos utilizados para estudar o efeito supraescrito.
Resultados: Apresenta-se a limitação atual da integração das abordagens psicoterapêuticas na compreensão do bem-estar e prevenção de saúde mental, bem como a necessidade da sua implementação na medicina geral e familiar e psiquiatria comunitária. A consciência do eu e a consciência de morbidade promove o bem-estar físico e mental e contribui para o desenvolvimento de traços emocionais não patológicos. A psicoterapia clássica, assim como a meditação, permite que o indivíduo tenha acesso às suas representações internas, proporcionando o espaço mental necessário para trabalhar afetos e cognições. O acesso clínico a essas representações internas é importante não apenas no tratamento de doenças incapacitantes, mas também indispensável nos cuidados de saúde primários.
Conclusões: O médico, através da promoção da saúde física e mental do indivíduo, da sua família e da comunidade, tem um papel preponderante na adesão aos pressupostos de boa prática clínica. A hipótese de investigação defendida por esta análise conceptual apresenta terreno para o debate de uma medicina da comunidade mais focada na indução de políticas para o bem-estar mental, para a consciência do eu e para a consciência de morbidade. Estas políticas de saúde mental na comunidade são atualmente consideradas como objetivos do programa de saúde de medicina geral e familiar, nomeadamente a abordagem centrada na pessoa e no seu contexto, direcionada para a capacitação e empoderamento do doente ou indivíduo saudável, como agente responsável pela própria saúde.

Keywords
Affective and Cognitive Neuroscience; General and Family Medicine; Meditation; Morbidity awareness; Psiconeuroimmunology; Psychotherapy; Self-awareness.

Abstract

Introduction: *Recently, neuropsychiatry has focused almost exclusively on the correction of pathophysiological changes, inevitably neglecting certain psychosomatic factors of chronic diseases in the community. It is thus necessary to develop and apply mental health that uses a more comprehensive approach in the treatment of disabling diseases and in primary health care.*
Aim: *This study aims to review the effect of self-awareness and awareness of morbidity, as described subjectively by patients during psychotherapeutic interventions or by healthy adults in meditative practices.*

Materials and Methods: *To assess the effect described, a conceptual analysis is proposed as a method. Reviewing the current literature on the «state of the art" of brain dynamics and the psychoneuroimmunological axis, the correlation between the patient's subjective experience and affective and cognitive neuroscience was tested. As a research hypothesis, it was intended to highlight the scientific paradigms used to study the effect described above.*

Results: *The current limitation on the integration of psychotherapeutic approaches in understanding well-being and mental health prevention is presented, as well as the need for its implementation in general and family medicine and in the community psychiatry. Self-awareness and awareness of morbidity promotes physical and mental well-being and contributes to the development of non-pathological emotional traits. Classical psychotherapy, as well as meditation, allows the individual to access his internal representations, providing the mental space necessary to work on affections and cognitions. The clinical access to these internal representations is important not only in the treatment of disabling diseases, but also indispensable in primary health care.*

Conclusion: *The physician, by promoting the physical and mental health of the individual, his family and the community, has a predominant role in the adhesion to the assumptions of good clinical practices. The research hypothesis defended by this conceptual analysis presents ground for the debate of a community medicine more focused on inducing policies for mental well-being, for self-awareness and for morbidity awareness. These mental health policies in the community are currently considered to be objectives of the general and family medicine health program, namely the person-centered approach, which aims at training and empowering the patient or healthy individual as the prime agent responsible for his own health.*

Introdução

A medicina ocidental, seguindo o progresso científico, tem-se centrado quase exclusivamente na correção das alterações fisiológicas, físico-químicas, enquanto descuida os fatores psicossomáticos das doenças. É necessária uma abordagem psicossomática, que cada vez mais integra a epigenética. É na senda desta realidade que na área da saúde muitos se têm dedicado ao desenvolvimento de uma medicina que utilize uma abordagem mais completa e compreensiva no tratamento da doença.[1]

A medicina contemporânea precisa de médicos mais humanizados, de abordagens e atitudes psicossomáticas, de valorizar a pessoa e a saúde em todas as suas dimensões. Dizia Barahona Fernandes que é preciso reconhecer a vantagem que a medicina e cirurgia têm ao atenderem à perspetiva psicossocial e à compreensão da personalidade dos doentes na sua relação com o médico e equipa de saúde e na sua situação, regressando às raízes hipocráticas do todo humano (bio-psico-sociocultural), resistindo à dualidade cartesiana mente-corpo.[1]

A consciência é facilmente compreendida como a realidade momentânea que o indivíduo experiencia de acordo com a sua vivência. O conceito de vivência (*Erlebnis*),[2] bem descrito na filosofia de Nietzsche, possui originariamente uma tripla significação: a imediatez (*Unmittelbarkeit*) entre homem e mundo; a significabilidade (*Bedeutsamkeit*) para o caráter global da existência; a incomensurabilidade (*Inkommensurabilität*) do conteúdo da própria vivência, conferindo a ela também uma dimensão estética.

O estado "normal" ou "ordinário" de consciência permite aceder à realidade ordinária, enquanto um estado mental de consciência ampliada (mais autoconsciência) permite o acesso a uma realidade menos ordinária, tanto interior como exterior. É com a finalidade de mecanismo alternativo de aquisição e / ou integração de conhecimento que estes estados mentais foram desde os primórdios da humanidade usados em práticas meditativas. Atualmente, os seus princípios são usados em variantes psicoterapêuticas.[1,3]

Material e Métodos

Como método de investigação, apresentamos um modelo de análise conceptual que, na nossa opinião, contribui para a exploração da consciência do eu e consciência de morbidade sob a ótica da neurociência afetiva e cognitiva.

Para avaliar o efeito descrito em pacientes durante intervenções psicoterapêuticas ou por adultos saudáveis em práticas meditativas, foi efetuada uma revisão de literatura sobre o "state of the art" de dinâmicas cerebrais e eixo psiconeuroimunológico.

PUBMED e Google Scholar foram usados como recurso para a revisão de literatura de livros e artigos científicos. Assim sendo, uma pesquisa bibliográfica sistemática foi realizada usando a ferramenta de pesquisa no portal do PUBMED, complementada pelos utilitários de pesquisa do Google Scholar. As seguintes palavras-chave foram usadas como filtros: "MeSH (Medical Subject Headings)", "doenças neurológicas", "doenças psiquiátricas", "saúde mental", "meditação" e "saúde comunitária". Para realizar uma

revisão ordenada com base nas publicações sobre o efeito estudado, foram pesquisadas correlações e associações adicionais de palavras-chave. As publicações de 2000 a 2019 foram selecionadas. Os artigos e livros com data anterior ao ano 2000 que foram incluídos justificam-se pela importância histórica das hipóteses levantadas. Da mesma forma, para realizar uma revisão ordenada da correlação entre experiência subjetiva do paciente e a neurociência afetiva e cognitiva, também foram aplicados os seguintes filtros de pesquisa: "Humans" e "Clinical trial", a fim de incluir, exclusivamente, artigos de natureza experimental relativos à espécie *Homo sapiens*.

No total, foram analisados 81 artigos e livros para as respetivas correlações de palavras-chave, que pretendem evidenciar os paradigmas científicos utilizados para a construção da nossa hipótese de investigação. Com base na análise dos artigos e livros filtrados e compilados, que apresentamos posteriormente nas referências bibliográficas, foi realizado um levantamento de uma amostra representativa de dados empíricos. A partir deste levantamento de dados, foi feita uma anotação dos correlatos entre a "consciência do eu", "consciência de morbidade", "medicina geral e familiar", "meditação", "neurociência afetiva e cognitiva", "psiconeuroimunologia", "psicoterapia" e doenças psiquiátricas como a esquizofrenia, perturbações da personalidade, perturbações do humor e perturbações de ansiedade.

Por fim, este método empírico desenvolve um desenho experimental, no que diz respeito às condições neuropsiquiátricas e da medicina geral e familiar da CID-10, que aplicámos na pesquisa empírica, para estudar o efeito entre uma medicina da comunidade mais focada na indução de políticas para o bem-estar mental, para consciência do eu e para a consciência de morbidade.

Para nosso entendimento, trata-se de um método adequado e que reúne informações sobre as respostas neurofisiológicas e psicofisiológicas presentes ou alteradas nos estados saudáveis *versus* patológicos de doentes seguidos em saúde comunitária. Esta atualização científica é importante para a publicação de estudos experimentais sobre a hipótese de investigação proposta por este trabalho, i.e., relação causal fisiológica do efeito da consciência do eu e consciência de morbidade em pacientes crónicos durante intervenções psicoterapêuticas ou adultos saudáveis em práticas meditativas.

Resultados

I. Meditação como modelo de estudo da neurociência afetiva

A meditação tem sido amplamente praticada em muitas civilizações há milhares de anos, como um meio de cultivar um estado de bem-estar e para fins religiosos. O objetivo geral tem sido alcançar uma maior compreensão e perceção da realidade pessoal e coletiva. Atualmente, é estudada em termos da sua influência sobre o cérebro e corpo.[4]

As práticas contemplativas que visam transcender o estado ordinário de consciência podem ser encontradas em sociedades em todo o mundo desde o Paleolítico.[5,6] Referências formais à meditação podem ser encontradas em textos antigos, do século III a.C., nas escrituras budistas de Abhidharma.[7,8] Hoje, "meditação" é usada como um termo genérico para se referir a uma ampla gama de práticas de autorregulação da emoção e atenção[9] e é considerada uma prática presente na sociedade contemporânea e nas atuais tradições filosóficas.

A interação mente-matéria parece beneficiar de técnicas de facilitação ou treino. Entre elas, encontramos técnicas motivacionais de *feedback*, a prática de *yoga* e meditação, relaxamento e fatores emocionais (humor positivo) ou treino de visualização.[10] Inúmeras experiências têm sido feitas com monges budistas com elevado treino de meditação, cujos resultados sugerem que as expectativas ou intenções podem levar à alteração de padrões de comportamento.[11]

As alterações neurofisiológicas induzidas pela meditação podem ser de dois tipos. As alterações que ocorrem durante a prática de meditação são referidas como *state changes*. As alterações que se acumulam ao longo dos meses ou anos e persistem mesmo quando a mente não é ativamente focada em meditação são referidas como *trait changes*.[12] Por um lado, o estudo da meditação é relevante para a investigação como um meio de explorar o cérebro em estados não patológicos. Por outro lado, é também importante para a abordagem dos efeitos benéficos da meditação sobre a saúde e o bem-estar, bem como com as suas potenciais aplicações clínicas.[4]

Sendo assim, propõe-se um quadro teórico[13] em que as práticas de meditação são classificadas em dois grupos principais: *focused attention* (vigília de concentração) e *mindfulness* (vigília de presença ou de atenção plena).

A meditação *focused attention* exige o estreitamento da vigília para que a mente contenha apenas o objeto de foco. A meditação *mindfulness* pode ser descrita como a vigília sustentável que visa uma consciência não reativa e não apegada à observação mental, sem interpretação cognitiva ou emocional, durante o momento a momento do desenrolar da experiência meditativa.[11-14]

Dado que a meditação é muitas vezes considerada como uma técnica de relaxamento, é razoável supor que a prática de meditação afeta o funcionamento do corpo. No entanto, a meditação induz mudanças de longa duração para a perceção do próprio corpo que podem ser observadas tanto em representações corticais *low level* como *high level* associado ao sentimento do self.[12]

Visto isto, a prática da meditação também induz mudanças na representação do *self*. Um estudo por ressonância magnética funcional[15] mostrou uma diminuição da conectividade entre o córtex insular, que está envolvido na perceção da dor e respostas internas do corpo, e o córtex pré-frontal medial, que está envolvido a nível mais elevado na cognição, após um programa de 8 semanas de meditação *mindfulness,* em relação ao grupo de controlo. Os resultados sugerem a presença de um estado de autoconsciência em praticantes de meditação,[12,16] caracterizado como um "estado de vigília hipometabólico".

A respiração, fator essencial na meditação, é uma das poucas funções autonómicas do corpo que podem ser controladas e assim afetar o funcionamento do sistema nervoso autónomo.[17,18] A respiração também tende involuntariamente a abrandar durante a tomada de consciência da respiração[19,20] utilizada na prática meditativa.

Assim, a redução de frequência respiratória induz alterações na atividade cardiovascular, que correspondem a um aumento da atividade restauradora do sistema parassimpático,[21] avaliada através da diminuição da frequência cardíaca basal em praticantes de meditação.[22] Por outro lado, a tomada de consciência da respiração também tem sido associada com aumento da sensibilidade ao reflexo barorreceptor.[23,24] A diminuição da pressão arterial é frequentemente relatada após a prática de meditação.[25,26] Um melhor controlo da pressão arterial é geralmente considerado um sinal de equilíbrio entre a atividade simpática e parassimpática.

Presentemente, há evidências crescentes de que a prática de meditação também afete o sistema imunitário. Estados psicológicos, como o *stress,* afetam o funcionamento do sistema imunitário.[27,28] O sistema imunitário está indiretamente sob a influência do sistema nervoso central através de sinalização hormonal e da atividade do sistema nervoso autónomo.[29,30] Um modelo de sincronização, descrito por Jerath *et al*,[31] entre o sistema cardiorrespiratório e o sistema nervoso central pode influenciar o sistema nervoso autónomo.

Este tipo de atividade hemisférica também com regulação emocional, afeta a maneira como emoções são experienciadas na mente e no corpo.[32] As emoções podem ser vistas como um conjunto de mudanças inter-relacionadas no corpo, em resposta a um estímulo. Estas mudanças inter-relacionadas são experimentadas como sentimentos e podem interromper o comportamento em curso ou ainda levar a processos mentais.[33]

Em indivíduos que, normalmente no dia a dia, tendem a reagir positivamente e sem respostas associadas ao *stress*, a atividade elétrica de base do cérebro deve-se à maior ativação anterior do lado esquerdo [atividade *gamma-band* (25-42 Hz)].[34] A relação entre afetividade positiva e atividade anterior das áreas do cérebro é fortemente apoiada, como avaliado por Craig,[32] pela conectividade anatómica entre o sistema nervoso autónomo e as áreas do cérebro anterior. Demonstra-se claramente como as partes mais antero-esquerdas do cérebro interagem mais diretamente com o sistema parassimpático, e as antero-direitas interagem mais diretamente com o sistema simpático.

Segundo Ochsner e Gross,[35] existem dois meios através dos quais pode ser alcançada a regulação das emoções: o controlo da atenção e o controlo cognitivo. O controlo da atenção envolve a manipulação da quantidade de atenção que é naturalmente disponibilizada para processar estímulos emocionais. O controlo cognitivo envolve a alteração das expectativas ou julgamentos sobre estímulos emocionais. Estes controlos estão presentes na meditação se a atenção é focada para longe da emoção (práticas de concentração) ou quando a emoção é simplesmente observada (práticas de *mindfulness* contemplativo).[36-38]

II. Psicoterapia como modelo de estudo da neurociência cognitiva

Há múltiplas evidências de que o cérebro pode influenciar o sistema imunitário através do eixo hipotálamo-hipófise, do sistema neuroendócrino e seus neuropéptidos, e através do sistema nervoso

autónomo e órgãos linfóides pela libertação de citoquinas. Mais ainda, o sistema imunitário influencia a atividade cerebral através de interleucinas e da síntese de péptidos como corticotrofina ou endorfinas.[1]

Vários estudos têm demonstrado o efeito imunossupressor de alguns estímulos *stressores*. Neste sentido, surgiram outros estudos indicativos de que intervenções psicológicas como a hipnose clínica ou práticas como a meditação são capazes de atenuar ou reverter esta imunossupressão, consequência do estímulo *stressor*.[39] O desafio tratar-se-á de compreender como a autoconsciência e uma maior perceção do eu pode influenciar o corpo, especificamente na prática psicoterapêutica.

A psiconeuroimunologia tem sido particularmente estudada em associação com o cancro. Neste contexto, esta ciência procura aferir os papéis que a personalidade, estado mental, atitude perante a doença, eventos de vida adversos, suporte social e outras variáveis psicológicas podem ter na indução e desenvolvimento do cancro.[1]

Inúmeros estudos[40,41] têm reportado um aumento da sobrevida em doentes oncológicos inseridos em grupos de suporte e sujeitos a treino de autoconsciência quando comparados a grupos de controlo, sem qualquer intervenção psicoterapêutica.[1] Mais ainda, outras investigações[42] provaram que os efeitos das intervenções psicológicas se mantêm estatisticamente significativos, e que um nível elevado de *stress* emocional de base, bem como estratégias de *coping* ativas, estão associados a uma sobrevida mais longa.

Vários estudos têm demonstrado o efeito imunossupressor de alguns estímulos *stressores*, como referido anteriormente. Exemplos destes são as épocas de exames académicos, o luto, o cuidar de um familiar doente, o próprio condicionamento em redor da quimioterapia e não o efeito direto da mesma, isto é, o ambiente hospitalar hostil que fica associado no tempo à toma dos imunossupressores.[1]

A par destas evidências, outros estudos surgem que avaliam intervenções psicológicas ou psicoterapêuticas, o treino de relaxamento e a imaginação guiada, que são capazes de atenuar ou reverter os efeitos imunodepressores acima descritos, em voluntários normais.[39] Na esteira da investigação em psiconeuroimunologia, procura-se compreender a psicobiologia por detrás da psicoterapia.

A teoria *deep psychobiology of psychotherapy*[43,44] pode ser definida como a exploração da experiência mente-corpo, desde o cultural e psicossocial ao molecular-genético-celular. Esta teoria procura explicar de que forma os nossos pensamentos, emoções, imaginação e experiência pessoal podem influenciar a saúde física; até que ponto podemos "dialogar biologicamente" de maneira a modular o modo como a sua informação é expressa no processo de tratamento.

Na década de 70, surge a *dream-protein hypothesis*, de Rossi. Esta teoria diz que o sonho é um processo psicofisiológico de crescimento que envolve a síntese e modificação de estruturas proteicas no cérebro, servindo como base orgânica para novos desenvolvimentos na personalidade.

Nesta sequência, a psicoterapia passa a ser interpretada como um processo facilitador de momentos criativos, como os do sonho. Estes momentos são aqueles em que o padrão de associação habitual é interrompido, seja por relaxamento, regressão a um tempo anterior de recordações, lapso espontâneo, ou privação sensorial, entre outros. Esta experiência criativa é então, simultaneamente, codificada em novas proteínas e redes neuronais no cérebro. Considera-se que, nesse instante, a diminuição do habitual estado de vigília promove o aparecimento de um outro estado ainda de vigília, mais profundo, ampliado e mais subtil.[45-47]

São esses momentos criativos a unidade base do *insight*, da descoberta de novos significados, da transformação da personalidade ou de acessos a autoconsciência. Estes correlacionam-se diretamente com alterações da estrutura molecular de proteínas no cérebro, associadas à criação de novas ligações celulares, memória e aprendizagem. Essas modificações moleculares são reconhecidas por Rossi[48] como refletidas no trabalho psicoterapêutico, que é suscetível de modular aquelas modificações. Segundo Rossi, a psicoterapia promove um estado psicológico adequado para que o doente reassocie e reorganize as suas complexidades psicológicas (modificando a informação presente até aí) segundo as suas capacidades e a sua experiência de vida, resultando na ressíntese interior do comportamento do doente. Para melhor compreender a relação psicoterapeuta-doente, temos segundo o autor,[48-50] sumariamente, quatro estádios:

(i) *Transdução de informação mente-cérebro:* o sistema límbico-hipotálamo-hipofisário é reconhecido como o maior transdutor de informação entre o cérebro e o corpo, onde as células do hipotálamo transformam os impulsos neurais eletroquímicos do cérebro,

que codificam a experiência fenomenológica da mente e emoções, nas hormonas mensageiras do sistema endócrino, que serão libertadas em circulação num *loop* de *feedback* de informação-transdução. Este *loop* de comunicação mente-corpo permite modular a ação dos neurónios e células de todo o corpo desde o nível das vias básicas de sensação e perceção à dinâmica intracelular de transcrição e tradução genética;

(ii) *Immediate-early genes:* esta classe de genes está permanentemente ativa, respondendo à sinalização dos mensageiros hormonais para a necessidade de adaptação a alterações do ambiente, desde estados psicológicos como choque e surpresa, toque e estimulação sexual, *stress* psicossocial a temperatura, alimentação, toxinas e trauma físico. Permitem assim que sinais do ambiente externo regulem outros genes no interior das células, por vezes dentro de 20 min a 1 h, consoante a intensidade do estímulo. Acredita-se que as memórias e novas experiências sejam codificadas no sistema nervoso central por alterações na estrutura e formação de proteínas nas sinapses neuronais;[51]

(iii) *Síntese proteica na memória, stress e tratamento:* o tempo requerido para o processo de transdução genética na produção de novas proteínas em resposta a estimulação psicológica ou *stress* físico oferece uma importante janela de informação dinâmica para novas abordagens na psicoterapia. Hoje sabe-se que, para além do ritmo circadiano de 24 h, existem ritmos ultradianos, mais rápidos do que o anterior, ao nível genético, endócrino e cognitivo-comportamental durante o sono, sonho e estados de vigília criativos, com importantes implicações para a psicoterapia;

(iv) *Moléculas mensageiras e memória estado-dependente:* as moléculas mensageiras que tiveram origem no processamento de proteínas de maior dimensão podem ser armazenadas dentro dos neurónios ou outras células como "memória molecular". São depois libertadas em circulação para completarem o *loop* de transdução de informação, atravessando a barreira hemato-encefálica para modular as redes neuronais e a experiência psicológica de cada evento, atingindo o eixo límbico-hipotálamo-hipofisário e outras áreas do córtex.

Estudos recentes indicam que a maioria das formas de aprendizagem, de Pavlov a Skinner, do *imprinting* à sensitização, envolvem estes mensageiros moleculares do corpo que atingem o cérebro para modularem as redes neuronais que codificam mente, memória, aprendizagem e comportamento, num componente dependente de um estado de consciência.[49,50]

O desafio que se coloca é, por exemplo, documentar como uma intervenção psicoterapêutica pode conduzir à facilitação da transcrição do gene do recetor de IL-2, que funciona como mensageiro do sistema imunitário. Linhas de investigação, como tal, oferecerão um novo meio de compreensão das possibilidades mente-cérebro.[52-54] Deste modo, a medição destes parâmetros em amostras de sangue, saliva ou outro tipo de amostra biológica, poderá ser um critério de avaliação e comprovação da eficácia dos métodos de psicoterapia.[49]

III. Bem-estar e promoção de saúde mental na comunidade: o papel da prática de meditação e da psicoterapia

A memória e a consciência, como recursos definidores da natureza humana, podem ser também encaradas como instrumentos terapêuticos de autoconsciencialização, *i.e.,* de promoção de um diálogo mente-corpo que tente substituir os sentimentos associados ao *stress* pelos positivos, mais benéficos à saúde.[55]

Retomando alguns pontos-chave, cada indivíduo participa na sua saúde e doença pela via dos sentimentos, das convicções, das atitudes em relação à vida, que influenciam igualmente a resposta à terapêutica. Os estados emocionais e mentais têm, assim, um papel na doença e na recuperação/ /tratamento, pelo que através de técnicas psicoterapêuticas de acesso ao inconsciente, se pode promover a necessária "ressíntese interior" implicada, por exemplo, na evolução significativa da sobrevida e outros fatores em doentes oncológicos.[56]

Técnicas que promovem a capacidade de autoconsciência induzem um processo mental de bem-estar. Este processo mental, que envolve um elevado nível atencional, é apoiado na imaginação e memórias autobiográficas do sujeito. Este estado mental é considerado um estado modificado de consciência em relação à consciência ordinária devido às alterações fisiológicas e mentais do estado de vigília

ordinária e flutuante, de imaginação simples, simulação, sono ou sonho.[57] É um procedimento durante o qual um profissional de saúde ou investigador sugere a um indivíduo uma experiência subjetiva que engloba alterações sensoriais, de perceção, no pensamento e/ou comportamentais.[58]

Para compreender este tipo de intervenções psicoterapêuticas, é necessário estudar as alterações psicofisiológicas que lhe estão adjacentes.[57] Estas podem ser definidas como indutoras de um estado psiconeurofisiológico natural, com suspensão relativa da consciência ou *awareness* periférico. O papel do terapeuta é apenas facilitar o processo terapêutico, produzir e guiar o paciente para a sua autoconsciência.[3,59,60]

O termo estado "modificado" de consciência considera estados menos profundos, induzidos por meios mais suaves como a meditação ou abordagens psicoterapêuticas.[61] Estes estados mentais podem ser espontâneos, induzidos (física, fisiológica, psicológica e farmacologicamente) ou patológicos[61] e têm variações socioculturais.[3]

Segundo Simões,[57] a neuroimagiologia moderna tem permitido visualizar as zonas neuroanatómicas em funcionamento em sujeitos que descrevem acessos de autoconsciência, revelando diferenças em relação à imaginação em vigília. A ressonância magnética funcional teve um papel preponderante na avaliação das alterações do fluxo sanguíneo cerebral regional, descritos pelos sujeitos como "consciência do eu".[62-64]

A autoconsciência parece afetar as funções integrativas do cérebro e alterar a formação da experiência consciente.[61,65,66] Do ponto de vista neuropsiquiátrico, a autoconsciência[67-72] comporta um conjunto de alterações neurofisiológicas temporárias nas quais se manifestam processos psicológicos no comportamento, atribuídos a agentes externos. Tem-se objetivado alteração da atividade cerebral nas áreas associadas à mediação dos processos subsequentes à evocação de memorias autobiográficas, admitindo-se que para o cérebro, imaginar é quase igual a fazer.[57,73] Vários fatores intervêm na resposta a estas técnicas psicoterapêuticas, *e.g.* o estado motivacional e expectativas do indivíduo e a sua experiência subjetiva.[74]

No campo da imunologia, podemos concluir que a autoconsciência é capaz de influenciar o sistema imunitário, reduzindo o impacto de emoções associadas ao *stress* e melhorando a imunocompetência, sendo os resultados dependentes do número ou duração das intervenções psicoterapêuticas. Apontou-se o *stress* como fator de imunossupressão, cuja gestão pode ser feita por intermédio destas, ou de técnicas de relaxamento ou meditação. Desta forma, otimiza-se o sistema imune, o que se refletiu na contagem de populações linfocitárias.[28,29]

Outros estudos apontam ainda para o potencial profilático da tomada de consciência do processo patológico. Este acesso de autoconsciência é capaz de influenciar diversos fatores psicossomáticos da doença, permitindo a melhoria da qualidade de vida, o aumento da sobrevida, menor impacto dos sintomas adversos das terapêuticas farmacológicas e melhoria das estratégias de *coping*.[42] A modulação de parâmetros imunológicos como, por exemplo, a função das células NK ou IL-2, com técnicas meditativas ou de imaginação guiada, pode também desencadear melhores respostas do sistema imune contra o cancro.[75-77]

Sendo assim, no campo da medicina da comunidade, demonstra-se a eficácia destas intervenções tanto na dor aguda, como na crónica, permitindo ainda controlar sintomas mentais acompanhantes de doenças incapacitantes, como a ansiedade, a insónia, náusea e vómitos.[53,54]

Conclusões

A autoconsciência promove o bem-estar físico e mental e contribui para o desenvolvimento de traços emocionais positivos.[78] De modo semelhante, a psicoterapia possibilita ao indivíduo aceder aos seus recursos internos de tratamento, trabalhando as suas emoções e cognições. É assim importante integrar os seus princípios na doença física, como na psicopatologia, e especificamente nas perturbações de ansiedade e do humor.[79] Tornar-se consciente da qualidade flutuante de fenómenos internos ajuda a reduzir as perturbações da perceção concomitante ao desenvolvimento patológico.

Atualmente, as intervenções baseadas em meditação, tais como a redução do *stress* baseado em *mindfulness* (MBSR), são abordagens cognitivo-comportamentais que já estão a ser utilizadas em doença física e mental incapacitante, mas que têm igualmente um campo de inserção na medicina somática preventiva. Diversos estudos são analisados, cujos resultados têm sido promissores e apoiam a sua utilização em práticas terapêuticas.[80]

O médico de família e comunidade, atuando sob o princípio de orientação e advocacia do doente para o seu superior interesse através da promoção da saúde física e mental do mesmo, da sua família

e da comunidade, tem um papel preponderante na adesão aos pressupostos defendidos nesta análise.

Assim, a proposta de uma medicina na comunidade baseada na indução de políticas para o bem-estar e autoconsciência poderá ser uma via para a concretização plena dos pilares da medicina geral e familiar, explanados pela WONCA (World Organization of National Colleges, Academies and Academic Associations of General Practitioners/ /Family Physicians).[81] Estes defendem uma abordagem centrada na pessoa e no seu contexto, direcionada para a capacitação e empoderamento do doente ou indivíduo saudável como agente responsável pela própria saúde.

Deste modo, releva-se a importância de se desenvolver mais e melhor investigação sobre a problemática de uma comunidade orientada para a promoção de saúde e prevenção de doença, permitindo o seu desenvolvimento em medicina e psiquiatria na comunidade. Esta abordagem poderá representar uma mais-valia significativa na qualidade de vida dos doentes e na abordagem terapêutica da doença.

Apesar dos diversos efeitos neurofisiológicos e psicofisiológicos das técnicas clássicas de psicoterapia e de meditação no corpo e nos processos cognitivos e afetivos, *guidelines* e protocolos clínicos necessitam de ser revisitados sob a ótica da saúde na comunidade. Esta análise conceptual levanta como objetivos de investigação a prevenção e tratamento de patologia somática e mental, sob a promoção do bem-estar a nível pessoal e social. Nos próximos anos, espera-se ver o desenvolvimento e validação de mais protocolos adaptados e estratificados a patologia específica.

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Annex G. *Self-Reflexive Consciousness: A Model For The Experimental Use Of Neurofeedback In Sensorial Immersion In A Center For Consciousness Knowledge*

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A state of self reflexive "flow" and trait of consciousness: a model for the experimental use of hypnosis and neurofeedback in sensorial immersion in a centre for consciousness knowledge

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Abstract

The present model aims to develop and optimize an innovative technological resource. Advanced biometric solutions and innovative technologies specifically designed are implemented, combining bio-direct and bio-indirect signals. The model underpinning is aiming to explore the relationship between states and traits of consciousness, meditative practices/clinical hypnosis and physiology. The additional major outcome is to test and optimize immersive technological systems by developing advanced algorithms in multimodal feedback environments.

States and traits of consciousness are converted into scientific data that will be measured by electroencephalogram, electrocardiogram, galvanic skin response, eye tracking and movement detection, and evaluate the impact of distinct immersive approaches. These full set of biosensing technology will collect these states and traits of consciousness and look for their commonalities and differences. The interest of this model is to study the tacit and explicit knowledge created, and applied in a real multidisciplinary site, the centre for consciousness knowledge, a translational laboratory for induction of different states and traits of consciousness, especially values linked to citizenship, happiness and the sense of self.

Keywords

States and traits of consciousness, happiness, self, brain-computer interface, immersive technologies, biosensing, citizenship

INTRODUCTION

There is at present no satisfactory, universally accepted definition of human consciousness and even less so of self-consciousness [1].

Consciousness is a momentary creation or configuration of neural patterns which describe a relation between the organism, on the one hand, and an object or event, on the other. This composite of neural patterns describes a state that, for lack of a better word, one calls the reflexive self or self-consciousness, possibly following the Buddhist definition [2]. That state is the key to subjectivity.

For the purposes of clinical neurosciences, consciousness consists of two basic elements: arousal (i.e., wakefulness, vigilance or level of consciousness) and awareness of environment and of self (i.e., content of consciousness) [3, 4]. By arousal it is referred the behavioral continuum that occurs between sleep and wakefulness. Awareness refers to the thoughts and feelings of an individual.

Compared to awareness of the environment, awareness of self (or self-consciousness) is an even more complex and ill-defined concept, requiring a representation of self vs other [5].

The creation of neural patterns of the self is accompanied by characteristic observable behaviors [6].

The study of consciousness - with induction techniques, like meditative practices - of a state, trait, process, and intervention has proven to be beneficial across a diverse group of psychological disorders as well as for general stress reduction [7].

Yet, there remains a lack of clarity in the operationalization of this construct, and underlying mechanisms [8], and the possibility of combining it with immersive biosensing technologies.

The neurophysiological changes induced by meditative practices are of two types: (1) state changes and (2) trait changes [9].

State refers to the altered sensory, cognitive, and self-referential awareness that can arise during an activity, whereas trait refers to the lasting changes in these dimensions that persist over time [10, 11, 12]. State changes after meditations are reported to include a deep sense of calm peacefulness and experiences of perceptual clarity and conscious awareness [13, 14, 15].

Trait changes - that persist over time - include a heightened awareness of the sensory field, thoughts, feelings, and the experience of self. This experience consists of contentless awareness that is independent of mental activities, that can be present during deep sleep, and produces the perception of an altered self-identity [12, 15, 16, 17]. This awareness is related to the isolated self-identity.

By that point of view, there are simple and complex degrees of consciousness, and therefore different states and traits of consciousness. The simplest kind - (1) "core consciousness" - provides the organism with a sense of self about one moment and generates the sense of "core self", a transient form of self. The complex kind - (2) "extended consciousness" - provides the organism with an elaborate sense of the historical self, known as the traditional notion of self [6].

This model offers the chance to study a possible intervention on the modulation of consciousness components. Moreover, the approach described in this model might generate solutions to the improvement in the average levels of happiness, citizenship and wellbeing in general populations, not accomplished by psychotropic drugs and traditional psychotherapy methods [18].

Unfortunately, for the time being, consciousness or self-consciousness cannot be measured objectively by any device [1], so there is therefore a problem to evaluate the subjective experience of self-consciousness in another person [19].

However, some progress has been done with (1) event-related potentials as a complimentary tool to objectively investigate cerebral mechanisms underlying the processing of self-referential stimuli [20]; or (2) neuroimaging studies results, suggesting that self-referential processing is mediated by cortical midline structures that constitutes the "core self" and is critical for elaborating experiential feelings of self [21].

To solve this theoretical and practical problem, a mixed set of different technologies involving: (1) brain-computer interfaces, (2) virtual and immersive environments, (3) augmented reality and (4) multimodal neurofeedback stimulation is proposed in this model. This follows the studies and technology published as The Sensorium [22], which has been used also for solving this problematic.

A brain–computer interface is a system that enables humans to interact with their environment by translating their brain signals into control commands for a external device [23].

Virtual environments are "synthetic sensory information that leads to perceptions of environments and their contents as if they were not synthetic" [24]. Immersive virtual environments in turn, are ones that 'surround' an individual and create the perception they are enclosed within and interacting with environments that provide a continuous stream of stimuli [25]. Immersive virtual environments are created through the integration of various hardware and software systems [24, 26].

Augmented reality is a technological system that allows inserting virtual contents in the real world in order to run in the same representation and, in real time, enhancing the user's sensory perception of reality [27]. They are compared to virtual reality systems but characterized by a computer-generated environment that elicits a strong user's experience of "presence" [28, 29].

Multimodal neurofeedback stimulation is a technique in which the subject, using awareness and auto neuromodulation of its own brain activity in real time, searches for a given desired outcome, for instance, the improvement of a given condition [30].

In this model, through the set of technologies previously described, individuals will be able to modulate and voluntarily control endogenous brain rhythms via multisensorial feedback in real-time [31].

The Sensorium [22], presented as proof of concept, is a multimodal neurofeedback environment which allows the user to self-experience the own ongoing brain and body signals in sound and light. This extended self-experience can simulate meditative practices and lead people into an enhanced body experience.

At this point, the authors postulate that immersive environments and brain computer interface techniques may be used to promote subjective happiness states and to train citizenship values. Those techniques might show neurophysiological and neuroimagiological evidences of full reward system activation: prefrontal cortex, nucleus accumbens and ventral tegmental area, as seen in meditation used as induction technique of a state and trait of consciousness [32].

In any case, brain waves have not been directly and actively manipulated. This may be achieved via brain stimulation using a multimodal neurofeedback approach, extending the recent work of Hinterberger [22].

Combined approaches, with (1) neurofeedback in immersive environments and with (2) meditation, might also promote active learning and training of a specific state and trait of consciousness [33, 34].

The previously described approach to states and traits of consciousness induction allows people to experience their non-perceptible body processes through the modeling of a series of stimuli such as soundscapes and lightscapes [22]. Also it is reported [22] an induction of a subjective state of happiness, as it is achieved through a mindful meditation experience.

Moreover, it is possible to create a parallel to the multisensorial stimulation provided by snoezelen rooms, where users report being stimulated and soothed [35]. This stimulation allows the achievement and maintenance of a subjective state of happiness and long term well-being, matching the concept of subject orientedness/centeredness, defined as an intervention developed for the subject and by the subject actively [36].

Concluding, virtual immersive realities induced by brain computer interfaces, as in lifelike experiences, create a full immersive state which is needed to achieve a desired state and trait of consciousness [37, 38].

The final aim of this model is therefore the creation of Centre of Consciousness Knowledge, modeled as mixed museum and laboratory with brain computer interface experimentations on states and traits of consciousness. The proposition in this paper is that happiness and self-consciousness, as a learnt skill, might be induced through these different but combined techniques, in different experimental activities.

With it, it is hoped to give contribution to the creation of a uniquely tailored system to address the needs of more citizenship and the population well-being in the future.

METHODOLOGY

Methodological Frameworks

In order to develop activities that integrate biosignals with states and traits of consciousness, a physiological model will be implemented. The purpose is to support the integration of biosignals in the different activities.

This neuroscientific approach of states and traits of consciousness look for neuronal correlates of different activities/tasks. With this set of technologies task specific occurrences are identified and correlated to specific human characteristics and values, e.g. empathy, compassion, self-consciousness, meditative and hypnotic states, subjective happiness states; focused attention and open-monitoring attention.

The integration of immersive and non-immersive technologies in the Centre of Consciousness Knowledge, comprising virtual and augmented realities, user dynamic interfaces, brain-computer interfaces, biosignals monitoring and biofeedback will comprise the following types of technology:

a) Virtual and augmented realities: are the most immersive technologies (e.g., Oculus Rift (https://www.oculus.com/rift/), holography-like immersive modelion systems). The first one allows the creation of a virtual world that users can interact with. The second one consists on the blending of virtual reality and real life. These two approaches have the potential to recreate completely virtual and mixed scenarios that enhance the user experimentation with a given universal value (e.g. empathy, compassion).

b) User dynamic interfaces: (e.g., Leap motion (https://www.leapmotion.com/) - hand movement detection sensor, Kinect for Windows (https://www.microsoft.com/en-us/kinectforwindows/default.aspx) - body movement detection sensor, EyeTribe (https://theeyetribe.com/) - eye movement and tracking sensor) can be used to improve the engagement between the user and the activity itself. For the purpose of this model several technologies will be used.

c) Biosignals monitoring and biofeedback: several biosignals will also be acquired in order to assess the physiolog-

ical response of the user during the activity performance including electroencephalo-graphy (EEG), electrocardiography (ECG), Galvanic Skin Response (GSR) and pulse oximetry. For that purpose three main devices will be used: Neurosky's Mindwave Mobile (http://neurosky.com/), a device that allows the recording of 1-channel EEG and the assessment of one's Attention and Meditation levels; an in-house developed headset, a device that allows the recording of 2-channel EEG integrated with an accelerometer and also an oximeter; and, finally, an in-house developed emotional device, a 1-channel ECG device integrated with a 1-channel GSR.

Tools Architecture, Development and Processing

A division between bio-direct and bio-indirect signals is considered in order to describe the processing of both types of signals. Bio-direct, in this model, are defined as those who are physiological signals (e.g. electro-encephalogram) and so, they are used as direct biological signals and the bio-indirect, in this model, are defined as signals acquired using sensors that track the human behavior (e.g. eye and body movement tracking).

a) Bio-direct signals: processing algorithms will be implemented to analyze the acquired data. For each one of the acquired biosignals a specific metric will be computed and used to get physiological information about the individual during each activity. In the case of EEG signals, focus will be given to its power spectrum since the analysis of each frequency band (known as Gamma, Beta, Alpha, Theta and Delta bands) allows distinguishing different levels of attention and meditation. Also, using EEG as a physiological response one can identify emotions during different activities. Once again, algorithms will be developed to reach and to make a distinction among positive, neutral and negative emotions.

Both ECG and GSR are commonly used to monitor stress levels of an individual and they are the basis of the well-known polygraph test. Using both measures one aims to reach an algorithm capable of evaluate the human conviction when facing an absolute truth and when facing a disguised preconception. This step forward is crucial to understand states and traits of consciousness in a neuroscience perspective. From pulse oximetry acquisition the goal is to extract the heart beat rate that also correlates with different activities which in turn enhance different emotions causing different biofeedback.

The final goal is to be able to use individual and/or group biofeedback to control sound/light and video environment.

b.) Bio-indirect signals:

Bio-indirect signals processing will be implemented for both body movement (using Leap motion and Kinect for Windows) and eye tracking (EyeTribe) technologies.

The data processing of the first one, body movement, will allow the identification of both facial and all-body movements regarding different activities and so, regarding different emotions. The output from this technology will provide the tracking of the face and body. The second one, eye tracking technology, will be used by the user to answer questions with the eyes by looking and focusing a specific region of interest (ROI) on a specific picture. The processing of gaze coordinates will allow getting the time spending by the user looking to each ROI which in turn will provide an answer given by the eyes.

The processing of both bio-direct and bio-indirect signals will be performed in C# and MATLAB languages using Windows OS and Android OS, for better adaptation to the Center of Consciousness Knowledge user's profile, i.e. children, students and museum attenders.

Multisensorial Stimulation in Neurofeedback environment

As an example of the previously described technologies, an application is described.

In an isolated room for sound and light, a set of audio-visual equipment is installed: Yamaha HS7 sound monitors; professional audio interface and Oculus Rift virtual reality goggles and BCI developed for mind music. Brain waves, recorded with EEG, will be addressed to a synthesizer via MindWave Mobile headset (Neurosky), device which, in turn, will transform brain waves into digital audio and visual stimuli. Brain wave patterns will drive the sound, sequence, and timbre of the musical performance and the correspondent video effects in the multisensorial stimulation in neurofeedback environment.

The sonification of the brain wave patterns will be made by attention and meditation algorithms, but also by EEG power spectrum bands modulation (Gamma, Beta, Alpha, Theta and Delta). The sonification is made by a conversion of EEG signals to MIDI, a musical instrument digital interface. The digital music composed controls, after, light and video atmospheres.

This sonification and visual immersion on the subjects own neurophysiological patterns should improve the perceptions of the "core-consciousness" and a better identification of the "core-self". With the algorithms and power bands modulation described before, an induction technique, namely mindful meditation, should be created in a virtual immersive environment. Depending on the subjects own orchestration, that is controlled by neurofeedback, the music created, can be more melodic, harmonic and rhythmic, generating a general attention and meditative practice. This meditative state might induce a subjective state of happiness controlled by the participant.

CONCLUSION

The overall scope of the model is to propose an innovative set of technologies of induction techniques of states and traits of consciousness. These induction techniques are made by (1) brain-computer interfaces, (2) virtual and immersive environments, (3) augmented reality and (4) multimodal neurofeedback stimulation. These different technologies are implemented in the Centre of Consciousness Knowledge*.

Concluding, states and traits of consciousness, e.g. subjective happiness or self-consciousness and citizenship consciousness, might be induced in a museum setting.

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Annex H. *Transcultural Perspective On Consciousness: Traditional Use Of Ayahuasca In Psychiatry In The 21st Century In The Western World*

Review; *Facsimile*
Published with PhD supervisors as complementing doctorate achievement

Cosmos and History: The Journal of Natural and Social Philosophy, vol. 12, no. 2, 2016

TRANSCULTURAL PERSPECTIVE ON CONSCIOUSNESS:

TRADITIONAL USE OF AYAHUASCA IN PSYCHOTHERAPY IN THE 21ST CENTURY IN WESTERN WORLD

T. Re, J. Palma, J.E. Martins & M. Simões

"…imagine if you could have the cathartic breakthroughs that ten years of hard therapy might give you in one afternoon."

– Jason Silva (2015)

ABSTRACT: New research on ayahuasca's potential applications in psychiatry have been undertaken in the last few years. It is relevant to start by pointing out that this south American brew has a very low potential for abuse, having no associated psychiatric or neuropsychological disorders. P.The most studied area in which ayahuasca may be applied is drug addiction. Interest for the understanding of ayahuasca's therapeutic potential in drug addiction is to determine by which mechanism the brew acts in order to promote behavioural and personality changes that allow freedom from addiction.

Some authors consider that the benefits provided by ayahuasca may arise from a symbiotic action between pharmacology and altered consciousness state, with some volunteers reporting an understanding of how some life happenings may have contributed to their depression and an achievement of a blissful state, which continued throughout time.

KEYWORDS: Drug addiction; Ayahuasca's potential applications; Antidepressant action; Anxiety

New research on ayahuasca's potential applications in psychiatry and psychoteraphy have been undertaken in the last few years. It is relevant to start by pointing out that this south American brew has a very low potential for abuse, having no associated psychiatric or neuropsychological disorders (Grob et al., 1996; Da Silveira et al., 2005; Gable, 2007; Halpern et al., 2008; Fábregas et al., 2010).

The most studied area in which ayahuasca may be applied is drug addiction. A Canadian study conducted by Thomas *et al.* assessed the impact of having ayahuasca ceremonies integrated in a rehabilitation programme in the form of a retreat (Gabor Maté's *Working with Addiction and Stress*). For that, 12 volunteers were selected and assessed by several psychometric instruments: *Difficulty in Emotion Regulation Scale* (DERS), *Philadelphia Mindfulness Scale* (PHLMS), *Empowerment Scale* (ES), *Hope Scale* (HS), the *McGill Quality of Life* questionnaire and the *4 Week Substance Use Scale* (4WSUS), which were given at baseline and repeated two and four weeks after the retreat, as well as monthly for five months (Thomas et al., 2013). Furthermore, the *State of Consciousness Questionnaire* (SOCQ) was given immediately after the retreat. The referred instruments were chosen because their analysis fits the bio-psycho-social model of human behaviour, trauma and drug addiction: though them it is possible to assess four factors which influence the drug abuse pattern (Emotion Regulation, Mindfulness, Empowerment and Hopefulness). Furthermore, while the *McGill Quality of Life* questionnaire is both a potential influencer of patterns of substance use and an outcome measure, the 4WSUS is the main outcome predictor, by assessing problematic substance use (Thomas et al., 2013).

Thomas *et al.* found out that the volunteers' score for the *McGill Quality of Life* questionnaire increased with statistical significance over time, and consistently after a slight decrease between the first and third follow-ups (figure 1). Both Hopefulness and Mindfulness criteria had a linear and statistically significant increase (figure 2) (Thomas et al., 2013).

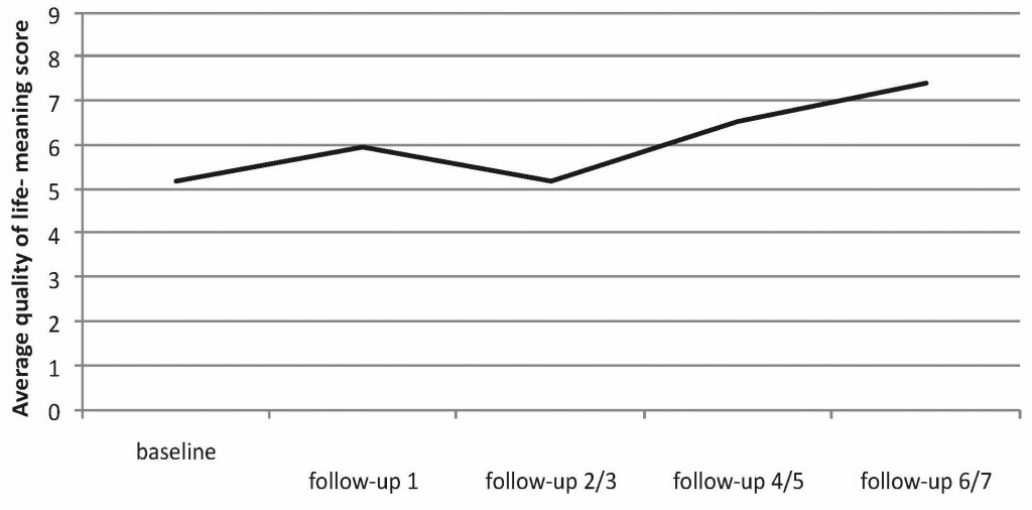


Fig. (**1**) – Average *McGill Quality of Life* scores through time (Thomas et al., 2013)

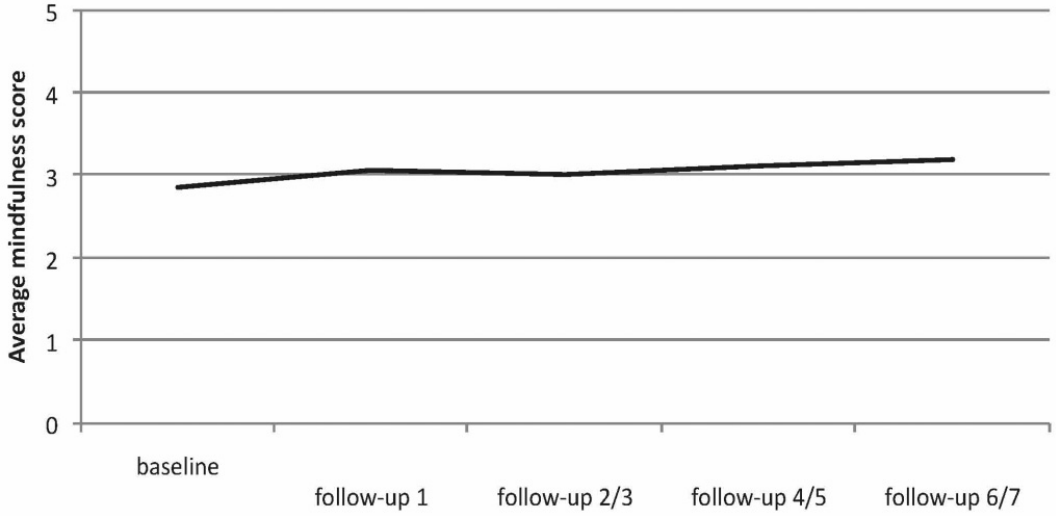


Fig. (**2**) – Average Mindfulness scores through time (Thomas et al., 2013)

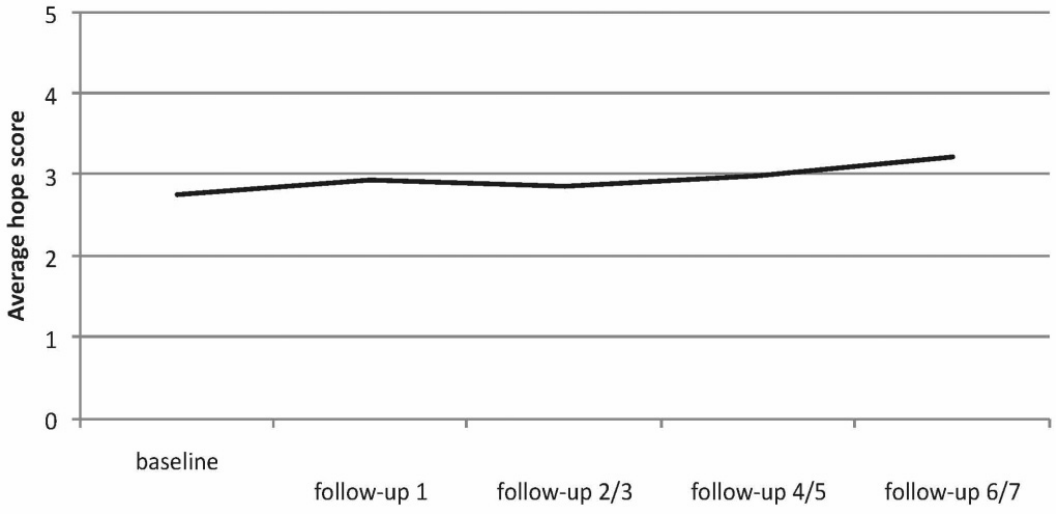


Fig. (**3**) – Average Hopefulness scores through time (Thomas et al., 2013)

Finally, it can be observed that the 4WSUS score decreased for all assessed drugs, having this decrease statistical significance for cocaine (figure 4). The only exceptions were *Cannabis*, sedatives and opiates, probably because these were medically prescribed to the subjects (Thomas et al., 2013).

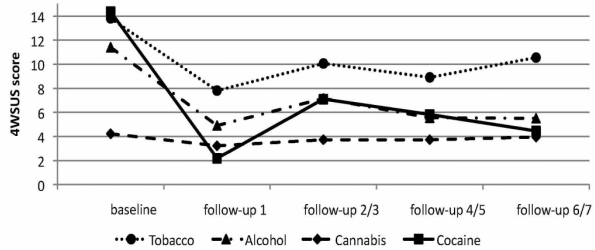


Fig. (4) – Average 4WSUS scores for tobacco, alcohol, *Cannabis* and cocaine (Thomas et al., 2013)

It can be concluded that this therapy model seems to be associated with statistically significant improvements in factors which hold an inverse relationship with drug addiction (Thomas et al., 2013).

Interest for the understanding of ayahuasca's therapeutic potential in drug addiction is to determine by which mechanism the brew acts in order to promote behavioural and personality changes that allow freedom from addiction.

One hypothesis lays on ayahuasca's pharmacology. Currently, the well-accepted hypothesis regarding neurochemical mechanisms of addiction suggests a dopaminergic hyperactivity in the mesolimbic pathway. This pathways is constituted by three critical brain areas, the ventral tegmental area (VTA), a neuronal group which releases dopamine when subjected to internal or external stimuli associated with addictive behaviour, the nucleus accumbens (NA), which receives dopaminergic signals from the VTA and the prefrontal cortex (PFC), directly connected to the VTA and indirectly connected to the amygdala. It is proposed that ayahuasca may reduce the dopamine levels in the mesolimbic pathway through its action upon the serotonin receptors (Liester and Prickett, 2012).

Firstly, while DMT is regarded to be a full agonist of $5HT_{2A}$ receptors, β-carbolines may also act as agonists of these receptors. It is also known that $5HT_{2A}$

stimulation reduces the dopaminergic levels in the mesolimbic, nigrostriatal and mesocortical pathways (Liester and Prickett, 2012), either because the agonism of postsynaptic $5HT_{2A}$ receptors in dopaminergic pathways has a direct inhibitory effect on the dopamine release, or because of the existence of connexions among dopaminergic and serotoninergic pathways established through GABAergic interneurons, which inhibit dopamine release (Liester and Prickett, 2012).

Secondly, it is known that dopamine is the primary inhibitor of prolactin synthesis. Since $5HT_{2A}$ agonism promotes prolactin synthesis (Liester and Prickett, 2012), it is fair to assume that this happens by dopamine inhibition. Regular ayahuasca users present high prolactin levels (McKenna, Callaway and Grob, 1998), which further supports this hypothesis.

Being so, it can be concluded that ayahuasca reduces dopaminergic activity in the mesolimbic pathway through the pharmacological actions of DMT and possibly β-carbolines.

Another therapeutic topic of interest resides on β-carbolines' action upon DATs, since these alkaloids inhibit DYRK1A, a kinase found in the striatum which act as an important modulator of dopaminergic transmission and in dopamine vesicles' endocytosis (Brierley and Davidson, 2012).

Considering that DYRK1A acts through the inhibition of DAT's membrane trafficking (Brierley and Davidson, 2012), β-carbolines may promote a balance between the intracellular and extracellular dopaminergic concentration by preventing the kinase's action. This happens since, with the inhibition of DYRK1A, a higher number of DAT will be available for dopamine reuptake, countering the kinetic turnover promoted by several drugs. This contributes to the decrease of excessive extracellular levels of this neurotransmitter and consequently to the compensatory increase of its maximum reuptake index.

Finally, some studies show that harmine and norharman can reduce morphine withdrawal's biochemical effects upon tyrosine hydroxylase, reducing the symptoms of its abstinence syndrome, which can be generalized for all opiates withdrawal (Aricioglu-Kartal, Kayir and Uzbay, 2003; Miralles et al, 2005). Despite its explanation not being clear yet, it encompasses these β-carbolines' high affinity for imidazoline receptors, namely I_2, since another drug selective for these receptors, LSL, obtained a similar effect (Miralles et al, 2005).

However, the pharmacological mechanisms appear not to fully explain all of ayahuasca's potential. Another theory rises, based on the psychosomatic effects of this brew. In a extensive ayahuasca projects review, Loizaga-Velder obtained results which indicated that the involvement of certain individuals in ayahuasca's sessions would

lead to a lack or substantial reduction of drug use (Loizaga-Velder, 2013). It was possible to conclude that this brew can act as a catalyst for psychotherapeutic processes obtaining results in a shorter time, with greater efficacy and, at times, when other therapeutic strategies fail (Loizaga-Velder, 2013), given that there is an adequate set, setting and integration process (Loizaga-Velder and Verres, 2014). In order to explain the mechanisms by which this happens, three effect groups should be considered:

The first group includes ayahuasca's somatic effects. Emetic episodes, denominated *purges*, are commonly recounted after the brew's intake. Purges are reported to be powerful inductors of deep altered states of consciousness which together with the emetic episode itself, helps releasing psychological burdens, leading to a sensation of redemption, relieve, clarity and reduction of craving (Loizaga-Velder, 2013). A volunteer of a studies carried by Santos *et al.* declared having vomited large quantities of alcohol and cocaine (marães dos Santos and ano Holanda, 2006). More important than to understand whether this statement derives from a synesthetic process, a metaphorical hallucination or if the volunteer really felt the smell of alcohol and saw cocaine, is to realize the transformative potential that this experience had on the individual, who claims it was fundamental to solve her addiction problem (marães dos Santos and ano Holanda, 2006).

The second group relates to personal experience (Loizaga-Velder, 2013), being subdivided in psychobiographical and emotional effects. In the first subgroup, there are episodes in which traumatic live happenings are relived by the patient, however from a new, more integrated and functional perspective. It is possible, through these experiences to grasp how past choices and behaviours influence the present life situation (Liester and Prickett, 2012). Some volunteers reported that, through ayahuasca, they understood they were embarking upon a "selfdestructive lifestyle, which would inevitably lead to their ruin or even death, unless they operated a radical change on their personal conduct and orientation" (marães dos Santos and ano Holanda, 2006). Another type of ayahuasca's psychobiographical effect is the visualization of hypothetical future life situation which derive from their choices in the present (Liester and Prickett, 2012). It is also relevant to stress the facilitation of introspection, since ayahuasca's altered state of consciousness can lower the psychological defence mechanisms. Being directly confronted with their own behaviours and feelings in such personal way (since they derive directly from the self) instead of these being exposed through therapy (which often lead to denial), the patient is incapable of refuting their reality, embracing change (Loizaga-Velder, 2013).

In the emotional effects subgroup, it is important to highlight an increase in empathy, which improves communication and interpersonal relationships,

contributing to a positive emotional feedback, which counteracts the isolation characteristic of drug addiction (Loizaga-Velder, 2013). Furthermore, ayahuasca is capable of generating catharses and abreactions (marães dos Santos and ano Holanda, 2006) with reparatory effects on the emotional level (Mabit, 2007).

Finally, the third group of effects act upon a transpersonal level, providing the patient with a connexion with Nature, contact with the Divine or the sense of being part of a Universal Consciousness (Loizaga-Velder, 2013), a feeling resembling the Hindu and Buddhist metaphorical concept of Indra's Net of Jewels. Generally, these experiences are preceded by an episode of quasi-death or ego death. Transpersonal experiences' efficacy lays on their capacity to elicit insights about the patients' personality: during ayahuasca's altered state of consciousness, the living of symbolic although seemingly real death followed by a contact with the Divine with properties of a *mysterium tremendum et fascinans*, promotes a reflexion of personal values (marães dos Santos and ano Holanda, 2006). Loizaga-Velder and Verres found out that 9 of the 14 interviewed stated that, with ayahuasca sessions, their cravings disappeared, having two thirds of them attributed this to the reflexion induced by transpersonal experiences (Loizaga-Velder and Verres, 2014). After the interviews, patients reach conclusions, which enable a growing sense that life has a meaning, usually connected with spirituality (Loizaga-Velder and Verres, 2014). Drug abuse is seen as a tragic mistake caused by a misunderstood spiritual longing for Transcendence (Berges and Fábregas, 2013). It can be argued that Transcendence is but an alteration of basal consciousness state, which is an integral part of human life, attainable without the use of psychoactive substances (though dreams, extreme pain, meditation…) (Mabit, 2007). Bearing in mind that these consciousness alterations are indispensable and represent a need for spiritual fulfilment, populations should be educated on how to access these Transcendence states in a safe and beneficial way. It is worthy noticing that despite a close relationship with entheogens, indigenous populations only suffered from collective drug abuse problems after contacting with Western societies and being subjected to acculturation (Mabit, 2007). This may have happened because indigenous people can easily integrate altered states of consciousness in their social, psychic and social structure, in a coherent manner for the individual and his reference group (Mabit, 2007). Such does not happen in Western culture, which tends to be self-referring and lacking the spiritual and psychosocial tools. Therefore, the individual is "imprisioned" in a alluring state in which daily reality becomes insipid. Thus begins the process of dissociative dependence (Mabit, 2007).

Another set of conditions possibly treated with ayahuasca sessions are anxiety and panic disorders. The currently accepted first line treatment from such are SSRIs

(Bandelow et al., 2012, Baldwin et al., 2014). It is possible to be understood through the neuropharmacological action of these drugs that the disorders' symptoms are attenuated by a serotoninergic activation, promoted also by ayahuasca. In a study conducted by Santos *et al.*, 9 volunteers with more than 10 years of experience with ayahuasca sessions taken each two weeks were surveyed through the State-Trait Anxiety Inventory (STAI), Anxiety Sensitivity Index (ASI-R) and Beck Hopelessness Scale (BHS) (Santos et al., 2007), R. G . The three instruments were filled by all at the beginning of the experiment, after the volunteers drank a placebo resembling ayahuasca. The methodology was repeated for the following two weeks, with either placebo or ayahuasca given to participants before answering the questionnaires. The results show a statistically significant decrease in despair and panic, measured respectively by BHS and ASI-R, without, however, affecting anxiety traits (STAI) (figure 5) (Santos et al., 2007). Regarding this, it can be argued that volunteers began the study with low levels of anxiety, therefore ayahuasca's potential to reduce anxiety would not be detected (Santos et al., 2007).

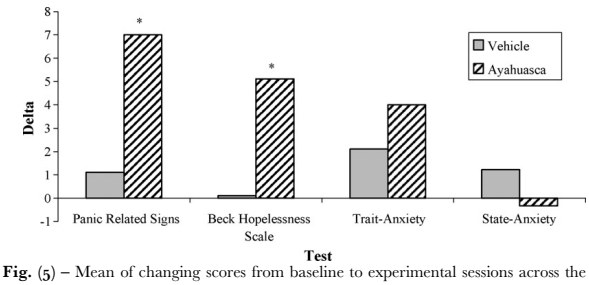


Fig. (5) – Mean of changing scores from baseline to experimental sessions across the four different test. A positive delta-value shows a reduction in score, while a negative value reflects its increase. Asterisk (*) indicates a statistically difference between vehicle and ayahuasca treatments with a significance level of P<0.05 (Santos et al., 2007)

The results can be caused by the pharmacological properties of both DMT and β-carbolines, namely their effect upon serotonin receptors and MAO-A, respectively (Santos et al., 2007).

Finally, there is an apparent benefit from ayahuasca's consumption for major depressive disorder. In a study conducted by Osório *et al.* on six volunteers with clinical diagnosis of treatment-refractory depression, several mood-related psychometric

parameters were assessed using the Brief Psychiatric Rating Scale (BPRS), Young Mania Rating Scale (YMRS), Hamilton Rating Scale for Depression (HAM-D) and Montgomery-Åsberg Depression Rating Scale (MADRS) before and several times after (from 40 minutes to 21 days) a single ayahuasca consumption (Osório et al., 2015). HAM-D and MADRS suffered a similar variation pattern, decreasing continuously until the fourteenth day and having a statistically significant score reduction at the last checkpoint (figure 6) (Osório et al., 2015). There was also reported a decrease in BPRS, manly the parameter which measures anxious depressive symptoms (BPRS-AD), supporting antidepressant and anxiolytic effects of ayahuasca (Osório et al., 2015).

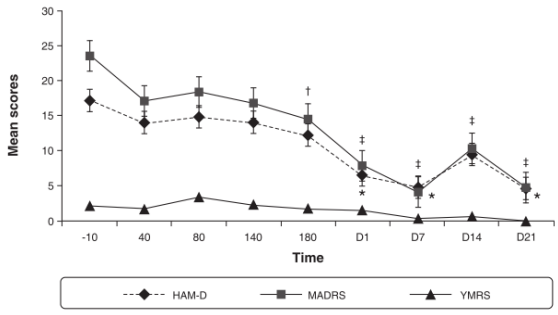


Fig. (6) – Temporal distribution of mean scores on the HAM-D, MADRS, and YMRS (Osório et al., 2015)

This acute antidepressant action, as well as the maintenance of results 21 days after a single dose intake is one of the most promising aspects of ayahuasca use in this context, since current medical therapy takes 2 to 4 weeks before reaching its peak effect and patients usually enter remission after 6 weeks (Liester and Prickett, 2012; Baldwin et al., 2014) (Osório et al., 2015). It is also worthy to highlight that ayahuasca had promising results in conditions which failed to respond to conventional treatment, being this demonstrated in other two studies (de Lima Osório, 2011; Sobiecki, 2013).

The mechanisms of action may again reside on ayahuasca's effect on the 5HT receptors (Ray, 2010) and on MAO-A inhibition by β-carbolines (Riba et al., 2003), which increase the availability of serotonin and other monoamines involved in the

etiology of depression. Furthermore, a study showed a decrease in BOLD signals in structures of the Default Mode Network (DMN), such as the anterior and posterior cingulate cortex, medial prefrontal cortex, precuneus and in the inferior parietal lobe after ayahuasca intake (Palhano-Fontes et al., 2014). A study by Sheline *et al.* reported a hyperactivation of DMN in major depressive disorder, probably because this condition is associated with a pathological inability for DMN to regulate self-referencial activity in an appropriate way (Sheline et al., 2009).

Some authors consider that the benefits provided by ayahuasca may arise from a symbiotic action between pharmacology and altered consciousness state, with some volunteers reporting an understanding of how some life happenings may have contributed to their depression and an achievement of a blissful state, which continued throughout time.

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Annex I. *Presença De Genes De Resistência A Antibióticos Na Cavidade Oral: Uma Revisão Sistemática*

Review; *Excerpt*
Published with PhD supervisors as complementing doctorate achievement

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#097. Presença de genes de resistência a antibióticos na cavidade oral: uma revisão sistemática

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Objetivos: O uso excessivo e, muitas vezes, desnecessário de antibióticos pode originar a seleção de genes de resistência a antibióticos. Os biofilmes, especificamente os orais, são conglomerados bacterianos que potenciam a preservação dos genes de resistência a antibióticos. Além disso, há estudos que destacam a propagação dos genes de resistência a antibióticos para outros locais do organismo. Deste modo, é de extrema importância compreender quais são os genes de resistência a antibióticos na cavidade oral, como se pode fazer a sua determinação e estimar o seu impacto na ecologia da cavidade oral. Pretendeu-se verificar: (i) quantos estudos foram realizados in vivo, em amostras com origem na cavidade oral; (ii) que métodos foram utilizados para a deteção dos genes de resistência a antibióticos; (iii) e quais os genes de resistência a antibióticos encontrados.

Materiais e métodos: A revisão bibliográfica foi realizada na base de dados PubMed® do NCBI (19-04-2016), com a seguinte estratégia: acrescentou-se sucessivamente cada grupo de palavras-chave: pesquisa 1 – «antibiotic resistant bacteria» AND «oral biofilm» AND «saliva» AND «mouth»; pesquisa 2 – «antibiotic resistance» AND «oral biofilm» AND «saliva» AND «mouth». Foi obtido um total de 254 artigos científicos, analisados quanto à metodologia utilizada e respetivos resultados. Adicionaram-se 20 artigos referenciados por um artigo da primeira pesquisa. Desse total de 274, foram excluídos os artigos com objetivo de testar terapias alternativas aos antibióticos, e estudos em Candida, ficando 135 artigos. Destes foram selecionados apenas os estudos realizados na cavidade oral, obtendo-se 50 artigos, dos quais 30 referem a presença de genes de resistência a antibióticos.

Resultados: Dos artigos selecionados a maioria utiliza exclusivamente técnicas de cultivo (46,7%), 6,7% usam a reação de polimerase em cadeia e 3,3% a versão quantitativa da reação da polimerase em cadeia. A título de exemplo, foram encontrados 18 genes de resistência a antibióticos ß--lactâmicos, na cavidade oral.

Conclusões: Dos poucos estudos focados na cavidade oral, verifica-se a existência de genes de resistência a antibióticos no biofilme oral. É, deste modo, de extrema importância realizar estudos de quantificação de genes de resistência a antibióticos, de forma a conseguir avaliar o impacto no microbioma oral.

http://dx.doi.org/10.1016/j.rpemd.2016.10.095

Annex J. *Transcultural Perspective On Consciousness: A Bridge Between Anthropology, Medicine And Physics*

Conceptual Analysis; *Excerpt*
Published with PhD supervisors as complementing doctorate achievement

Cosmos and History: The Journal of Natural and Social Philosophy, vol. 11, no. 2, 2015

TRANSCULTURAL PERSPECTIVE ON CONSCIOUSNESS: A BRIDGE BETWEEN ANTHROPOLOGY, MEDICINE AND PHYSICS

ABSTRACT: The Unesco Chair "Anthropology of Health, biosphere and Healing System" inside the University of Genoa (IT) is a unique experience inside the University of Genoa that stems from a cultural necessity to fill and a wealth of knowledge to preserve health, environment and treatment strategies considered strictly connected in modern medicine. This new, integrated approach contradicts and overcomes the traditional separation between humanities and scientific medicine and treatments. Health and approach to treatment strategies are not uniform around the worlds; the universal baseline is quality assurance of investigation in science

The need to establish connections between Medicine, especially in the therapeutic aspect (healing), and all the information already obtained from the mind-matter phenomenology has led to much experimentation and theorizing in this border and transcultural area.

The research group formed by anthropologist who have studied altered states of consciousness in different cultures, medical doctors, quantum physicists and molecular biologists will try to define a transcultural perspective on consciousness merging anthropology, medicine and physics.

In particular, the research field site is located in Mayantuyacu, a traditional healing center located in the Peruvian Amazon where the ancient art of *ashanika* healing is set.

Mayantuyacu is situated on the bank of a river with thermal water at 100 ° flowing in the middle of the forest. Around the central Maloca, where is the common life, were built to accommodate malocas other people who come to Mayantuyacu to know and to seek treatment from knowing millennial ashanika and properties of thousands of plants including plants teacher.

The following elements were firstly analyzed and considered the bridge from a traditional healing system to a new paradigm in medicine:

1. music called *icaros*,
2. master plants like *ayahuasca* involved during the healing ceremonies.

KEYWORDS: Ethnomedicine; Teaching plants; Electromagnetic energy; Consciousness

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Annex K. *External Support*

Molecular Cognition Strategy (2016, October 16). **(- 6 -)**
https://www.limmit.org/uploads/2/6/8/4/26841837/mindbraincollege26out2016molecularcogstrategy.pdf



Molecular Cognition Group Strategy

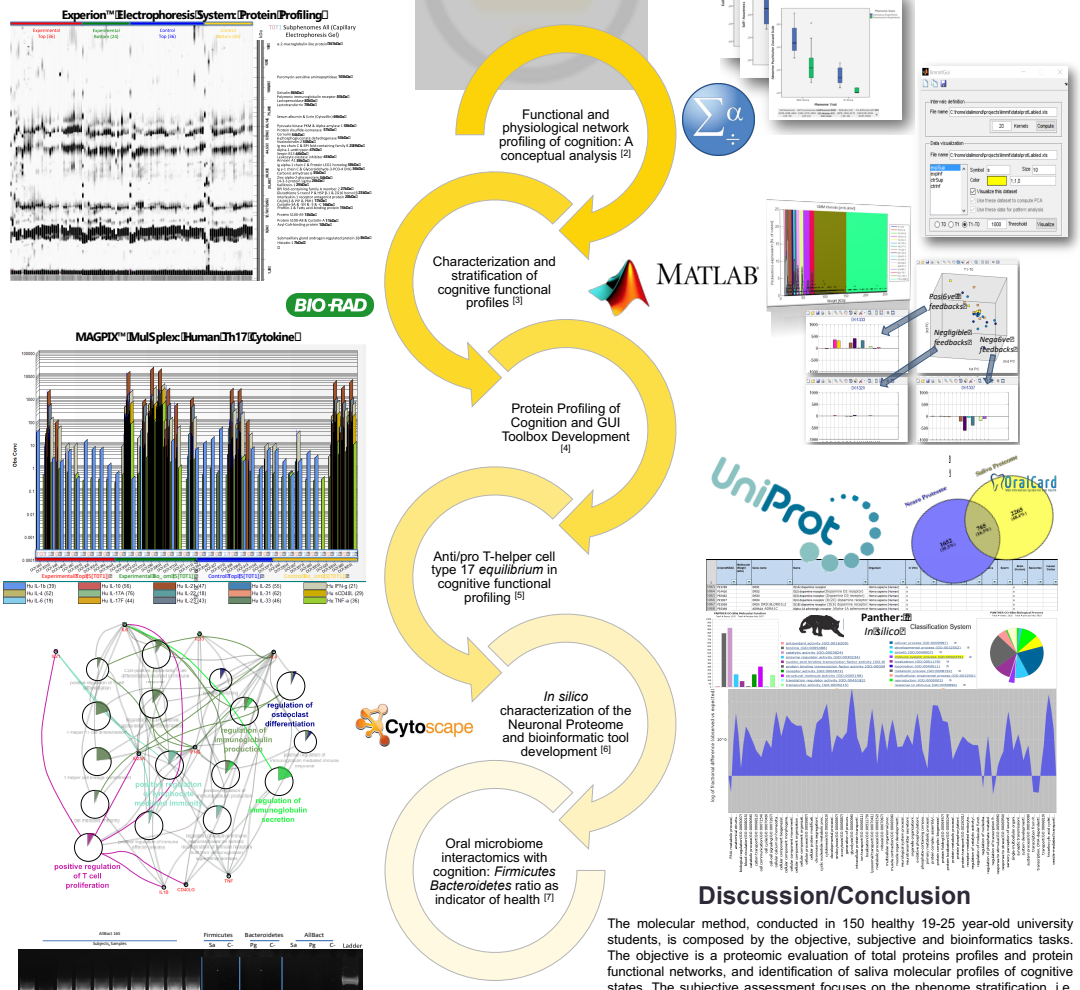
[1,2]Martins, J. E., [1,2]Simões, J.F., [1,2]Sousa, S., [1,2]Hipólito, I., [1,2]D'Alimonte, D., [1,2]Rodrigues, T., [1]Rosa, N., B., [1]Correia, M.J., [1]Simões, M. , [1]Barros, M.

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Introduction

Neuronal functional protein networks found in the oral proteome are possibly useful to characterize a healthy phenome state and trait. Proteomics have extensively and objectively epitomized the understanding of healthy and pathological physiological states or conditions.[1] Our group aims at the molecular characterization of cognition.

Strategy



Discussion/Conclusion

The molecular method, conducted in 150 healthy 19-25 year-old university students, is composed by the objective, subjective and bioinformatics tasks. The objective is a proteomic evaluation of total proteins profiles and protein functional networks, and identification of saliva molecular profiles of cognitive states. The subjective assessment focuses on the phenome stratification, i.e. the psychological and phenomenological characterization. The bioinformatics assessment is a systems biology data correlation between the objective and subjective assessments, thru the development of a graphical user interface toolbox for data exploration and visualization.
The main outcomes of this study are the neuronal-oral proteome characterization, the identification of salivary healthy cognitive molecular networks and multi-omics data-mining solutions. [8]

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[8]Martins, J.E., Hipólito, I., Simões, J.F., Sousa, S., Rosa, N., D'Alimonte, D., Marques, T., Manadas, B., Correia, M.J., Simões, M., Barros, M. (2016/2017). MultiOmics Data visualization INtoGration: Functional and physiological healthy network profiling of cognition. *Frontiers in Integrative Neuroscience*, in redaction

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Extract

	A	B	C	D
1	**Label 1 English**	Note	Note	Note
2	**Label 2 Português**	Proceeding	Proceeding	Proceeding
3	**Label 3 English**	Type	Place	Course
4	**Variable**	***Note_Proc_Type***	***Note_Proc_Place***	***Note_Proc_Course***
5	**VALUE LABELS "**	***VALUE LABELS 'Note_Proc_Type'***	***VALUE LABELS 'Note_Proc_Place'***	***VALUE LABELS 'Note_Proc_Course'***
6	**VALUES English**	1' 'Experimental', '2' 'Control'	1' 'Medicine Faculty', '2' ISCSP', '3' 'Nova SBE', '4' 'Faculty of Sciences', '5' 'SANA Metropolitan'	1' 'Science of Consciousness', '2' 'Psychiatry I', '3' 'Mental Health', '4' ' Service Nucleus', '5' 'Sociology/Human Resources and Public Administration', '6' 'Economy', '7' 'Health Sciences', '8' 'Informatics Engineering' , '9' 'ETPA Congress'
7	Subject 1	1	1	
8	Subject 2	1	1	
9	Subject 3	1	1	
10	Subject 4	1	1	
11	Subject 5	1	1	
12	Subject 6	1	1	
13	Subject 7	1	1	
14	Subject 8	1	1	
15	Subject 9	1	1	
16	Subject 10	1	1	
17	Subject 11	1	1	
18	Subject 12	1	1	
19	Subject 13	1	1	
20	Subject 14	1	1	
21	Subject 15	1	1	
22	Subject 16	1	1	
23	Subject 17	1	1	
24	Subject 18	1	1	

	BO	BP	BQ	BR	
1	Psychological Assessment	Psychological Assessment	Psychological Assessment	Psychological Assessment	Psyc
2	Subjective Happiness Scale	Subjective Happiness Scale	Subjective Happiness Scale	Subjective Happiness Scale	Subj
3	Comparing with my pairs, I consider myself:	Some people are generally very happy. They apreciate life independently of what is passing around them, ceasing the best of everything. How do you consider that person?	Some people are not generally happy. Hey never seem as happy as they should. How do you consider that person? (REVERSE POINT)	Total	Perc
4	***PA_SHS_Comp***	***PA_SHS_Happy***	***PA_SHS_NHappy***	***PA_SHS_Total***	
5	***VALUE LABELS 'PA_SHS_Comp'***	***VALUE LABELS 'PA_SHS_Happy'***	***VALUE LABELS 'PA_SHS_NHappy'***	***VALUE LABELS 'PA_SHS_Total'***	*VAL*
6	1' 'A person not very happy' ... '7' 'A person very happy'	1' 'A person not very happy' ... '7' 'A person very happy'	1' 'A person not very happy' ... '7' 'A person very happy'		Valu
7	5	7	3	22	
8	6	6	4	22	
9	6	7	3	24	
10	7	6	2	26	
11	4	7	4	19	
12	6	6	2	24	
13	5	7	3	22	
14	6	7	2	25	
15	6	7	4	23	
16	3	6	2	15	
17	5	7	2	23	
18	6	7	2	25	
19	6	7	2	25	
20	6	7	3	24	
21	5	6	3	22	
22	4	6	4	18	
23	6	3	2	21	
24	6	7	3	24	

Extract

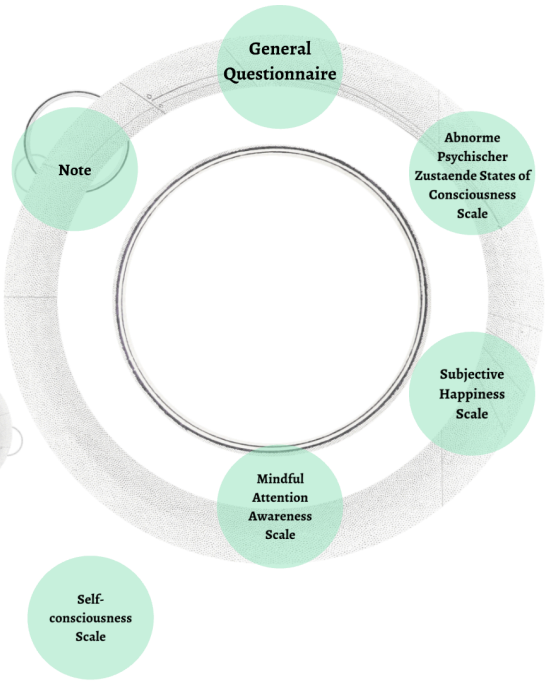
Prezi

Neurotypical DB

Variables

https://tinyurl.com/NeurotypicalDB

Hipólito, I., & Martins, J. (2017). Mind-life continuity: A qualitative study of conscious experience. *Progress in Biophysics and Molecular Biology*. Volume 131, p. 432-444, doi: 10.1016/ j.pbiomolbio.2017.09.004



Prezi

Subjective Happiness Scale

1. Generally, I consider myself:
2. Comparing with my pairs, I consider myself:
3. Some people are generally very happy. They apreciate life independently of what is passing around them, ceasing the best of everything. How do you consider that person?
4. Some people are not generally happy. Hey never seem as happy as they should. How do you consider that person?

'1' 'A person not very happy'
...
'7' 'A person very happy'

Neuro.OralCard (2020). **(- 127 -)**
Full support on:
https://www.limmit.org/uploads/2/6/8/4/26841837/neuro.oralcard.xlsx

LIMMIT
Pursuing a translational model of consciousness

Neuro.OralCard

Embedded type database of literature review of the nervous system

A bioinformatic tool to be used as a salivary database system for neuronal knowledge-based discovery.

In silico protein biobank of 2000 to 2017 neuroproteomics studies.

Neuropsychiatric and non-pathological healthy conditions were annotated via UniProtKB coding of information.

2416 neuronal proteins were gathered, from which 765 are present in saliva. 85% of the neuronal proteins were initially considered as a possible disease state biomarker. Whilst 33% were considered as a possible biomarker of a mental health state or of neurotypical subjects. From those, only 13% were in fact identified, quantified and proposed as disease biomarkers and almost none as a biomarker of a mental health state or of neurotypical subjects.

Neuro.OralCard.xlsx
Download File

Extract

	A	B	C	D	E	F	G	H
1	**UniprotKB**	**Mollecular Weight (kDa)**	**Gene name**	**Name**	**Organism**	**In Vitro**	**In Vivo (Post Surgical)**	**Post-Mortem**
2	O15527		OGG1 MMH,MUTM,OGH	N-glycosylase/DNA lyase [Includes: 8-oxoguanine DNA glycosylase (EC 3.2.2.-); DNA-(apurinic or apyrimidinic site) lyase (AP lyase) (EC 4.2.99.18)]	Homo sapiens			
3	P12821		ACE DCP,DCP1	Angiotensin-converting enzyme	Homo sapiens			
4	P15884		TCF4BHLHB19,ITF 2,SEF2	Transcription factor 4 (TCF-4) (Class B basic helix-loop-helix protein 19) (bHLHb19) (Immunoglobulin transcription factor 2) (ITF-2) (SL3-3 enhancer factor 2) (SEF-2)	Homo sapiens			
5	P21964		COMT	Catechol O-methyltransferase (EC 2.1.1.6)	Homo sapiens			
6	P35228		NOS2 NOS2A	Nitric oxide synthase, inducible	Homo sapiens			
7	Q96HC4		PDLIM5 ENH,L9	PDZ and LIM domain protein 5	Homo sapiens			
8	Q01484		ANK2	ankyrin 2, neuronal	Homo sapiens			

	A	B	C	D	E	F	G	H
1	**UniprotKB**	**Mollecular Weight (kDa)**	**Gene name**	**Name**	**Organism**	**In Vitro**	**In Vivo (Post Surgical)**	**Post-Mortem**
5032	Q9Y3Q8		TSC22D4 THG1 TILZ2	TSC22 domain family protein 4 (TSC22-related-inducible leucine zipper protein 2) (Tsc-22-like protein THG-1)	Homo sapiens			x
5033	Q9Y3R5		DOPEY2 C21orf5 KIAA0933	Protein dopey-2	Homo sapiens			x
5034	Q9Y3R5		DOPEY2 C21orf5 KIAA0933	Protein dopey-2	Homo sapiens			x
5035	Q9Y3S1		WNK2 KIAA1760 PRKWNK2	Serine/threonine-protein kinase WNK2 (EC 2.7.11.1) (Antigen NY-CO-43) (Protein kinase lysine-deficient 2) (Protein kinase with no lysine 2) (Serologically defined colon	Homo sapiens			x
5036	Q9Y484		WDR45 WDRX1 WDRXI4 WIPI4	WD repeat domain phosphoinositide-interacting protein 4 (WIPI-4) (WD repeat-containing protein 45)	Homo sapiens			x
5037	Q9Y490		TLN1	talin 1	Homo sapiens		x	
5038	Q9Y496		KIF3A KIF3	Kinesin-like protein KIF3A (Microtubule plus end-directed kinesin motor 3A)	Homo sapiens			x

Neuro.OralCard

Composition of Literature Review (79 articles)

Extract

Abell, J. G., Stalder, T., Ferrie, J. E., Shipley, M. J., Kirschbaum, C., Kivimäki, M., & Kumari, M. (2016). Assessing cortisol from hair samples in a large observational cohort: the Whitehall II study. *Psychoneuroendocrinology*, 73, 148-156.

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...

Extract

Entry	Entry name	Protein names	Gene names	Involvement in disease	Pharmaceutical use	Gene ontology (molecular function)	Gene ontology (biological process)	Gene ontology (GO)	PubMed ID
A0AVF1	IFT56_HUMAN	Intraflagellar transport protein 56 (Tetratricopeptide repeat protein 26) (TPR repeat protein 26)	D4 TTC26 IFT56				cilium assembly [GO:0060271]; intraciliary transport [GO:0042073]; intraciliary transport involved in cilium assembly [GO:0035735]; smoothened signaling pathway [GO:0007224]; spermatid development [GO:0007286]	centrosome [GO:0005813]; ciliary basal body [GO:0036064]; ciliary tip [GO:0097542]; cilium [GO:0005929]; intraciliary transport particle B [GO:0030992]; cilium assembly [GO:0060271]; intraciliary transport [GO:0042073]; intraciliary transport involved in cilium assembly [GO:0035735]; smoothened signaling pathway [GO:0007224]; spermatid development [GO:0007286]	14702039; 12690205; 12853948; 15489334; 17974005
P60981	DEST_HUMAN	Destrin (Actin-depolymerizing factor) (ADF)	DSTN ACTDP DSN			actin filament binding [GO:0051015]	actin filament depolymerization [GO:0030042]; actin filament fragmentation [GO:0030043]; actin filament severing [GO:0051014]; actin polymerization or depolymerization [GO:0008154]; positive regulation of actin filament depolymerization [GO:0030836]	actin cytoskeleton [GO:0015629]; cortical actin cytoskeleton [GO:0030864]; extracellular exosome [GO:0070062]; actin filament binding [GO:0051015]; actin filament depolymerization [GO:0030042]; actin filament fragmentation [GO:0030043]; actin filament severing [GO:0051014]; actin polymerization or depolymerization [GO:0008154]; positive regulation of actin filament depolymerization [GO:0030836]	8399167; 14702039; 11780052; 15489334; 12665801; 11812157; 17081983; 16815975; 19413330; 19369195; 19608861; 20068231; 21269460; 21406692; 22223895; 22814378; 23186163; 25944712; 16959974
Q9Y608	LRRF2_HUMAN	Leucine-rich repeat flightless-interacting protein 2 (LRR FLII-interacting	LRRFIP2			LRR domain binding [GO:0030275]	regulation of transcription, DNA-templated [GO:0006355]; Wnt signaling	LRR domain binding [GO:0030275]; regulation of transcription, DNA-templated [GO:0006355];	10366446; 14702039; 17974005; 16641997; 15489334; 21220426; 15677333; 17081983; 17693683; 18220336; 19367720; 18669648;

VENNY Database Neuro.OralCard (2020). **(- 145 -)**
Full support on: https://tinyurl.com/VENNYDatabaseNeuro-OralCard

Extract

Neuronal Proteome	Salivary Proteome	Neuronal Proteome Exclusive	Salivary Proteome Exclusive	Neuronal & Salivary Proteomes
O15527	A0A5E4	O15527	A0A5E4	P21964
P12821	A0N5G3	P12821	A0N5G3	P35228
P15884	A0N5G5	P15884	A0N5G5	Q96HC4
P21964	A1XBS5	8TAS1	A1XBS5	A8K2U0
P35228	A2J1M4	A0AVF1	A2J1M4	O00139
Q96HC4	A2J1N6	A0FGR8	A2J1N6	O00231
8TAS1	A2MYC9	A0JNW3	A2MYC9	O00410
A0AVF1	A2MYD4	A0JNW5	A2MYD4	O00429
A0FGR8	A2N0U4	A0MZ66	A2N0U4	O00483
A0JNW3	A2RTY6	A1KZ92	A2RTY6	O00571
A0JNW5	A5D903	A1L0V1	A5D903	O14548
A0MZ66	A6MUU8	A1XP52	A6MUU8	O14639
A1KZ92	A6NC17	A1YPR0	A6NC17	O14686
A1L0V1	A6NCD1	A2RRP1	A6NCD1	O14732
A1XP52	A6NCN2	A3KMH1	A6NCN2	O14880
A1YPR0	A6NFH5	A3KN83	A6NFH5	O14974
A2RRP1	A6NJ16	A4D0S4	A6NJ16	O14983
A3KMH1	A6NJS3	A4D1E1	A6NJS3	O15015
A3KN83	A6NNF4	A4UGR9	A6NNF4	O15020
A4D0S4	A6QL64	A5A3E0	A6QL64	O15021
A4D1E1	A8K008	A5YM72	A8K008	O15031
A4UGR9	A8K2U0	A6NC98	A8MQC9	O15144
A5A3E0	A8MQC9	A6NDB9	A8TX70	O15145
A5YM72	A8TX70	A6NKT7	B2R577	O15400
A6NC98	B2R577	A6NMS7	B2ZDQ1	O43169
A6NDB9	B2ZDQ1	A6NNZ2	B3EWG3	O43175
A6NKT7	B3EWG3	A7E2V4	B3EWG4	O43390
A6NMS7	B3EWG4	A7E2Y1	B3EWG5	O43396
A6NNZ2	B3EWG5	A7KAX9	B3KS75	O43488
A7E2V4	B3KS75	A8K7I4	B3KU16	O43617
				...
P26640	P08519	Q14257	P16219	Q9H444
P26641	P08571	Q14289	P16233	Q9H4B7
P27105	P08574	Q14457	P16401	Q9H4G4
P27338	P08575	Q14524	P16402	Q9H4M9
P27348	P08582	Q14527	P16444	Q9H8W5
P27361	P08590	Q14643	P16499	Q9H9B4
P27448	P08603	Q14653	P16520	Q9HAV0
P27449	P08603-2	Q14669	P16562	Q9HC84
P27708	P08637	Q14676	P16870	Q9HDC9
P27816	P08670	Q14689	P16930	Q9NP97
P27824	P08708	Q14690	P16989	Q9NQ38
P27986	P08709	Q14692	P17014	Q9NQC3
P27987	P08727	Q14703	P17036	Q9NQI0
P28221	P08729	Q14738	P17081	Q9NRV9
P28222	P08754	Q14781	P17213	Q9NRW1
P28223	P08758	Q14789	P17540	Q9NRX4
P28288	P08779	Q14982	P17661	Q9NSB2
P28289	P08865	Q14C86	P17693	Q9NSC7

Cytoscape Database Neuro.OralCard (2020). **(- 153 -)**
Full support on: https://tinyurl.com/CytoscapeDBNeuroOralCard

Extract

	A	B	C	D	E	F	G	H	I	J	K
1	**Type of Sample**				**Tissue and Structure**						
2					Non-Brain						
3									Anatomical		
4	In Vitro	In Vivo (Post Surgical)	Post-Mortem	Other	Blood	Saliva	Sperm	All Brain	Neocortex	Insular Cortex	Whole Brain
5	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**
6	O43169	A0JNW3	A0AVF1	O15527	O15527	A8K2U0	Q93065	Q01484	Q01484	A0AVF1	A0FGR8
7	P00387	A1L0V1	A0FGR8	P12821	P12821	P01036		8TAS1	A0JNW3	A0JNW5	A0MZ66
8	P07550	A8MV58	A0JNW5	P15884	P15884	P01591		A0AVF1	A1L0V1	A1KZ92	A1YPR0
9	P08172	O00139	A0MZ66	P21964	P21964	P01766		A0FGR8	A8MV58	A2RRP1	A3KMH1
10	P08173	O00186	A1KZ92	P35228	P35228	P01779		A0JNW3	O00139	A3KMH1	A7E2Y1
11	P08588	O00401	A1YPR0	Q96HC4	Q96HC4	P01834		A0JNW5	O00186	A3KN83	A7KAX9
12	P08908	O00408	A2RRP1	Q01484	8TAS1	P01871		A0MZ66	O00401	A4D0S4	A8K7I4
13	P08912	O00410	A3KMH1	8TAS1	A1XP52	P01876		A1KZ92	O00408	A4D1E1	A8MT70
14	P08913	O00429	A3KN83	A1XP52	O00141	P01877		A1L0V1	O00410	A4UGR9	A8MWA6
15	P11229	O00445	A4D0S4	A8K2U0	O00327	P02538		A1YPR0	O00429	A5A3E0	A8MXJ8
16	P14416	O00483	A4D1E1	O00141	O00626	P04080		A2RRP1	O00445	A6NC98	A8MXZ1
17	P18089	O00571	A4UGR9	O00327	O14732	P04406		A3KMH1	O00483	A6NDB9	B1ANS9
18	P18825	O14490	A5A3E0	O00499	O14746	P06870		A3KN83	O00499	A6NKT7	O00154
19	P19404	O14531	A5YM72	O00626	O14798	P0CG05		A4D0S4	O00571	A6NMS7	O00170
20	P20309	O14548	A6NC98	O14732	O43169	P12273		A4D1E1	O14490	A6NNZ2	O00186
21	P21554	O14576	A6NDB9	O14746	O43612	P13645		A4UGR9	O14495	A7E2V4	O00213
22	P21728	O14578	A6NKT7	O14798	O43914	P13647		A5A3E0	O14531	A7E2Y1	O00231
23	P21917	O14594	A6NMS7	O15020	O75365	P21964		A5YM72	O14548	A8K2U0	O00305
24	P21918	O14936	A6NNZ2	O15228	O75444	P28223		A6NC98	O14576	A8K8P3	O00408
25	P23975	O14974	A7E2V4	O15513	O94759	P54108		A6NDB9	O14578	A8MTJ3	O00442

	Q	R	S	T	U	V	W	X	Y	Z	AA
1	**Brain Type**					**Biomarker**					
2	Pathological Brain		Healthy Brain			Disease State Biomarker					
3											
4	All	Colloid cyst, Glial neoplasm or Lesional Epilepsy	All	Cardiopulmonary Arrest, Myocardial Infarct or Mesenteric Bleeding	Receptoromics (Psychoactive Drug)	All	PNS Disease Only	CNS Disease Only	PNS & CNS Disease	Non NS	Health State Biomarker
5	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**	**UniprotKB**
6	O15527	A0JNW3	A0AVF1	A0MZ66	P07550	O15527	O60313	O15527	O43175	Q01484	A0JNW3
7	P12821	A1L0V1	A0FGR8	A5YM72	P08172	P12821	P04792	P12821	P12235	O00499	A0MZ66
8	P15884	A8MV58	A0JNW5	O00429	P08173	P15884	P07196	P15884	P48643	O15228	A1L0V1
9	P21964	O00139	A0MZ66	O43426	P08588	P21964	P22748	P21964	P60201	O15513	A5YM72
10	P35228	O00186	A1KZ92	O60831	P08908	P35228	P40939	P35228	Q14203	O43707	A8K2U0
11	Q96HC4	O00401	A1YPR0	O60888	P08912	Q96HC4	P51149	Q96HC4	Q58I22	P00367	A8MV58
12	8TAS1	O00408	A2RRP1	O75077	P08913	Q01484	P55084	8TAS1	Q9H2M9	P00558	O00139
13	A0JNW3	O00410	A3KMH1	O75122	P11229	8TAS1	Q86UR5	A1XP52	Q9NQX3	P00918	O00186
14	A1L0V1	O00429	A3KN83	O75964	P14416	A0AVF1	Q9UHD8	O00141	Q9P2R7	P02511	O00401
15	A1XP52	O00445	A4D0S4	O95202	P18089	A0FGR8		O00327		P04075	O00408
16	A8MV58	O00483	A4D1E1	P0CG47	P18825	A0JNW5		O00626		P04899	O00410

LIMMIT
Pursuing a translational model of consciousness

Extract

MolecularCog

MolecularCog(G) Atlas

OPEN

https://prezi.com/view/6onOjN5x1HAlpgRUBOWx/

The following maps were obtained by Cytoscape extraction with the ontology databases

Maps

1. Type of Sample: *In Vitro*
2. Type of Sample: *In Vivo* (Post Surgical)
3. Type of Sample: *Post-Mortem*
4. Type of Sample: Other
5. Tissue and Structure: Blood
6. Tissue and Structure: Saliva
7. Tissue and Structure: All Brain
8. Tissue and Structure: Neocortex
9. Tissue and Structure: Insular Cortex
10. Tissue and Structure: Whole Brain
11. Tissue and Structure: Central Anterior Frontal, Medial Anterior Parietal, Lateral Temporal, Posterior Superior Frontal, Inferior Parietal, Inferior Temporal Gyrus, Anterior Superior Frontal
12. Tissue and Structure: Dorsolateral Prefrontal
13. Tissue and Structure: Postsynaptic Density (hPSD)
14. Tissue and Structure: Myelin (Frontal Lobe)
15. Tissue and Structure: Membrane Proteins (Ontology)
16. Brain Type: Pathological Brain (All)
17. Brain Type: Pathological Brain (Colloid cyst, Glial neoplasm or Lesional Epilepsy)
18. Brain Type: Healthy Brain (All)
19. Brain Type: Healthy Brain (Cardiopulmonary Arrest, Myocardial Infarct or Mesenteric Bleeding)
20. Brain Type: Healthy Brain (Receptoromics (Psychoactive Drug))
21. Biomarker: Disease State (All)
22. Biomarker: Disease State (CNS Disease Only)

Ontology || Type || Updated on || Pathways/Genes

REACTOME || Pathways || 17.02.2018 || 2155/10609 (Croft et al., 2010)

KEGG || COMPOUND || 16.02.2018 || 267/3657 (Hattori et al., 2003)

KEGG || KEGG || 16.02.2018 || 316/7325 (Hattori et al., 2003)

GO || Molecular Function || 16.02.2018 || 4612/17556 (Hill et al., 2008)

GO || Cellular Component || 16.02.2018 || 1924/18620 (Hill et al., 2008)

GO || Immune System || 16.02.2018 || 1170/3152 (Diehl et al., 2007)

GO || Biological Process || 16.02.2018 || 15785/17540 (Botstein et al., 2000)

References

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Croft, D., O'kelly, G., Wu, G., Haw, R., Gillespie, M., Matthews, L., ... & Jupe, S. (2010). Reactome: a database of reactions, pathways and biological processes. *Nucleic acids research*, 39(suppl_1), D691-D697.

Diehl, A. D., Lee, J. A., Scheuermann, R. H., & Blake, J. A. (2007). Ontology development for biological systems: immunology. *Bioinformatics*, 23(7), 913-915.

Hattori, M., Okuno, Y., Goto, S., & Kanehisa, M. (2003). Development of a chemical structure comparison method for integrated analysis of chemical and genomic information in the metabolic pathways. *Journal of the American Chemical Society*, 125(39), 11853-11865.

Hill, D. P., Smith, B., McAndrews-Hill, M. S., & Blake, J. A. (2008, April). Gene Ontology annotations: what they mean and where they come from. In *BMC bioinformatics* (Vol. 9, No. 5, p. S2). BioMed Central.

Raw Protein Profile Population (2020). **(- 184 -)**

Full support on: https://tinyurl.com/RawProteinProfilePopulation

Extract

RawProteinProfilePopulation.csv

	A	B	
1	MW;Individuals; [P];;;MW;1;2;3;4;5;6;7;8;9;10;11;12;13;14;15;16;17;18;19;20;21;22		
2	9	7;#1;7	27;;;9
3	13		31;;;9
4	16;#1;70	85;;;9	4;#N/A;#N/A;7
5	22	4;#1;2	82;;;9
6	28	8;#1;59	27;;;9
7	37;#1;14	75;;;9	7;7
8	46	8;#1;278	43;;;9
9	61	4;#1;666	04;;;9
10	70	1;#1;495	43;;;11
11	101	5;#1;1	03;;;11
12	149	8;#1;4	04;;;12
13	9	5;#2 manhã;6	11;;;13;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A
14	13	1;#2 manhã;6	87;;;13
15	16;#2 manhã;106	32;;;13	2;#N/A;#N/A;#N/A;5
16	22	2;#2 manhã;5	19;;;13
17	28	5;#2 manhã;62	55;;;13
18	34	5;#2 manhã;118	85;;;13
181	76	4;#15 manhã;16	62;;;93;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A
182	154	6;#15 manhã;28	89;;;93
183	9	8;#16 manhã;98	62;;;95
184	11	8;#16 manhã;15	76;;;95
185	13	1;#16 manhã;39	19;;;97
186	15	9;#16 manhã;125	59;;;97
187	26	8;#16 manhã;33	44;;;100
188	34	5;#16 manhã;9	1;;;100
189	46;#16 manhã;274	77;;;101	5;1
190	61	1;#16 manhã;709	73;;;101
191	69	8;#16 manhã;367	58;;;101
192	102;#16 manhã;2	13;;;101	96;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A;#N/
193	131;#16 manhã;0	3;;;102;#N/A;#N/A;#N/A;#N/A;#N/A;0	61;#N/A;#N/A;#N/A;0
194	9	7;#17 manhã;33	08;;;102;#N/A;#N/A;#N/A;#N/A;#N/A;#N/A;0
195	13;#17 manhã;8	79;;;102	1;#N/A;#N/A;#N/A;0
196	16;#17 manhã;47	15;;;102	1;#N/A;#N/A;#N/A;0
197	26	9;#17 manhã;4	03;;;102
198	28	7;#17 manhã;2	46;;;102
199	34	8;#17 manhã;37	19;;;105
200	46	6;#17 manhã;103	61;;;105
257	130	4;#50 manhã;0	49;;;;;;;;;;;;;;;;;;;;;;;;;;
258	9	1;#52 manhã;10	22;;;;;;;;;;;;;;;;;;;;;;;;;;
259	9	8;#52 manhã;36	01;;;;;;;;;;;;;;;;;;;;;;;;;;
260	13	1;#52 manhã;39	37;;;;;;;;;;;;;;;;;;;;;;;;;;
261	15	6;#52 manhã;74	88;;;;;;;;;;;;;;;;;;;;;;;;;;
262	28	1;#52 manhã;17	39;;;;;;;;;;;;;;;;;;;;;;;;;;
263	32	2;#52 manhã;7	68;;;;;;;;;;;;;;;;;;;;;;;;;;
264	46	2;#52 manhã;363	84;;;;;;;;;;;;;;;;;;;;;;;;;;
265	61	5;#52 manhã;363	2;;;;;;;;;;;;;;;;;;;;;;;;;;
266	69	3;#52 manhã;153	74;;;;;;;;;;;;;;;;;;;;;;;;;;
267	74;#52 manhã;48	92;;;;;;;;;;;;;;;;;;;;;;;;;;	
268	79	1;#52 manhã;21	9;;;;;;;;;;;;;;;;;;;;;;;;;;
269	9	7;#55 manhã;35	52;;;;;;;;;;;;;;;;;;;;;;;;;;

Expected Protein Profile Strategy (2020). **(- 198 -)**

Full support on: https://tinyurl.com/EPPStrategyDataExport

Extract

RUN	Project N	Run Nam	Instrume	Date	Time	Well	Sample N	Peak Nu	Mig.Time	Mol. Wt. (	Correctec	Area Rati	Concentr	Calib.Con	% Total	Sample P	Total Con	FWHM
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	1	18.55	1.20	1730.52	0.0000	0.00	0.00	0.00	0	0.00	0.47
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	2	21.45	4.85	121.67	0.0000	0.00	0.00	0.00	0	0.00	0.42
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	3	22.65	6.35	1136.27	0.0000	0.00	0.00	0.00	0	0.00	0.66
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	4	23.65	7.61	1014.31	0.0000	0.00	0.00	0.00	0	0.00	0.65
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	5	24.30	8.43	75.36	0.0000	0.00	0.00	0.00	0	0.00	0.51
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	6	25.55	10.00	495.93	0.0000	0.00	0.00	0.00	0	0.00	0.34
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	7	28.70	20.00	629.83	0.0000	0.00	0.00	0.00	0	0.00	0.32
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	8	30.10	25.00	562.30	0.0000	0.00	0.00	0.00	0	0.00	0.32
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	9	32.65	37.00	164.86	0.0000	0.00	0.00	0.00	0	0.00	0.35
1	Imported	1st (070∠	4170	7/4/2016	6:02:37P	0	Ladder	10	35.15	50.00	358.87	0.0000	0.00	0.00	0.00	0	0.00	0.40
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	11	39.20	75.00	392.83	0.0000	0.00	0.00	0.00	0	0.00	0.48
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	0	Ladder	12	42.25	100.00	175.44	0.0000	0.00	0.00	0.00	0	0.00	0.73
1	Imported	1st (070∠	4170	7/4/2016	6:02:37P	0	Ladder	13	47.75	150.00	135.65	0.0000	0.00	0.00	0.00	0	0.00	1.15
1	Imported	1st (070∠	4170	7/4/2016	6:02:37P	0	Ladder	14	57.20	260.00	204.02	0.0000	0.00	0.00	0.00	0	0.00	1.12
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	1	13.02	0.00	6.58	0.0000	0.00	0.00	0.00	7	916.78	0.22
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	2	15.71	0.00	211.38	0.0000	0.00	0.00	0.00	7	916.78	0.19
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	3	16.27	0.00	5.34	0.0000	0.00	0.00	0.00	7	916.78	0.25
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	4	16.72	0.00	38.48	0.0000	0.00	0.00	0.00	7	916.78	0.42
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	5	17.28	0.00	39.33	0.0000	0.00	0.00	0.00	7	916.78	0.43
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	6	18.55	1.20	2084.56	0.0000	0.00	0.00	0.00	7	916.78	0.39
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	7	21.24	4.58	11.10	0.0000	0.00	0.00	0.00	7	916.78	0.41
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	8	22.66	6.36	381.92	0.0000	0.00	0.00	0.00	7	916.78	0.39
1	Imported	1st (0704	4170	7/4/2016	6:02:37P	1	D01309	9	23.62	7.58	959.42	0.0000	0.00	0.00	0.00	7	916.78	0.75

	Run	Well	Sample Name	Mol. Wt. (kDa)	Concentration (ng/µl)	Order	Subphenom	Mol. Wt. (kDa) Color	Molecular Ban
	1	1	D01309	9.6	91.46	1	EI	9.6	9.1
	1	1	D01309	12.6	57.59	2	EI	12.6	9.3
A4	1	1	D01309	15.6	247.24	3	EI	15.6	9.4
	1	1	D01309	32.0	34.59	4	EI	32.0	9.5
	1	1	D01309	48.1	374.01	5	EI	48.1	9.6
	1	1	D01309	59.9	40.15	6	EI	59.9	9.7
	1	1	D01309	63.4	71.75	7	EI	63.4	9.8
	1	1	D01309	260.0	75	8	EI	260.0	9.9
	1	2	D01309R	9.4	41.01	9	EI	9.4	10.0
	1	2	D01309R	11.7	17.35	10	EI	11.7	10.1
	1	2	D01309R	13.9	8.5	11	EI	13.9	10.4

Molecular Ban	D01309	D01309R	D01334	D01334R	D01348	D01348R	D01353	D01353R	D01365	D01365R	D01330	D01330R	D01350	D01350R	D01354	D01354R	D01356	D01356R
9.1	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.3	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.3	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.3	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.4	#N/A	41.01	93.23	#N/A	50585.31	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A
9.5	#N/A	#N/A	#N/A	30.81	#N/A	#N/A	37.47	173.94	#N/A	2972.96	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A	#N/A

Export for MOdeLING.Vis (2020). **(- 198 -)**

Full support on: https://tinyurl.com/ExportForMOdeLINGVis

Extract

Present in the following subphenomes								
Experimental Superior	Experimental Superior	Experimental Inferior	Experimental Inferior	Controlo Superior (t	Controlo Superior (t	Controlo Inferior (t	Controlo Inferior (t	Molecular Bands Sum
		x						9.1
x		x		x				9.3
x		x	x	x	x	x	x	9.4
x	x		x	x	x	x	x	9.5
x	x	x		x	x	x	x	9.6
x	x	x	x	x	x			9.7
x	x	x	x	x	x	x	x	9.8
x	x	x	x	x	x			9.9
			x		x	x	x	10
x	x				x			10.1
							x	10.4
	x	x						10.5
	x							11.1
	x							11.3
			x					11.7
	x				x			11.9
					x			12
x	x			x				12.1
x				x				12.2
x								12.3
		x			x			12.4
		x		x	x			12.5
		x						12.6

01309	D01309R	D01334	D01334R	D01348	D01348R	D01353	D01353R	D01365	D01365R	D01330	D01330R	D01350	D01350R	D01354	D01354R	D01356	D01356R	D01372	D01372
	41.01	93.23		50585.31															
			30.81			37.47	173.94		2972.96										41.6
91.46													326.76						
																		2101.32	
										J9	62.19			4.84	668.37				
																162.93	653.93		
					3183.86														
	17.35																		
							46.64												
		48.03		3096.84															
																		257.88	
			9.62												423.89				
57.59																			

D01308	D01308R	D01310	D01310R	D01347	D01347R	D01368	D01368R	D01384	D01384R	D01326	D01326R	D01327	D01327R	D01333	D01333R	D01337	D01337R	D01340	D013
										10.78									
			33.59								11.64								
																	10.14		
4.18	27.46							20.05											
		21.86			1499.27									22.54				82	
						80.19	61.82												4
									30.79										

MOdeLING.Vis (2020). **(- 199 -)**
Full support on:
https://www.limmit.org/uploads/2/6/8/4/26841837/modeling.vis.zip

MODeLING.Vis

Graphical User Interface Matlab Toolbox

MOdeLING.Vis comprehends 3 components: (i) Data Visualization, (ii) Data Exploration and (iii) Data Mining.

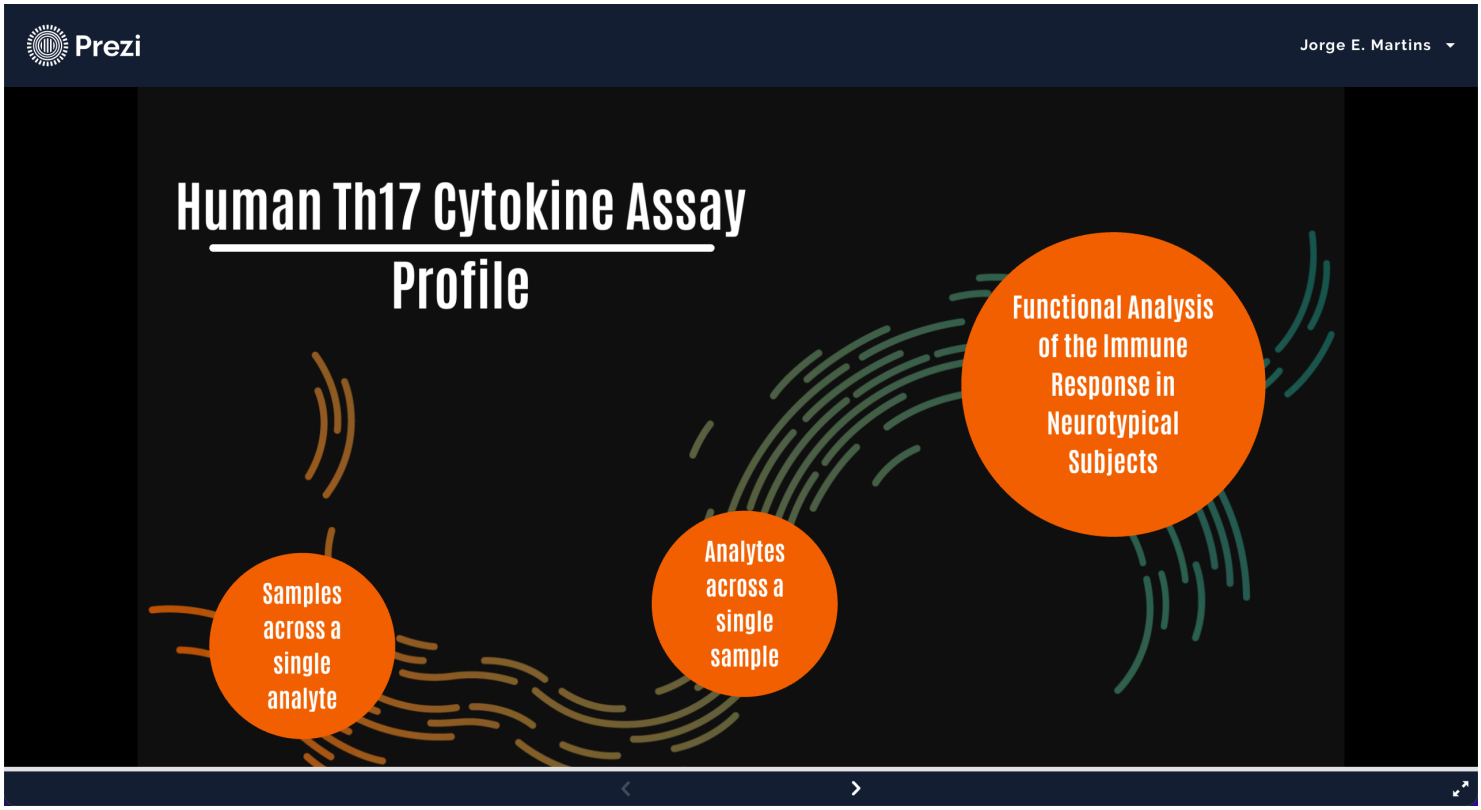
These components encompasses a data processing toolbox, which aims to i) implement and test a visualization software that allows for the interactive exploratory of information content embedded in multi-layered and multi-source data; and ii) service the scientific community by distributing, instructing and providing support to software users, i.e. researchers and clinicians.

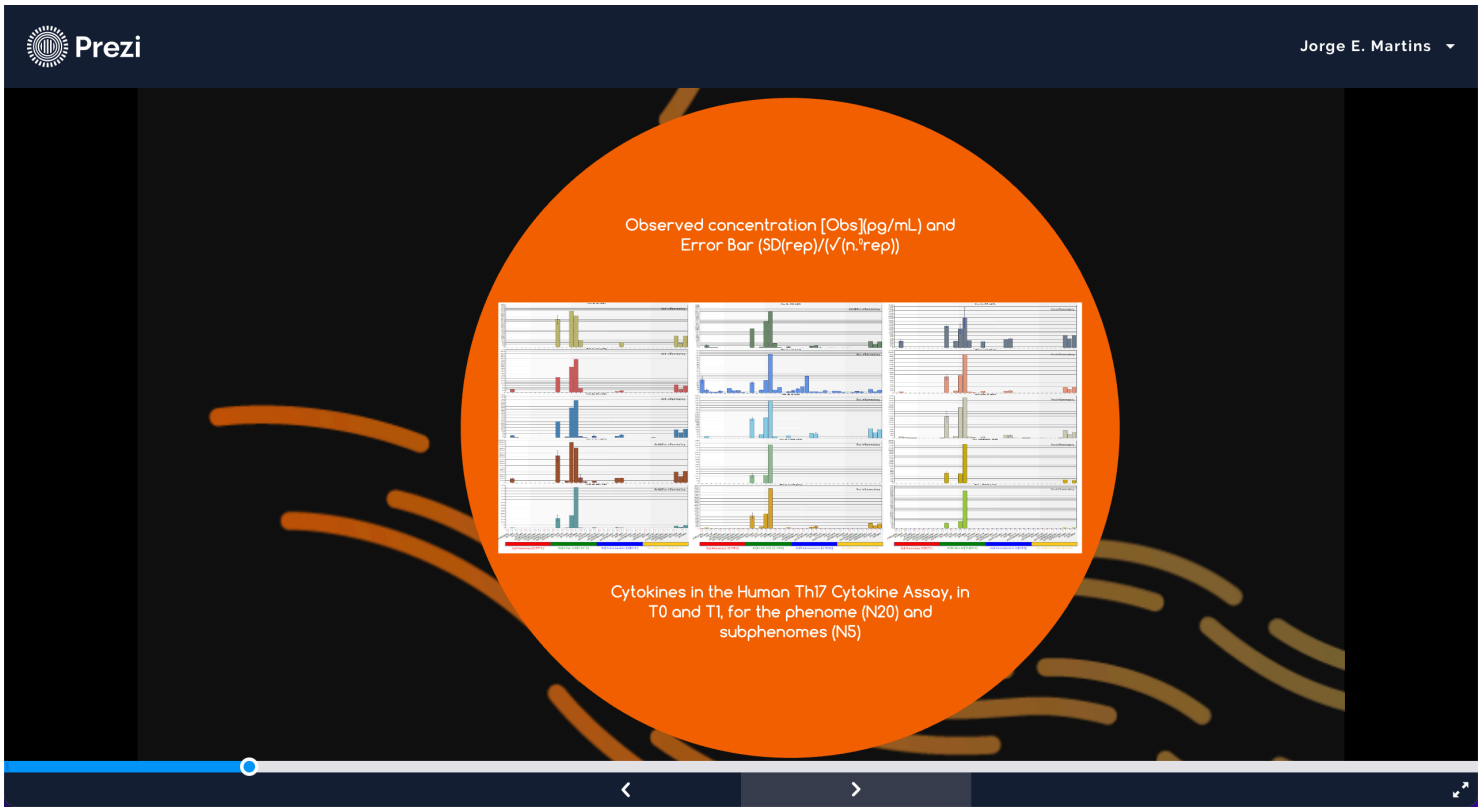
MODeLING.Vis
Download File

code

Nome	Data de alteração	Tamanho	Tipo
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limmiLtn3DVis.m~	17/12/2016, 13:55	4 KB	Documento
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t1t0.limmit	17/12/2016, 13:54	787 bytes	Documento
utils	21/01/2018, 06:36	--	Pasta

Extract


Prezi
Jorge E. Martins
Human Th17 Cytokine Assay
Profile
Functional Analysis of the Immune Response in Neurotypical Subjects
Analytes across a single sample
Samples across a single analyte


Prezi
Jorge E. Martins
Observed concentration [Obs](pg/mL) and
Error Bar (SD(rep)/(√(n.°rep))
Cytokines in the Human Th17 Cytokine Assay, in
T0 and T1, for the phenome (N20) and
subphenomes (N5)

Human Th17 Cytokine Database (2020). **(- 252 -)**
Full support on:
https://www.limmit.org/uploads/2/6/8/4/26841837/humanth17cytokinedb.xlsx

Human Th17 Cytokine DB

Embedded type database of literature review of the Th17 Cytokine system

A bioinformatic tool for knowledge-based discovery of the cytokines interaction network in neurotypical subjects.

2 main cell source types were distinguished: T helper cell type and myeloid monocytes.

HumanTh17CytokineDB.xlsx
Download File

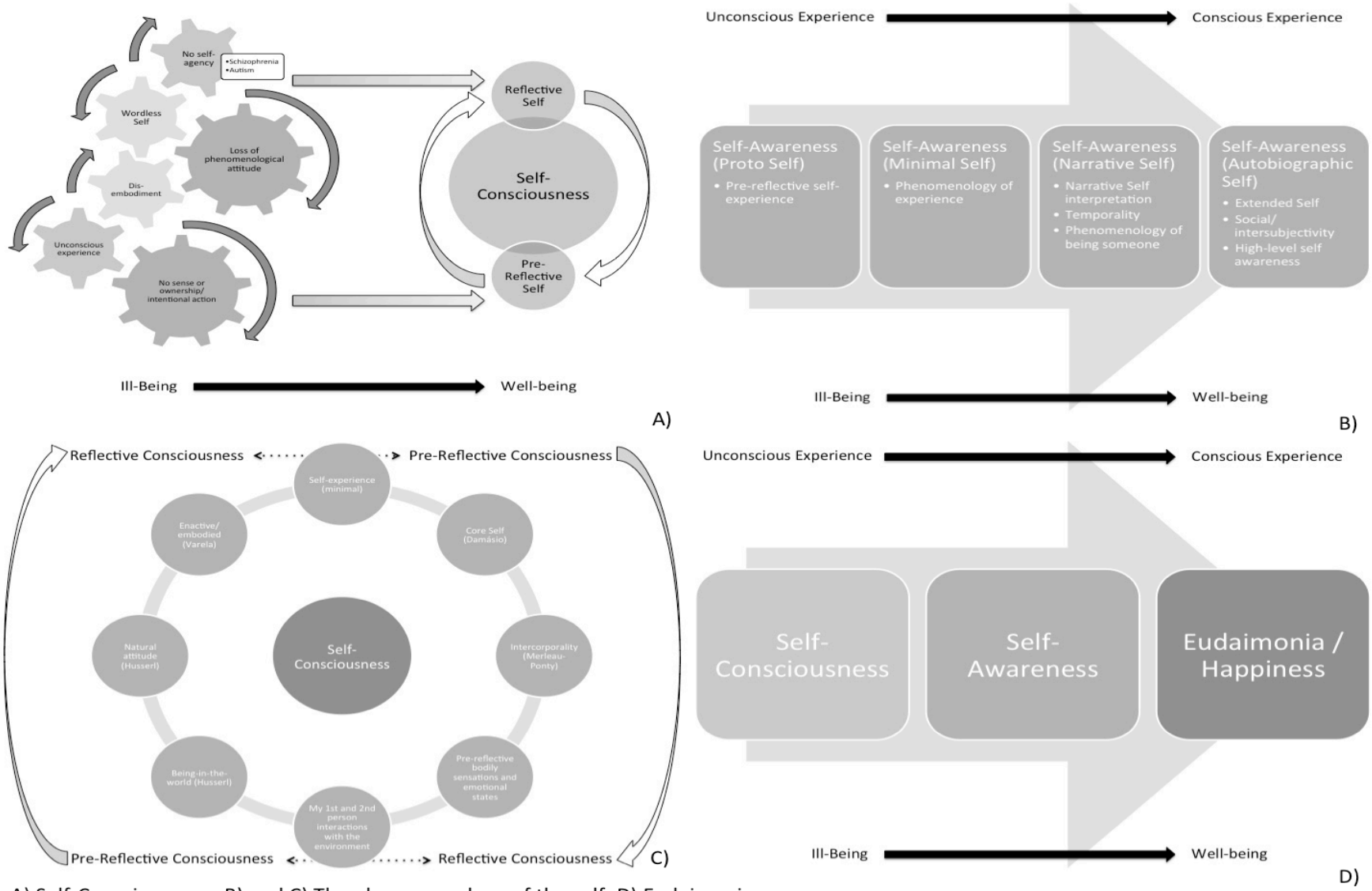
Extract

	A	B	C	D	E	F	G	H	I	J	K
1 2 3	Protein Characterization (*In silico*)					Protein Histophysiology (Literature Review)					
4	Uniprot	Entry name	Protein name	Gene name	Mass (Uniprot)	Cell sources	Cell source type	Anti/Pro Inflammatory (0/1/2)	Size molecular weight	Cell targets	T Cell type T_H1
5	P22301	IL10_HUMAN	Interleukin-10(IL-10) (Cytokine synthesis inhibitory factor) (CSIF)	IL10	20,517	B cells	Lymphoid B cell	0	20.5 kd	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	0
6	P22301	IL10_HUMAN	Interleukin-10(IL-10) (Cytokine synthesis inhibitory factor) (CSIF)	IL10	20,517	T cells	Lymphoid Double Positive (DP)	0	20.5 kd	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	0
7	P22301	IL10_HUMAN	Interleukin-10(IL-10) (Cytokine synthesis inhibitory factor) (CSIF)	IL10	20,517	DCs	Myeloid Monocyte	0	20.5 kd	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	0
8	P22301	IL10_HUMAN	Interleukin-10(IL-10) (Cytokine synthesis inhibitory factor) (CSIF)	IL10	20,517	macrophages	Myeloid Monocyte	0	20.5 kd	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	0
9	P22301	IL10_HUMAN	Interleukin-10(IL-10) (Cytokine synthesis inhibitory factor) (CSIF)	IL10	20,517	monocytes	Myeloid Monocyte	0	20.5 kd	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	0
10	Q9H293	IL25_HUMAN	Interleukin-25(IL-25) (Interleukin-17E) (IL-17E)	IL25IL17E,UNQ3120/PRO10272	20,33	epithelial cells	Epithelial Progenitor Cells	0	17 kd	T_H2 memory cells	0
11	Q9H293	IL25_HUMAN	Interleukin-25(IL-25) (Interleukin-17E) (IL-17E)	IL25IL17E,UNQ3120/PRO10272	20,33	mast cells	Hematopoietic Stem Cell	0	17 kd	T_H2 memory cells	0
12	Q9H293	IL25_HUMAN	Interleukin-25(IL-25) (Interleukin-17E) (IL-17E)	IL25IL17E,UNQ3120/PRO10272	20,33	T_H2 cells	Lymphoid Double Positive (DP) Helper (T_H)	0	17 kd	T_H2 memory cells	0
13	Q9H293	IL25_HUMAN	Interleukin-25(IL-25) (Interleukin-17E) (IL-17E)	IL25IL17E,UNQ3120/PRO10272	20,33	basophils from atopic individuals	Myeloid	0	17 kd	T_H2 memory cells	0

CytokinesInteractionNetwork +

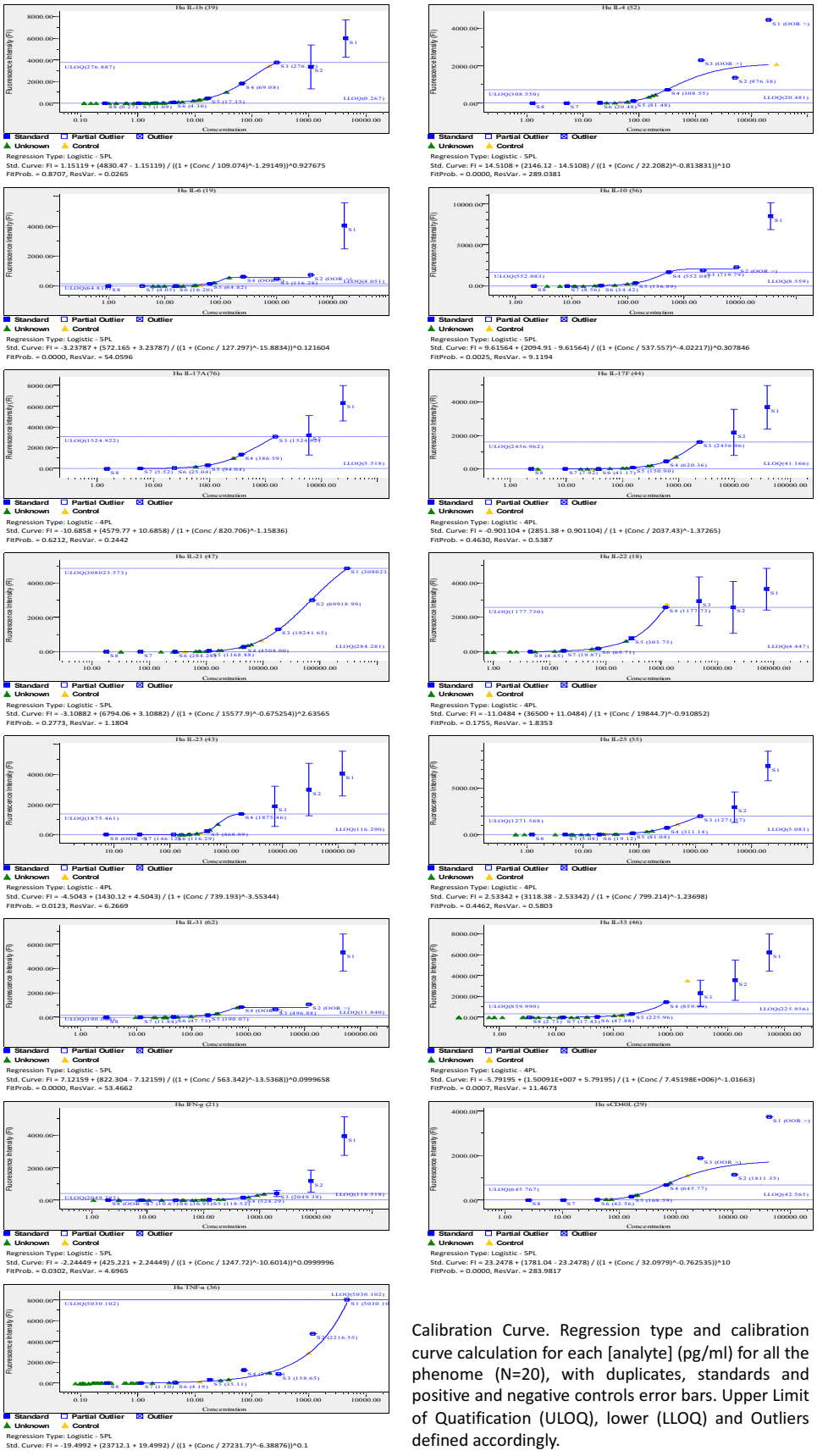
Supplementary Figures and Complementary Data

Supplementary Figure 1. Sample Characterization And Neurophenomenology
4 graphical representations of the cognition of the neurotypical self are presented. In A., the different constructs of self-consciousness are introduced. In B., self-awareness is shown as a progression in a 2-dimensional axis: a) unconscious to conscious experience and b) ill-being to well-being. In C., the self-consciousness trait is shown as a prerequisite to self awareness. In D., it is presented a conceptual analysis of well-being.



A) Self-Consciousness; B) and C) The phenomenology of the self; D) Eudaimonia

Supplementary Figure 2. Calibration Of The *Human Th17 Cytokine Assay, Bio-Rad®, Using Bio-Plex Manager™*



Calibration Curve. Regression type and calibration curve calculation for each [analyte] (pg/ml) for all the phenome (N=20), with duplicates, standards and positive and negative controls error bars. Upper Limit of Quatification (ULOQ), lower (LLOQ) and Outliers defined accordingly.

Complementary Data 1. Post-translational Modifications Of The Analytes Present In The Merck *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay*

Orexin (13,363 Da) has a post-translational modification with specific enzymatic cleavages at paired basic residues yielding the different active peptides[80]. This was taken in account in the Merck *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the analyte was fully detectable in salivary samples (Manzardo et al., 2016).

β-Endorphin (29,424 Da) has a post-translational modification with specific enzymatic cleavages at paired basic residues yielding the different active peptides, but also O-glycosation of N-acetylgalactosamine[81]. This was taken in account in the Merck *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the analyte was fully detectable in salivary samples (Manzardo et al., 2016).

Oxytocin (13,000 Da) has a post-translational modification characterized by an acetylation (N(alpha)-acetyl-oxytocin) and glycosylation (Chen et al., 2011), which may control the bioactivity of the neurohypophyseal hormone. This was taken into account in the Merck *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the analyte was fully detectable in salivary samples (Manzardo et al., 2016).

Substance P (13,000 kDa) has a post-translational modification characterized by glyoxal-derived advanced glycation (side chain guanidine moiety of the arginine residue) (Lopez-Clavijo et al., 2012). This was taken into account in the Merck *HNPMAG-35K | MILLIPLEX MAP Human Neuropeptide Assay* and the analyte was fully detectable in salivary samples (Manzardo et al., 2016).

Complementary Data 2. Post-translational Modifications Of The Analytes In The Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay*

IL-1β (17,300 Da) has a post-translational modification characterized by a CASP1-catalyzed proteolytic cleavage. Processing and secretion are temporarily associated (Andrei et al., 2004). This was taken in account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-4 (15,100 Da) has a post-translational modification characterized (Carr et al., 1991) by disulfide bonds and glycosylation (N-linked (GlcNAc…) asparagine). This was taken in account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-6 (20,900 Da) has a post-translational modification characterized (May et al., 1991) by glycosylation (N- and O-glycosylated). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-10 (18,600 Da) has no post-translational modifications yet characterized.

IL-17A (31,300 kDa) has a post-translational modification characterized (Gerhardt et al., 2009) by disulfide bonds and glycosylation (N-linked (GlcNAc…) asparagine). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-17F (15,100 Da) has a post-translational modification characterized (Ely, Fischer and Garcia, 2009) by disulfide bonds and glycosylation (N-linked (GlcNAc…) asparagine). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-21 (16,000 kDa) has a post-translational modification characterized (Bondensgaard et al., 2007) by disulfide bonds and glycosylation (N-linked (GlcNAc…) asparagine). This was

[80] retrieved August 20, 2020. UniProt Consortium. (2015). UniProt: a hub for protein information. *Nucleic acids research*, 43(D1), D204-D212

[81] retrieved August 20, 2020. UniProt Consortium. (2015). UniProt: a hub for protein information. *Nucleic acids research*, 43(D1), D204-D212.

taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-22 (33,600 Da) has a post-translational modification characterized (Xu et al., 2005) by disulfide bonds and glycosylation (N-linked (GlcNAc…) asparagine). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-23 (33,600 Da) has a post-translational modification characterized (Parham et al., 2002) by phosphorylation and glycosylation (N-linked (GlcNAc…) (high mannose) asparagine). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-25 (16,000 Da) has a post-translational modification characterized by a disulfide bond and glycosylation (N-linked (GlcNAc…) asparagine)[82]. This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-31 (30,000 Da) has a post-translational modification characterized by glycosylation (N-linked (GlcNAc…) asparagine)[83]. This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

IL-33 (17,900 Da) has different post-translational modifications. The full-length protein can be released from cells and is able to signal via the IL1RL1/ST2 receptor. However, proteolytic processing by CSTG/cathepsin G and ELANE/neutrophil elastase produces C-terminal peptides that are more active than the unprocessed full-length protein. They may also be proteolytically processed by calpains (Hayakawa et al., 2009). Proteolytic cleavage mediated by apoptotic caspases including CASP3 and CASP7 results in IL33 inactivation (Lüthi et al., 2009). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

INF-γ (19,348 Da) has a post-translational modification characterized (Pan et al., 1987) by proteolytic processing, producing C-terminal heterogeneity, with proteins ending alternatively at Gly-150, Met-157 or Gly-161. This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

sCD40L (16.3 kDa) has different post-translational modifications. The soluble form derives from the membrane form by proteolytic processing (Pietravalle et al., 1996). N-linked glycan is a mixture of high mannose and complex type. Glycan structure does not influence binding affinity to CD40 (Khandekar et al., 2001). This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

TNF-α (25,644 Da) has a post-translational modification (Pócsik et al., 1995). The soluble form derives from the membrane form by proteolytic processing. The membrane form, but not the soluble form, is phosphorylated on serine residues. Dephosphorylation of the membrane form occurs by binding to soluble TNFRSF1A/TNFR[84]. This was taken into account in the Biorad® *Bio-Plex Pro™ Human Th17 Cytokine Assay* and the analyte was fully detectable in salivary samples (Geng, Ma and Roen, 2012).

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Curriculum Vitae

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