UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE QUÍMICA E BIOQUÍMICA

Using iPSCs-derived microglia from Alzheimer´s disease patients to assess early cell deficits and neurotoxic properties

Daniela Barbosa Lopes

Mestrado em Bioquímica
Especialização em Bioquímica Médica

Dissertação orientada por:

Profª Doutora Dora Maria Tuna de Oliveira Brites
Profª Doutora Margarida Henriques da Gama Carvalho

2021

This work was performed in Neuron Glia Biology in Health and Disease Group at iMed.ULisboa, Faculty of Pharmacy, University of Lisbon, headed by Professor Dora Brites. It was funded by EU Joint Programme-Neurodegenerative Disease Research (JPND) project under grant agreement No 643417, supported by Fundação para a Ciência e a Tecnologia (JPco-fuND/0003/2015 to DB) and, in part, by iMed.ULisboa (UIDB/04138/2020 and UIDP/04138/2020). iPSCs from AD patients and healthy controls were obtained under the JPND-MADGIC-project: Jari Koistinaho from the University of Helsinki (patient iPSCs) and Tarja Malm from University Eastern Finland (microglia precursors).

Para a minha família

Agradecimentos

Em primeiro lugar, à Professora Doutora Dora Brites por me ter aceitado desde o primeiro contacto, tornando assim possível a realização deste trabalho. Obrigada por me deixar integrar o seu grupo e por toda a confiança em mim depositada ao longo de todo este percurso. Obrigado por me ter demonstrado incansavelmente como boa Ciência é feita, pela exigência, frontalidade e preocupação. Obrigada pela sua constante disponibilidade, dedicação e toda a compreensão. Obrigado pela sua frontalidade e boa disposição misturados.

Quero também agradecer à Adelaide Fernandes. Obrigada pelo apoio, disponibilidade e carinho demonstrado, sempre pronta para nos ajudar. A tua boa disposição tornou esta caminhada muito mais leve.

Ao Gonçalo Garcia, que me acompanhou em todo o processo do laboratório e mesmo depois disso, que esclareceu todas as dúvidas e que tem um espírito crítico apuradíssimo. É também um amigo com um coração gigante e sem dúvida um elemento de ligação muito importante no grupo. Muito obrigado!

À Geci, Rita, à Andreia, ao Professor Doutor Rui Silva e à Professora Doutora Alexandra Brito, um agradecimento pelo espírito crítico e por toda a disponibilidade que foram constantemente demonstrados.

Aos meus colegas de laboratório que sempre se mostraram recetivos em especial à Laura, à Isabel, ao Paulo, ao Hermes e ao Alexandre por todo o companheirismo, amizade, disponibilidade e compreensão.

Aos meus amigos que estiveram sempre lá para me animar quando a parte prática não resultava e que compreenderam a minha falta de disponibilidade e que nunca me deixaram desanimar. Obrigado pelo vosso carinho, compreensão e apoio!

À minha família, que me permitiu tudo o que consegui realizar até hoje. Que sempre me ajudou e incentivou durante a realização deste trabalho, a eles posso agradecer a força e persistência que me ensinaram a ter e que se tornaram fundamentais para a conclusão deste projeto. Aos meus pais que sempre acreditaram em mim e que espero um dia conseguir recompensar todo aquilo que me dão. Em particular agradeço aos meus avós que são o exemplo perfeito de como o trabalho, perseverança e esforço compensa sempre.

Abstract

Alzheimer's disease (AD) is the most common, progressive, and irreversible neurodegenerative disorder is characterized by memory loss, cognitive impairment, and behavioural abnormalities. As most of the diseases, this pathology results from a mixture of genetic and environmental factors. The major mark of AD is the secretion of Amyloid-β (Aβ) forms, and the associated senile plaques composed of aggregated Aβ and other misfolded proteins. Such abnormalities may have a genetic basis, as the existence of one mutation either in Amyloid Precursor Protein (APP) or in its proteolytic machinery. Other abnormality is the gain or loss of function of presenilin 1 (PSEN1) or 2 (PSEN2) that causes early-onset familial AD. On the other hand, the sporadic AD, despite not having a standard rule may be related, for example, with a mutation in Apolipoprotein E (APOE). During many years, the amyloid cascade hypothesis was the best theory to explain AD pathophysiology. So, it is normal that the metabolic pathways involved in the formation of amyloid plaques are the object of multiple studies. AD has no cure, the research continues, but the disease still presents several obstacles to its full understanding.

Microglia, the innate immune cells of the central nervous system are responsible for many functions to sustain brain homeostasis, including those involved in the reparative response, which seems to fail in AD, namely in advanced stages of the disease. Steady-state microglia exhibit a particular phenotype, characterized by having a cellular body of small dimensions and ramifications that extend in all directions. This ramified morphology allows microglia to scan the environment, without interfering with neuronal activities. Depending on the stimuli and the time of exposure, microglia acquire several modifications in their phenotypes. These phenotypes can vary from inflammatory and cytotoxic to a more pro-regenerative one, being in most cases a blend of phenotypes that are hard to describe. This indicates that the activated, dysfunctional, or degenerative microglia may differently contribute to the onset and progression of such disorder.

So, it becomes crucial to understand the interplay between neurons and microglial cells in the AD disease from its onset. However, human brain is a highly complex structure that cannot be easily explored *in vivo* and has proven difficult to model *in vitro*. Many of models (human and animal cell lines, as well as invertebrate, zebrafish, and rodent animal models) represent key events in human brain and cell-cell interactions understanding. The isolation of microglia from *post-mortem* tissue or surgery specimens may introduce technical artefacts, and the impact of confounding factors. As a result, recent cellular models generated from patient somatic cells by using the induced pluripotent stem cell (iPSC) reprogramming technology.

Here, we aimed to develop a human model able to recapitulate the pathological potential of AD microglia, thus surpassing species-specific differences and allowing translation to clinic. For that, iPSC-derived microglia from healthy (control) and AD individuals with Presenilin 1 (PSEN1) and Swedish (Swe) mutations were obtained by first differentiating iPSCs into EMPs and then into primitive microglia at the University of Eastern Finland by Prof[a] Tarja Malm. As the erythromyeloid cells (EMPs) can be frozen, they were sent to us and matured in our laboratory for 2 days until reaching the stage of maturation proper for the studies we carried out - induced microglia (iMicroglia). The method as a whole is achieved relatively quickly (only 24 days), requiring few reagents when compared to others and being relatively easy. An isogenic control was also used, derived from the same patient with PSEN1 mutation, but where the correction was made. The ability to work with iMicroglia differentiated from iPSCs of patients with AD opens space for a better understanding of the disease and for the development of new clinical strategies. The difficulties arise from the high heterogeneity of the cell population that is obtained, the different functional maturation and regional specificity. After maturation, the cells were

characterized by specific markers and associated with microglia. Then, we investigated changes in the expression of reactive markers and inflammatory-associated miRNAs (miRNAs) in all differentiated cell lines and in an isogenic control in which the PSEN1 gene was corrected (ISO PSEN1).

In the present work we demonstrated that iMicroglia can be efficiently generated from iPSCs following a defined protocol. It is important to note that iMicroglia showed the usual microglia markers based on the gene expression and immunofluorescence protein data, while also revealed phagocytic ability. We tested the phagocytic capacity of the generated iMicroglia, since one of the problems pointed out to the microglia in the case of AD is its inefficiency in clearing the Aβ plaques. As expected, the iMicroglia spontaneously phagocytosed the fluorescent latex beads. We also identified changes in cell morphology of the different cell lines, results demonstrated no differences between the Control and PSEN1 or Swe mutation in relation to their perimeter or Feret's diameter. Regarding the area, while Swe mutated cells showed increase in cell area, ISO PSEN1 showed a reduction *vs.* Control line. We additionally analysed the expression of cellular markers in iMicroglia that have currently been linked with neuroinflammation and related with cell behaviour in AD, *e.g.*, iNOS, CX3CR1, TREM2, MFGE8 and Arg1, in the PSEN1 microglia. Some of these alterations disappeared in the ISO PSEN1 line. Results demonstrate an overall deficiency of the phagocytic and inflammatory machinery, especially in PSEN1 iMicroglia. Certain cell markers were more notorious after activation with inflammatory stimuli, such as interferon (IFN)-γ and lipopolysaccharide (LPS). In what concerns miRNAs, we observed an overexpression of miRNA (miR)-146a and miR-21 in cells bearing Swe and PSEN1 mutations, when compared to control, while isogenic cells showed overexpressed miRNAs, probably due to the genetic manipulation, together with a low mean cell area. The naïve PSEN1 iMicroglia revealed $Arg1^{low}$, $TREM2^{low}$ and $MFGE8^{high}$, and when stimulated with IFN-γ + LPS, the signature was still $Arg1^{low}$ and $MFGE8^{high}$, but this time with $iNOS^{high}$, as well. Inflammatory stimuli led to miR-125b^{high} in Swe and PSEN1 cells, but not in ISO PSEN1 ones. Thus, Swe and PSEN1 mutated iMicroglia show deregulated polarized markers before and after IFN-γ + LPS stimulation. When stimulated, ISO PSEN1 iMicroglia showed higher Arg1 and MFGE8, together with lower iNOS and miR-146a, relatively to the matched naïve cells, suggesting sustaining of functional capacity.

Our study was innovative in identifying changes in inflammatory microglia signature in familiar AD patient subpopulations that may help in their stratification, and in highlighting that isogenic correction only restore some of steady-state microglia markers, while exacerbate inflamma-miRNAs. A pioneer finding was to observe that Swe and PSEN1 cells were not able to respond to an additional stimulus, sustaining the inflamma-miRNA profiling, while ISO PSEN1 appear to have a machinery to suppress further priming. We obtained many heterogeneous results in terms of markers and miRNAs, also observed in other studies, reinforcing that patients with AD have specific inflammatory signatures thus benefiting from precision medicine. In conclusion, while promising, iPSCs-derived microglia deserve to be further studied to push forward our efforts to combat AD. Indications on the revitalization of such non-functional microglia may be more important that simply propose anti-inflammatory compounds that have systematically failed or even worse the patient AD progression.

Key words: Alzheimer's diseases; iPSCs-derived microglia; Microglial activation; miRNAs regulating neuroinflammation.

Resumo

A doença de Alzheimer (DA) é a doença neurodegenerativa progressiva e irreversível mais comum, sendo caracterizada pela perda de memória, deficiência cognitiva e anomalias comportamentais. Como a maioria das doenças, esta patologia resulta de uma mistura de fatores genéticos e ambientais. A marca principal da DA é a secreção das formas amiloide-β (Aβ), e das placas senis associadas compostas por agregados de Aβ e outras proteínas *misfolded*. Tais anomalias podem ter uma base genética, como a existência de uma mutação quer na Proteína Precursora Amiloide (APP) quer na sua maquinaria proteolítica. Outra anormalidade é o ganho ou perda de função da presenilina 1 (PSEN1) ou 2 (PSEN2) que causa a DA familiar precoce. Por outro lado, a DA esporádica, apesar de não ter uma regra padrão pode estar relacionada, por exemplo, com uma mutação em Apolipoproteína E (APOE). Durante muitos anos, a hipótese da cascata amiloide foi a melhor teoria para explicar a fisiopatologia da DA. Por isso, é normal que as vias metabólicas envolvidas na formação de placas amiloides sejam objeto de múltiplos estudos. A DA não tem cura, a pesquisa continua, mas a doença ainda apresenta vários obstáculos à sua compreensão completa.

Microglia, as células imunitárias inatas do sistema nervoso central são responsáveis por muitas funções para sustentar a homeostase cerebral, incluindo as envolvidas na resposta reparadora, a qual parece falhar na DA, nomeadamente em estádios avançados da doença. A microglia num estado estacionário exibe um fenótipo particular, caracterizado por ter um corpo celular de pequenas dimensões e ramificações que se estendem em todas as direções. Esta morfologia ramificada permite que a microglia monitorize o ambiente sem interferir com as atividades neuronais. Dependendo dos estímulos e do tempo de exposição, a microglia adquire várias modificações nos seus fenótipos. Estes fenótipos podem variar de inflamatórios e citotóxicos a mais pró-regenerativos, sendo na maioria dos casos uma mistura de fenótipos que são difíceis de descrever. Isto indica que a microglia ativada, disfuncional ou degenerativa pode contribuir de forma diferente para o início e progressão de tal desordem.

Assim, torna-se crucial compreender a interação entre neurónios e células microgliais na doença de DA desde o seu início. No entanto, o cérebro humano é uma estrutura altamente complexa que não pode ser facilmente explorada *in vivo* e tem-se revelado difícil de modelar *in vitro.* Muitos dos modelos (linhas de células humanas e animais, invertebrados, peixe-zebra e modelos de animais roedores) representam momentos chave na compreensão do cérebro humano e nas interações celulares entre vários tipos de células. O isolamento da microglia a partir de tecidos *post-mortem* ou amostras cirúrgicas pode introduzir artefactos técnicos. Como resultado, modelos celulares recentes gerados a partir de células somáticas do paciente, utilizando a tecnologia de reprogramação de células estaminais pluripotentes induzidas (iPSC) forneceram ferramentas promissoras para compreender os mecanismos da doença humana, incluindo a DA.

Neste trabalho, pretendíamos desenvolver um modelo humano capaz de recapitular o potencial patológico da microglia, ultrapassando assim as diferenças específicas das espécies e permitindo a aplicação na clínica. Para isso, usámos a microglia derivada de iPSCs de indivíduos saudáveis (controlo) e de doentes AD com mutações na presenilina 1 (PSEN1) e na Swedish (Swe), primeiro pela sua diferenciação a partir de iPSCs em EMPs e depois em microglia primitiva, geradas na University of Eastern Finland pela Profª Tarja Malm. Como as células eritromielóides (EMPs) podem ser congeladas, foram essas que nos foram enviadas e maturadas no nosso laboratório durante 2 dias até atingir o estádio de maturação própria para os estudos que realizámos - microglia induzida (iMicroglia). O método na sua globalidade é conseguido de forma relativamente rápida (apenas 24 dias), requerendo poucos

reagentes quando comparados com outros e sendo relativamente fácil. Foi também usado um controlo isogénico, derivado do mesmo doente com mutação PSEN1, mas onde se fez a correção da mesma. A capacidade de trabalhar com iMicroglia diferenciada de iPSCs dos pacientes com DA abre espaço para uma melhor compreensão da doença e para o desenvolvimento de novas estratégias clínicas. As dificuldades surgem da heterogeneidade muitas vezes elevada da população de células que se obtém, da diferente maturação funcional e da especificidade regional. Após maturação as células foram caracterizadas por marcadores específicos e associados à microglia. De seguida, investigámos alterações na expressão de marcadores reativos e miRNAs associados a inflamatórios (miRNAs) em todas as linhas celulares diferenciadas e num controlo isogénico em que o gene PSEN1 foi corrigido (ISO PSEN1).

Neste trabalho demonstrámos que a iMicroglia pode ser gerada de forma eficiente a partir de iPSCs seguindo um protocolo definido. É importante notar que a iMicroglia mostrou os habituais marcadores de microglia com base na expressão genética e dados proteicos da imunofluorescência, ao mesmo tempo que revelou a capacidade fagocítica. Testámos a capacidade fagocítica da iMicroglia gerada, uma vez que um dos problemas apontados à microglia no caso da AD é a sua ineficiência na eliminação das placas Aβ. Como esperado, a iMicroglia espontaneamente fagocitou as beads fluorescentes. Também identificámos alterações na morfologia celular das diferentes linhas celulares, os resultados não demonstraram diferenças entre o Controlo e as mutações PSEN1 e Swe em relação ao seu perímetro ou ao diâmetro de Feret. No que diz respeito à área, enquanto as células Swe mostraram um aumento na área celular, as ISO PSEN1 mostraram uma ligeira redução *vs.* Controlo. Analisámos ainda a expressão de marcadores celulares na iMicroglia que estão atualmente ligados à neuroinflamação e relacionados com o comportamento celular em AD, *e.g.*, iNOS, CX3CR1, TREM2, MFGE8 e Arg1, na microglia PSEN1. Algumas destas alterações desapareceram na linha ISO PSEN1. Os resultados demonstram uma deficiência geral da maquinaria fagocítica e inflamatória, especialmente em iMicroglia PSEN1. Certos marcadores celulares eram mais notórios após a ativação das células com o interferão (IFN)-γ e com o lipopolissacarídeo (LPS). No que diz respeito aos miRNAs, observámos uma sobre-expressão de miRNA (miR)-146a e miR-21 em células com mutações Swe e PSEN1, relativamente ao controlo, enquanto nas isogénicas todos os miRNAs foram encontrados elevado e a área celular reduzida por provável manipulação genética. A iMicroglia PSEN1 revelou $Arg1^{baixo}$, $TREM2^{baixo}$ e $MFGE8^{alto}$, e após estimulação com IFN-γ + LPS, manteve $Arg1^{baixa}$ e $MFGE8^{alta}$, mas $iNOS^{alto}$. A estimulação causou $miR\text{-}125b^{alto}$ nas células Swe e PSEN1, mas não nas ISO PSEN1. Assim, as mutações Swe e PSEN1 desregulam os marcadores de polarização microglial tanto antes, como após estimulação com IFN- γ + LPS. As células ISO PSEN1 mostraram aumento de Arg1 e MFGE8, e diminuição de iNOS e de miR-146a, após estimulação relativamente às não estimuladas, mantendo a sua capacidade funcional.

O nosso estudo foi inovador na identificação de alterações na assinatura inflamatória microglial em subpopulações de doentes com AD familiar, as quais podem ajudar na estratificação dos doentes. Serviu também para evidenciar que a correção isogénica apenas restaura parte do fenótipo saudável, enquanto exacerba a expressão dos miRNAs inflamatórios. Outra descoberta importante e pioneira foi a de que os miRNAs nas células Swe e PSEN1 não respondem a estímulo adicional e nas ISO PSEN1 até os regulam negativamente. Obtivemos muitos resultados heterogéneos em termos de marcadores e miRNAs, que também observados noutros estudos, reforçando que os pacientes com DA têm assinaturas inflamatórias específicas, e por isso, beneficiariam muito de uma medicina individualizada. Em conclusão, os resultados obtidos para a microglia derivada dos iPSCs, apesar de promissores deverão ser complementados com mais estudos de modo a fomentar os esforços no combate à DA. A possibilidade de revitalização da microglia disfuncional pode ser mais importante que os sucessivos

compostos anti-inflamatórios propostos que sistematicamente falharam ou pioram a progressão da DA do paciente.

Palavras-chave: Doença de Alzheimer; Microglia derivada de iPSCs; Ativação microglial; miRNAs reguladores de neuroinflamação.

Table of Contents

Figure Index

Table Index

Abbreviations

AD	Alzheimer´s disease
ADAM	Disintegrin and metalloproteinase or α secretase
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Arg1	Arginase 1
Aβ	Amyloid β
BACE1	Beta-site APP-cleaving enzyme 1 or β secretase
BSA	Bovine serum albumin
Cas	CRISP-associated proteins
cDNA	Complementary DNA
Clu	Clustering
CNS	Central nervous system
CR1	Complement receptor 1
CRISP	Clustered regularly interspaced short palindromic repeats
CSF	Cerebrospinal fluid
CX3CL1	CX3C chemokine ligand 1
CX3CR1	Fractalkine receptor
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylenediaminetetraacetic acid
EMPs	Erythromyeloid progenitor cells
EVs	Extravesicular bodies
fAD	Familiar Alzheimer's disease
GFAP	Glial fibrillar acid protein
GWAS	Genome wide association studies
HDL	High density lipoprotein
HMGB1	High mobility group box 1
Iba1	Ionized calcium binding adaptor molecule 1
ICC	Immunocytochemistry
IFN-β	Interferon-β

IFN-γ	Interferon-γ
IL	Interleukin
iMicroglia	Induced microglia differentiation from iPSCs
iNOS	Inducible nitric oxide synthase
iPSCs	Induced pluripotent stem cells
IRAK1	IL-1 receptor-associated with kinases
LPS	Lipopolysaccharide
mABs	Monoclonal antibodies
MFGE8	Milk fat globule-EGF factor 8
MHC-II	Major histocompatibility complex class II
miRNA	MicroRNA
MP	Microglial progenitor
mRNA	Messenger RNA
MVBs	Multivesicular bodies
NCSTN	Niscatrir
NF-kB	Nuclear Factor kappa B
NFTs	Neurofibrillary tangles
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
PBS	Phosphate-buffer saline
PDL	Poly-D-lysine
Pen/strep	Penicillin/streptomycin
PM	Primitive macrophage
Poly IC	Polyriboinosinic-polyacidic acid
PRRs	Pattern recognition receptors
PSEN	Presenilin
RAGE	Receptor for advanced glycation end products
RealTime - qPCR	Real-time quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species

sAD	Sporadic Alzheimer’s disease
SOCS-1	Suppressor of cytokine signalling 1
SPN	Single nucleotide polymorphism
Swe	Swedish mutation
TALENs	Transcription activator-like effector nucleases
TGF- β	Transforming grow factor
Th	T helper cells
TLR4	Toll-Like Receptor 4
TLR6	Toll-Like Receptor 6
TMEM119	Transmembrane protein 119
TNF-α	Tumour necrosis factor alfa
TRAF6	Tumour necrosis factor of receptor associated factor 6
Treg	T regulatory cells
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
ULA	Ultra-low attachment

INTRODUCTION

1. Alzheimer´s disease

Alzheimer's disease (AD), first described by Alois Alzheimer, is a degenerative brain pathology that affects most part of the 50 million dementia patients globally diagnosed. Disorders grouped under the final term "dementia" are caused by atypical brain changes, which trigger a decline in cognitive abilities, severe enough to impair patients' way of life and independency (Alzheimer's Association 2019). This neurodegenerative disorder is characterized by a severe decline of cognitive abilities leading to confusion, language disturbance, visual complaints, agitation, hallucinations, and memory loss. From the several macroscopic hallmarks, the most important is a loss of brain volume, termed cerebral atrophy. The major hallmarks of AD are dysregulation in the secretion of Amyloid-β (Aβ) forms and the associated senile plaques composed of aggregated Aβ and other misfolded proteins (Fernandes *et al.*, 2018; Fujita *et al.*, 2020). Such abnormalities may have a genetic basis, as the existence of one or multiple mutations either in Amyloid Precursor Protein (APP) or in its proteolytic machinery. Other abnormality is the gain or loss of function of presenilin 1 (PSEN1) or 2 (PSEN2) that causes early-onset familial AD (fAD). In addition, neurofibrillary tangles (NFTs) of TAU protein, neurodegeneration, synaptic deterioration, as well as dystrophic neurites are critical contributors for AD pathology (Bird, 2018; Konttinen *et al.*, 2019; Jessell, 2000).

1.1. Familial and sporadic AD

Multiple risk factors contribute for AD, including feminine gender, low education level, smoking, obesity, diabetes mellitus, but increasing age persists as the greatest of them. The prevalence of AD is higher in women, but in men the congenital decline is more severe and occurs earlier. Hormonal and metabolic differences in the brain may explain these distinctions between sexes (Majolo *et al.*, 2019). Despite not being only a disease of old age, many people diagnosed with AD are 65 years or older. Besides, like most of the diseases, this pathology results from a mixture of genetic and environmental factors. Although advanced age is responsible for approximately 95% of the sporadic form of the disease, AD may also result from a family history (≥45-65 years). Presence of specific genetic mutations is related to enhanced susceptibility for AD development (Table 1). The sporadic AD (sAD), despite not having a standard rule may be related, for example, with a mutation in Apolipoprotein E (APOE), while fAD can be related with mutations on APP and autosomal dominant mutations PSEN1 and PSEN2 that promote the generation of Aβ peptides susceptible to aggregation (Fernandes *et al.*, 2018; Hansen, Hanson, & Sheng, 2017; Carlyle *et al.*, 2015).

Table 1. Protein/genes and mutations involved in Alzheimer's disease.

Protein/Gene	Function	Mutation	Reference
Apolipoprotein E (ApoE)	Repair neuron damage	E4*	(Davidson *et al*., 2006)
Amyloid precursor protein (APP)	Neuronal growth	D678N c-209g E693G A692G V717I K670M D671L	(Wakutani *et al*., 2004) (Theuns & Van Broeckhoven, 2000) (Nilsberth *et al*., 2001) (Nilsberth *et al*., 2001) (Van Tol, 1991) (Mullan *et al*., 1992)
Presenilin 1 (PSEN1)	Inflammatory process and cell apoptosis	A246E L424R M139V E280A L166P c-48t c-4.752t Del éxon 9	(Sherrington *et al*., 1998) (Kowalska *et al*., 2003) (Rippon *et al*., 2003) (Lopera *et al*., 1997) (Moehlmann *et al*., 2002) (Ophir *et al*., 2005) (Thinakaran *et al*., 1996) (Verkkoniemi *et al*., 2000) (Maertzdorf *et al*., 2001)
Presenilin 2 (PSEN2)	Neurodegeneration process	N141I D90N M139V T122R	(Tomita *et al*., 1997) (Rogaev *et al*., 1995)
TAU Protein	Polymerization of tubulin, aggregation of microtubules	R406W	(Connell *et al*., 2001) (Rademakers *et al*., 2003) (Y. Saito *et al*., 2002)

*not a mutation, a polymorphism

During many years, the amyloid cascade hypothesis was the best theory to explain AD pathophysiology. So, it is normal that the metabolic pathways involved in the formation of amyloid plaques are the object of multiple studies. This hypothesis defends that the accumulation and aggregation of Aβ peptide in the brain defines the beginning of the disease (Heppner *et al.*, 2015). Aβ is a short peptide that is produced through the endoproteolytic processing of APP, a type I membrane glycoprotein synthesized in the endoplasmic reticulum of neuronal cells (Querfurth & Laferla, 2010). Several studies refer that, under normal circumstances, APP plays an essential role in the formation of the neuronal network due to its high levels during synaptogenesis. This protein is involved in multiple processes including cell-to-cell interaction and cell-substrate linkage occurring in different cellular responses (Nalivaeva & Turner, 2013; Tai *et al.*, 2014).

The processing of APP occurs by two divergent pathways that involve a sequential cleavage by a group of enzymes called secretases (Figure 1). Under physiological conditions, the non-amyloidogenic

pathway is the most common, and includes a two-step cleavage mediated by α- and γ-secretases that originate a p3 fragment (Tai *et al.*, 2014). Importantly, α-secretase (ADAM) cleaves APP within the Aβ domain preventing the formation, release, and accumulation of Aβ peptide in the brain, which indicates a potential neuroprotective role for this via (Konttinen *et al.*, 2019; Tai *et al.*, 2014;Irene Piaceri & Benedetta Nacmias, 2013; L. Sun *et al.*, 2017). The alternative amyloidogenic pathway is very similar to the canonical one, but the aberrant APP cleavage by β secretase (BACE1) originates a fragment that gets anchored in the cell membrane. Subsequently, this fragment is cleaved by γ-secretase complex [PSEN1 and PSEN2, nicastrin (NCSTN), Aph-1 and PEN-2 (PS-enhancer-2)] on three different sites, originating several Aβ peptides with a length of 39-43 amino acids; the most common are - Aβ-38, Aβ-40 and Aβ-42 – with distinct propensities for aggregation in oligomers due to their hydrophobic nature (Dá Mesquita *et al.*, 2016). The Aβ1-42 peptide is the most hydrophobic and prone to fibril formation, being the predominant isoform found in cerebral plaques (Gu & Guo, 2013).

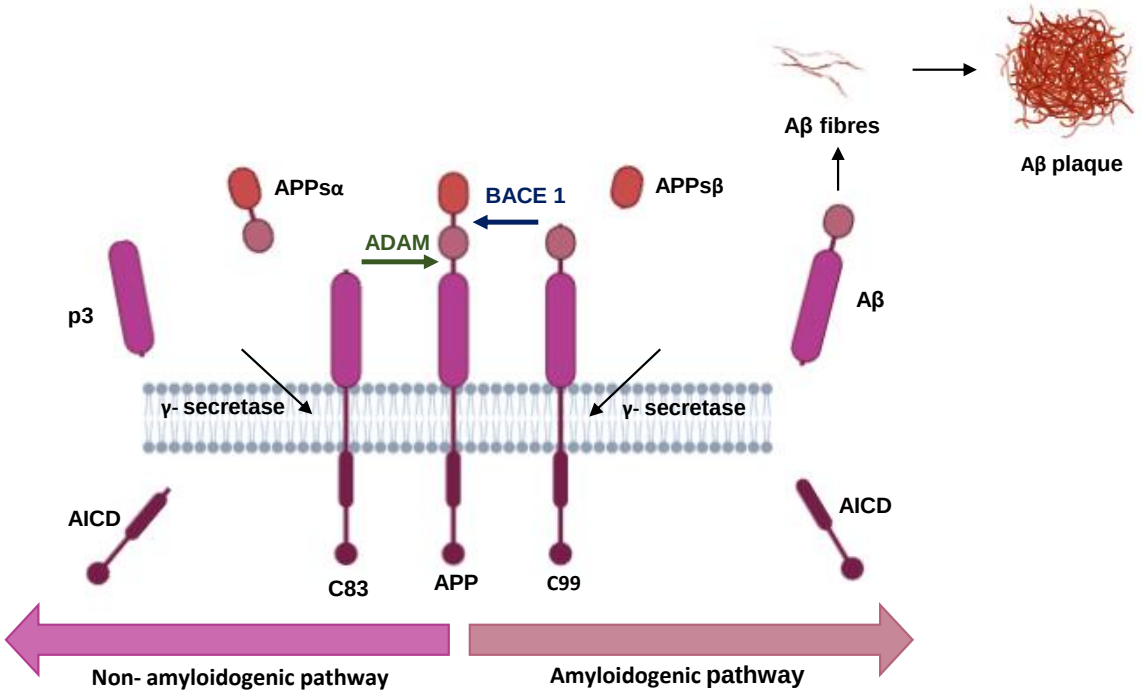

Figure 1. Amyloidogenic and non-amyloidogenic processing of the Amyloid Precursor Protein (APP). In the non-amyloidogenic route (left), α-secretase (ADAM) cleaves APP within the amyloid-beta (Aβ) domain, precluding the generation of this peptide. This cleavage releases a large soluble APP ectodomain (APPsα). γ-secretase-complex [PSEN1 and PSEN2, nicastrin (NCSTN), Aph-1 and PEN-2 (PS-enhancer-2)] mediated cleavage of the C83 and liberates the APP intracellular domain (AICD) into the cytosol and a non-pathogenic p3 peptide to the luminal side of the membrane. In the amyloidogenic pathway (right), the APP cleavage by β-secretase (BACE1) originates a membrane-bound C99 APP C-terminal fragment. The further cleavage by γ-secretase releases the Aβ peptide to the lumen and the AICD into the cytosol. In Alzheimer's disease, excessive amounts of Aβ peptide starts to abnormally accumulate, oligomerizes and aggregates eventually as Aβ plaques in the brain.

In AD, there is also a breakdown of post-translation modifications of TAU protein, namely in phosphorylation, leading to its hyperphosphorylation. In this state, TAU exhibits excessive aggregation with cytoskeletal proteins and weaker interactions with microtubules, which further increases free TAU, favouring aggregation and fibrillization, with the consequent malfunction of axonal transport (Kosik *et al.*, 1989). TAU filaments posteriorly accumulate in dystrophic neurites forming intracellular NFTs that become extracellular after the death of the neuron (Querfurth & Laferla, 2010). Under physiological

conditions, TAU appears to regulate cytoskeleton dynamics. It promotes the assembly of tubulin into microtubules and stabilizes their structure, contributing to the maintenance of neuronal polarity, axonal transport, and neurite outgrowth (Zhang *et al.*, 2016). In the central nervous system (CNS), TAU represents itself six isoforms that come from the alternative splicing of three exons, which differ by the presence or absence of one or two inserts in the amino-terminal portion and the presence of 3 to 4 repeated domains in the carboxyl-terminal portion. It is abundantly found in the distal portions of neuronal axons (Ballatore, Lee, & Trojanowski, 2007; Kosik *et al.*, 1989).

Other challenge in AD is a polymorphism in the ApoE gene. ApoE2, ApoE3, and ApoE4 variants are the most-recognized susceptible genes for AD. ApoE gene encodes the major protein component of high-density lipoprotein (HDL)–like lipoprotein particles that transport lipids, cholesterol, and other hydrophobic molecules in the CNS, participating in the clearance (avoiding amyloid deposition) and presenting an important function in repairing excessive damage to neurons. In this respect, it participates in the redistribution of lipids to axons and regenerating Schwann cells, restoring synaptic-dendritic connections (Hansen, Hanson, & Sheng, 2018; Bird, 2018). Additionally, by dysregulating the nuclear factor-κB (NF-κB) signalling cascade and prolonging the increase of cytokine levels, ApoE4 also plays a significant role in brain inflammation. The most common ApoE3 variant, appears to have a protective effect, limiting the spread of TAU and neurodegeneration, while presence of E4 isoform is ineffective in the mediation of the cellular repair and was described to unable Aβ clearance (Ophir *et al.*, 2005).

Over 60% of all sAD cases are not associated with APOE, suggesting that other genetic and environmental factors may contribute to determining the disease and it has been estimated that the genes mentioned above account for less than 30% of the genetic variation in AD, indicating that numerous additional AD-associated genes may exist (Liddelow, Marsh, Bennett, 2020). In the past years, a human genome-wide association study (GWAS) has identified over 20 genetic loci that robustly associate with AD risk. Many of these genes have a role in brain development, cytoskeletal organization, and immune function (Hansen *et al.*, 2018).

1.1.1. Mutations in familial AD

FAD mutations, either increase the total production of Aβ, shift the production to more toxic and aggregation-prone Aβ-42, or increase the aggregation propensity of the peptide, depending whether the mutation is localized around the β-secretase cleavage, at the γ–secretase cleavage site or within the Aβ sequence (De Leeuw & Tackenberg, 2019). The genes of PSEN1 and PSEN2 are crucial for gamma-secretase activity. These two genes are very similar, and they encode for two proteins that have around 67% of homology. Most of the presenilin mutations lead to increased production of Aβ-42, altering the ratio of Aβ-42 to Aβ-40. Most of PSEN1 and PSEN2 mutations are missense causing amino acid substitution in proteins and, like APP mutations, are autosomal dominant (Irene Piaceri1 *et al.*, 2013; Dá Mesquita *et al.*, 2016). PSEN1 mutations cause the most severe forms of the disease. Until now the median age of disease onset in families carrying PSEN1 mutations is near 45 years with a range from 28 to 79 years, depending on the position of the mutation in a particular region of the gene *(Hansen et al.*, 2018; Xiao *et al.*, 2017; Irene Piaceri1 *et al.*, 2013). PSEN2 families also show a wide range in age of onset (39-85 years), and therefore the age of onset is extremely variable among PSEN2-affected members of the same family, which may be due to the influence of other factors (Sun *et al.*, 2017). Other important mutations are the Swedish (Swe) mutation that is a specific modification in APP gene, at the

APP β-secretase cleavage site (codons 595 and 596 in APP695), making APP a preferable substrate for BACE1 (Fernandes *et al.*, 2018).

These Aβ peptides, resulting from the malfunction of the γ-secretase, aggregate and form insoluble oligomers with β-sheet conformation, originating the senile plaques that will spread to different areas of the brain (Kumar *et al.*, 2015). Although Aβ plaques seem to be important in AD pathophysiology, there is no evidence that the level of the pathology and plaque density are correlated. Thus, the idea of amyloid deposits being biologically inert is getting an increased support (Tai *et al.*, 2014).

It is also important to realize that the massive rise of cytokine levels is one of the causes for the lack of microglia phagocytosis of apoptotic cells, Aβ and other deposited proteins. These events trigger an increase of Aβ accumulation due to the downregulation of their phagocytic receptors, which leads to reduced clearance of Aβ (Hickman *et al.*, 2008). This theory is supported by the identification of mutations that compromise microglia function and have been correlated with high risks of AD development, for example in the extracellular domain of the triggering receptor expressed in myeloid cells 2 (TREM2), complement receptor 1 (CR1), ApoE4, and CD33 (Abud *et al.*, 2018; Heneka *et al.*, 2015; Pimplikar, 2014).

Mutations in TREM2 that is highly expressed by microglia, lead to a loss of their function in phagocytosis by impairing interactions with ligands, suggesting that TREM2 may have a protective role in AD and a neuroprotective character for normal functioning of microglia in AD (Hsieh *et al.*, 2009; Kober *et al.*, 2016; Hansen *et al.*, 2018). TREM2 stimulation initiates pathways that promote microglial chemotaxis, phagocytosis, survival, and proliferation. TREM2 binds to APOE and clustering (CLU; also known as apoJ), which are themselves encoded by AD risk genes (Y. Wang *et al.*, 2015; Hansen *et al.*, 2018). In the case of surface receptor CD33, a single-nucleotide polymorphism (SNP) in the gene reduces Aβ phagocytosis by peripheral macrophages, thus promoting the deposition of Aβ (Heneka *et al.*,2015).

1.2. The challenge of AD

In the last decades, life expectancy has increased substantially, due to general improvements in lifestyle. However, the subsequent prominent demographic upward shift in age distribution has led to an increased prevalence of age-related disorders. AD has no cure, but treatments for symptoms are available while the research continues. Though current AD treatments cannot stop the disease from advancing, they can temporarily slow the worsening of dementia symptoms and improve quality of life for patients and their caregivers (Alzheimer's Association 2019). The absence of therapies in AD are mainly related to the lack of knowledge on the small mechanisms that lead to AD. This approach to the details has been increasingly developed, but another problem is the unavailability of reliable models since most pre-clinical studies are done in mice that do not fully mimic what happens in humans. With a rapidly aging population, the number of AD patients is projected to reach 16 million by 2050. This will have a huge impact on modern societies, unless a treatment to cure the disease or halt its progression can be found (Pimplikar, 2014; Posner *et al.*, 2017).

2. Therapy

2.1 Current therapy

In the clinical practice, the treatment for AD patients is realized, first, with Rivastigmine, Galantamine, Tacrine or Donepezil and, secondly, when these are less effective, Memantine (Yiannopoulou & Papageorgiou, 2013). Donepezil enhances the cholinergic transmission since it is a cholinesterase inhibitor that will delay the degradation of acetylcholine at the synaptic cleft, while Memantine acts as an uncompetitive, moderate-affinity, N-methyl-D-aspartate antagonist believed to protect neurons from excitotoxicity. Combination of these two drugs show benefits on patient symptomatology. However, these two medications are unable to slow or prevent the progression of the disease. Antidepressants (serotonin reuptake inhibitors) and antipsychotic medications are often needed to treat symptoms that are observed in the later stages of the disease, such as depressed mood, sleep disturbances, delusions, and hallucinations (Yiannopoulou & Papageorgiou, 2013).

What is also indicated to control associated neuroinflammation, is the administration of nonsteroidal anti-inflammatory drugs (NSAIDs). Nevertheless, treatment with NSAIDs has only some efficacy in AD patients, when administered early in the disease. Several studies prove that long-term use of these drugs minimizes the risk of developing the pathology, but only if they are prescript in an early stage of the disease. Moreover, NSAIDs though decreasing microglial activation, may be responsible for their abnormal function and unresponsiveness (Caldeira *et al.*, 2017; Pimplikar, 2014).

2.2. Clinical trials

Until nowadays, all clinical trials have failed or produced very limited success, as described in Table 2. During many years, the therapeutic strategies were focused on Aβ production, degradation, and prevention of its accumulation in the brains of patients. In the last years, the major focus has been to inhibit the activity of BACE1. Although these approaches had a huge success in mouse models and seemed very promising, they consecutively failed in the human clinical trials relatively to the reduction of amyloid plaques or not being able to recover cognitive decline. BACE1 inhibitors aim to reduce Aβ and despite analysed for years, none have passed in clinical trials. Similar to what happens in other diseases early intervention at stages before symptoms are more efficient than after cognitive decline in consequence of amyloid plaques or TAU pathologies, lasting for 2 or 3 decades (Yiannopoulou & Papageorgiou, 2013; Pimplikar, 2014; Zhang *et al.*, 2016).

The major immunotherapy for AD is Aβ-targeting monoclonal antibodies (mAbs). There are many examples of BACE1 inhibitors and mAbs, (Table 2) though many failed in clinical trials. Together, the failure of these trials strongly suggests that it is better to handle Aβ deposits as a pathological feature than as part of a major mechanistic hypothesis (P. Liu *et al.*, 2019). Based on these failures in targeting Aβ, there has been a shift to different strategies, namely those that target TAU pathology. Ongoing studies evidence the use of vaccination for passive immunization to reduce TAU levels. This approach is based in a mouse model of AD where the inhibition of hyperphosphorylation kinases, as glycogen synthase kinase 3, has revealed promising results by stabilizing TAU and inhibiting its aggregation, or by preventing toxic post-translation modification (Yiannopoulou & Papageorgiou, 2013; Pimplikar, 2014; Zhang *et al.*, 2016).

Table 2. Status of some AD drugs in clinical trials.

Drug	Developer	Mechanism of action	Stage
Gantenerumab (RO4909832)	Roche/Genentech	Aβ-specific mAb	Phase III (failed to meet endpoint)
Solanezumab (LY2062430)	Eli Lilly	Aβ-specific mAb	Phase III (failed to meet endpoint)
AAB-003 (PF-05236812)	Janssen/Pfizer	Aβ-specific mAb	Phase I (ended)
Lanabecestat (AZD3293)	AstraZeneca/Eli Lilly	BACE1 inhibitor	Phase III (ended)
Verubecestat (MK-8931)	Merck & Co	BACE1 inhibitor	Phase III (ended)
Elenbecestat (E2609)	Eisai	BACE1 inhibitor	Phase III (ended)

Aβ, Amyloid-β; mAb, monoclonal antibody; BACE1, β secretase.

These failures in the clinical trials emphasize the importance of biomarkers to help early diagnosis since studies indicate that the therapeutic strategies are more effective during the early stages of disease development (Yiannopoulou & Papageorgiou, 2013).

3. AD-associated neuroinflammation

To date, new treatments have been clinically ineffective in preventing the loss of cognition. Because of the need to identify effective treatments for AD, neuroinflammation has become a renewed research interest (Lih-Fen Lue, 2019). Although acute neuroinflammation plays a protective role in the body, chronic neuroinflammation is always considered detrimental and damaging to nervous tissue. Neuroinflammation is characterized as a complex brain immune response aimed to protect the CNS against all the harmful changes that may occur. It involves a set of cellular and molecular alterations, recruitment of peripheral immune cells, induction of intracellular signalling pathways, and the release of inflammatory mediators (Heneka *et al.*, 2015). Under normal circumstances, neuroinflammation executes a reparative function that is beneficial to the organism and restores tissue health. Yet, exacerbated neuroinflammation is a common hallmark in many neurologic disorders including AD, multiple sclerosis, and stroke (Freilich, Woodbury, & Ikezu, 2013). *In vivo* studies have shown that inflammation can be found in AD patients even before the appearance of amyloid deposition, when there is only a mild cognitive impairment (Varnum & Ikezu, 2012). The novel genes identified from GWAS indicate that neuroinflammation has a causative role in neurodegeneration along with its secondary effects. However, neuroinflammation cannot be the only cause, otherwise the therapy that already exists would be sufficient and efficient (Heneka *et al.*, 2015).

3.1. Cellular players

Beyond neurons, the brain comprises other cells that are also critical for the establishment and maintenance of neural networks (Perea, Sur, & Araque, 2014). Since the discovery of the glia, researchers start realizing their functions, and despite such investigation, the total properties of glial cells remain unclear (Jäkel & Dimou, 2017). Currently, the whole glial cell population is subdivided into four major categories: (1) microglia, (2) astrocytes, (3) oligodendrocytes, and (4) progenitor cells, the $NG2^{+}$-glia (Jäkel & Dimou, 2017). Interactions among these multiple cell types orchestrate the functions for the homeostasis of the nervous tissue by executing a good spectrum of housekeeping functions (Verkhratsky & Parpura, 2016). There is a consensus that glial cells have an energetic role in brain function and cognition, both during development and in adulthood (Barres, 2008).

Microglia, the resident immunocompetent phagocytes, represent approximately 10% of total cells in a healthy brain and are the most abundant mononuclear phagocytes of the CNS (Colonna & Butovsky, 2017; Salter & Stevens, 2017). These innate immune cells of the CNS are originated from erythromyeloid progenitor cells (EMPs) in the embryonic yolk sac and migrate into the brain (Hansen, Hanson & Sheng, 2017). EMPs further develop to early primitive macrophages that migrate into the developing neural tube and become microglial progenitors that play crucial roles in CNS development, as well as in tissue maintenance, injury response, and pathogen defence (Konttinen *et al.*, 2019; Abud *et al.*, 2018). In fact, microglia are multifunctional cells that interact with numerous other cells in the CNS, including neurons, astrocytes, and oligodendrocytes, being important in the inflammatory response and pathogenesis. Microglia participate in normal brain development, strengthening neuronal connections through synaptic pruning, and are involved in oligodendrocyte and neuronal survival. They use their variety of pattern recognition receptors (PRRs) to respond to danger signals. These cells are involved in neuronal maintenance and support, while are also responsible for the inflammatory response in the brain following an injury or pathogenic infection (Abreu *et al.*, 2018a; Prinz *et al.*, 2019; Garcia-Reitboeck *et al.*, 2018).

Steady-state microglia exhibit a particular phenotype, characterized by having a cellular body of small dimensions and ramifications that extend in all directions. This ramified morphology allows microglia to scan the environment, without interfering with neuronal activities, monitoring all the brain parenchyma every few hours. Under stress stimuli, activated microglial cells assume high plasticity and acquire a reactive profile that compromise cellular homeostasis (Caldeira *et al.*, 2014; Hatch, Wei, Xia, & Götz, 2017). Depending on the stimuli and the time of exposure, microglia acquire several modifications in their phenotypes. These phenotypes can vary from inflammatory and cytotoxic to a more pro-regenerative one, being in most cases a blend of phenotypes that are hard to describe (Deczkowska *et al.*, 2018; Brites & Fernandes, 2015b). Once activated by pathological triggers, like debris from neuronal death or protein aggregates, microglia suffer morphological changes, converting their shape to an activated amoeboid and mobile cell. During this process, microglia release not only anti-inflammatory cytokines [*e.g.*, interleukin-4 (IL-4), IL-13, IL-10, and transforming growth factor beta (TGF-β)] but also pro-inflammatory cytokines and cytotoxic factors [*e.g.*, IL-1β, IL-12, IL-6, tumour necrosis factor (TNF-α)], as well as other pro-inflammatory markers, as inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS), while the receptor for advanced glycation end products (RAGE) is also increased (Figure 2) (Hansen *et al.*, 2018).

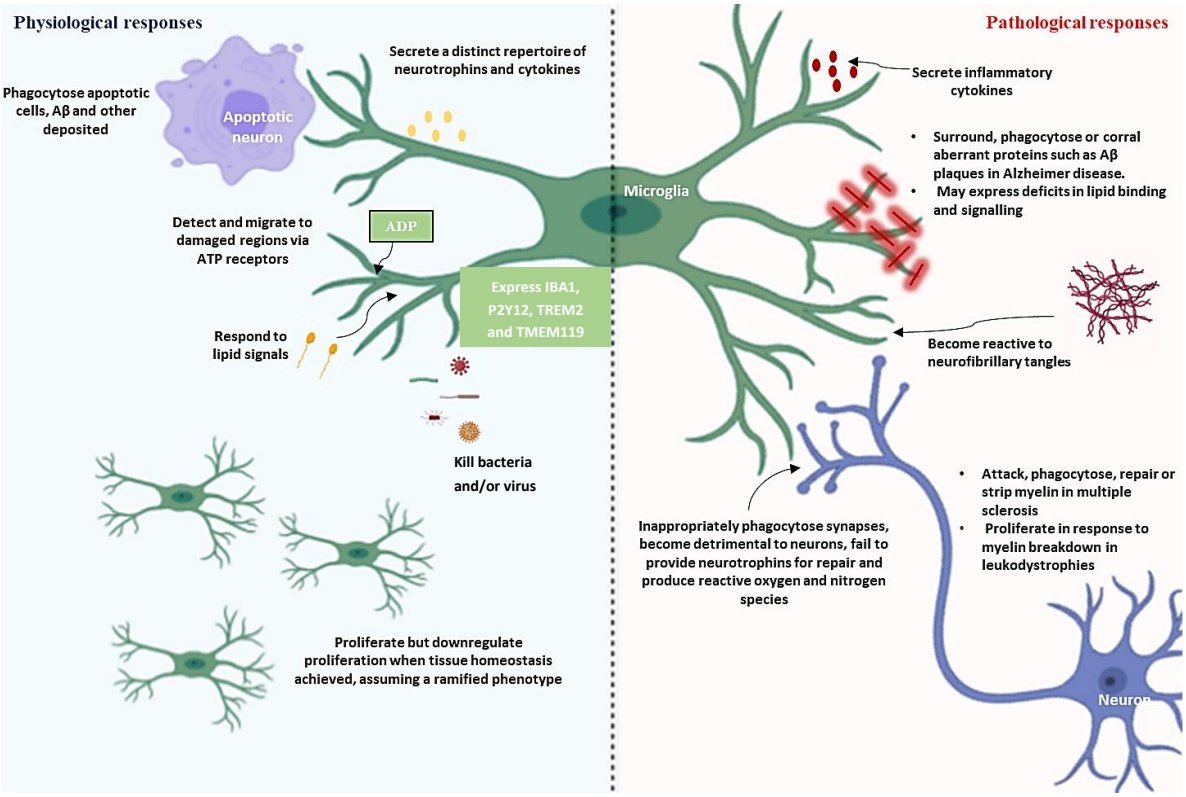

Figure 2. The physiological and pathological functions of microglia in AD. In physiological functions microglia contribute to healthy nervous system homeostasis in several ways. They provide cues and remove inappropriate synapses during development, and they secrete neurotrophins and cytokines to support and maintain neural networks in the mature nervous system. Moreover, they rapidly sense ATP signalling via receptors such as P2Y purinoceptor 12 (P2Y12) and migrate to areas of damage, where they proliferate and phagocytose apoptotic cells and any other damaged tissue to aid repair. **Recent research has focused in signaling targets in microglia in neurological diseases, including Alzheimer disease**. Such signalling is mediated by putative membrane-associated receptors, including triggering receptor expressed on myeloid cells 2 (TREM2), and mutations in TREM2 are genetic risk factors for Alzheimer disease. Microglia also influence nervous system pathology in a number of disorders. Microglia migrate to and surround amyloid-β (Aβ) plaques in Alzheimer disease in an attempt either to phagocytose this aberrant protein or to corral and contain it to prevent neuronal damage. In the ageing brain microglia appear dystrophic and become reactive, senescent and dysfunctional. In addition, their numbers are altered during ageing, decreasing in some areas of the brain and increasing in others. Adapted from Pocock & Piers, 2018.

Particularly in AD, microglia are capable to recognize Aβ oligomers via receptors such as CD36, toll-like receptor 4 (TLR4), TLR6 and TREM2. Moreover, *in vitro* studies in mouse models of neurodegenerative diseases with a deficiency in TREM2 demonstrated that its signalling is essential for microglia to detect and respond to neurodegeneration cues (Deczkowska *et al.*, 2018). This binding leads to the activation of microglia and differentiation in distinct microglia phenotypes. When incubated with Aβ-42, primary cultures of microglia show key-aging associated responses, with a progressive loss of reactivity, which course with reduced phagocytosis, migration, and lower expression of microRNAs (miRNAs) associated to neuroinflammation (Caldeira *et al.*, 2017; Konttinen *et al.*, 2019; Abud *et al.*, 2018; Ising *et al.*, 2019). A recent *in vitro* study referred that human CHME3 microglia gradually lose such protective property in chronic stressed conditions and upregulate both pro- and anti-inflammatory gene associated markers, while also evidence increased senescence-associated β-galactosidase

expression although participating in the early elimination of extracellular APP and Aβ accumulation when in co-culture with neuroblastoma Swe cells (Fernandes *et al*., 2018).

Exposure of microglia to the TLR4 ligand lipopolysaccharide (LPS) alone, or in combination with the immunomodulatory cytokine interferon-γ (IFN-γ), resulted in an upregulation of the pro-inflammatory cytokines (Brownjohn *et al.*, 2018). LPS has been widely used as an inducer of neuroinflammation and neurotoxicity in various models to study neurological disorders and is a powerful stimulator of microglia pro-inflammatory activation. Furthermore, inflammation-related neurodegeneration induced by LPS is a common approach that allows the study mechanisms of cellular neuroimmunology. LPS binds on microglia TLR4 to activate NF-κB and increase production of cytokines including IL-1, IL-6 and TNF-α (Abreu *et al.*, 2018). Due to the blood-brain barrier increased permeability in AD, LPS accumulates in patient's brains, associated with amyloid plaques, perivascular amyloid, and neurons. There are evidence that it causes downregulation of ADAM and upregulation of BACE1 what powers Aβ levels in brain (Sweeney *et al.*, 2018). In fact, several different patterns of co-localization of LPS and Aβ40/42 were detected in AD brains (Zhao *et al.*, 2019; Zhan, Stamova, & Sharp, 2018).

Other cells that play an important role in neuroinflammation are astrocytes since, together with the activation of microglia, the accumulation of reactive hypertrophic astrocytes around senile plaques has also been found. The response of astrocytes is represented by reactive astrogliosis, a reaction that takes place in several phases with a complete remodelling of astrocytes that normally results in neuroprotection and recovery of injured neural tissue (Olabarria *et al.*, 2010). These reactive astrocytes are characterized by overexpression of glial fibrillar acid protein (GFAP) and signs of functional impairment, without loss of organization. Astrocytes also play a very important role in the degradation of Aβ, through the lipidation of ApoE, adding to the microglia the ability to clean Aβ and the positive regulation of extracellular Aβ degrading proteases after exposure (Olabarria *et al.*, 2010). Further to this is the fact that a recent study describes that the pro-inflammatory cytokines produced by microglia activate astrocytes in a neurotoxic reactive state, aggravating the brain inflammatory status (Liddelow *et al.*, 2017).

3.2 Regulators of neuroinflammation: Inflammation- associated miRNAs

MiRNAs are a class of small, non-coding RNA molecules that broadly regulate specific gene expression, playing critical roles in multiple biological mechanisms, such as embryogenesis, neural and immune system development, host defense mechanism, and carcinogenesis (Freilich *et al.*, 2013). Emerging research shows that miRNAs can be secreted and delivered into recipient cells to inhibit their translation of target genes, thereby affecting their activities (Chen *et al.*, 2020; Freilich *et al.*, 2013). Cumulating data have pointed miRNAs as a new class of potential biomarkers for the diagnosis, prognosis, and treatment monitoring of a variety of diseases (*e.g.*, cancer, cardiovascular diseases, and neurological disorders) (Mishra, 2014).

The biogenesis of miRNAs is largely regulated by multiple steps and can occur through canonical or non-canonical pathways (Li & Rana, 2014). In the canonical pathway (Figure 3), independent genes or introns of protein-encoding genes are normally transcribed in the nucleus by RNA polymerase II to produce a primary precursor miRNA (pri-miRNA) in a hook. The pri-miRNAs are then processed by Drosha, from the RNase III family, giving rise to a ~70 nucleotide pre-miRNA molecule that is exported to the cytoplasm via exportin 5 and subsequently digested by Dicer in a ~22-stranded

double base pairs miRNA. After processing, the miRNA duplex guide strip binds to Argonaute proteins present in the RNA-induced silencing complex (RISC), while the other strip is released and discarded. As part of RISC, the miRNA base-pair targets mRNA and activates downstream gene regulatory mechanisms, regulates mRNA degradation, mRNA deadenylating, and / or translational repression (Bekris & Leverenz, 2015; Li & Rana, 2014). Alternatively, in the case of the non-canonical pathways, miRNA processing does not require all the proteins described above. An example of this is the involvement of splicing machines in the direct production of pre-miRNAs, bypassing the Drosha processing in the nucleus (Graves & Zeng, 2012).

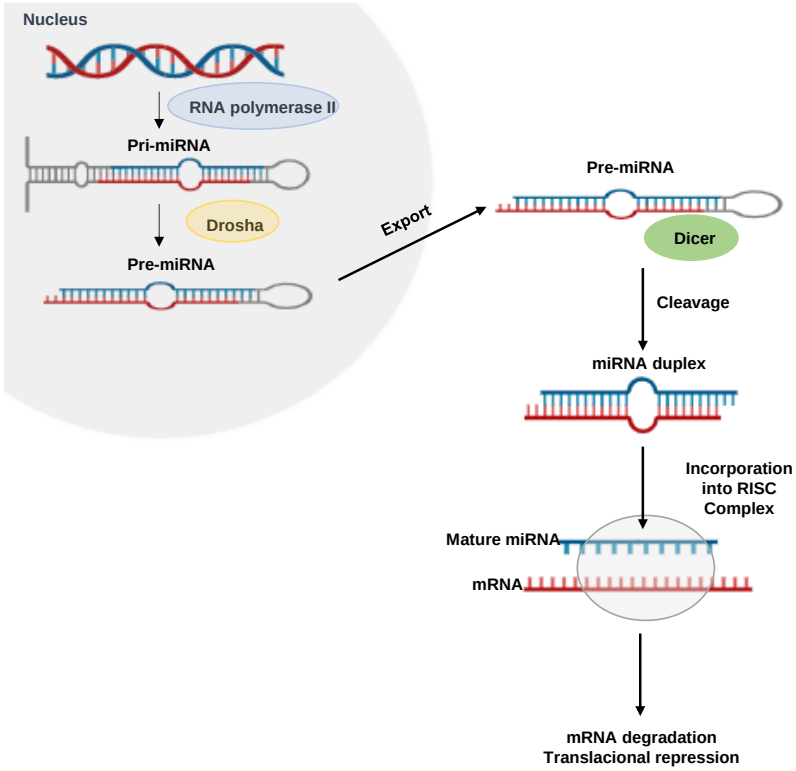

Figure 3. Schematic representation of miRNA biogenesis through canonical pathway. In the nucleus, micro-RNA (miRNA) gene is initially transcribed into a primary miRNA (pri-miRNA) by RNA polymerase II. The pri-miRNA is then processed by the Drosha complex to generate a precursor miRNA (pre-miRNA). Into the cyto-plasm, this precursor miRNA is again cleaved by Dicer into a mature miRNA duplex. The guide miRNA strand is incorporated in the RNA induced silencing complex (RISC) while the complementary miRNA strand is released and degraded. In RISC, the mature single-strand miRNA interacts with the 3'-UTR of the target mRNA and, depending on the level of binding complementary, induces mRNA degradation or inhibits its translation.

Returning to their bioactivities, miRNAs are reported to regulate the acute inflammatory response. For instance, many miRNAs are significantly enhanced upon different TLR stimulations, with miR-155 and miR-146a being two of the most famous among several ones. Other miRNAs are involved in other biological processes, such as miR-124 that regulates neuronal and microglial differentiations, while was also demonstrated ability to suppress the inflammatory response mediated by infiltrating microglia in an experimental autoimmune encephalitis mouse model. However, little is known about the global miRNA response, as well as their full array of targets under skewing conditions in microglia (Freilich *et al.*, 2013).

Expression levels of miRNAs can be changed in response to internal and external signals (Graves & Zeng, 2012). In AD, the research of miRNAs as biomarkers has become increasingly studied. It is evident that brain and circulating miRNAs play a direct role in the disease pathophysiology, being implicated in the expansion of the neurodegenerative process (Bekris & Leverenz, 2015). Multiple reports have pointed out that miRNAs can regulate the expression of genes that encode proteins involved in APP processing (*e.g.*, BACE1) (Long *et al.*, 2014).

MiR-124 and miR-128 are primarily expressed in neurons, while miR-23, miR-26, and miR-29 exist in large amounts in astrocytes, supporting a differential nature of expression. Some miRNAs are associated with neurological functions, such as learning and memory and miR-132, and miR-134 play a crucial role in the formation and plasticity of synapses, and miR-124a and miR-125b have been associated to the axonal outgrow (Brites & Fernandes, 2015a).

From the perspective of the pathology, the miRNAs that are considered important regulators of microglial function are: miR-21, -124, -125b, -146a and -155. These miRNAs are crucial contributors to neuroinflammation that occurs during AD pathology.

3.2.1 miR-21

MiR-21 is found mostly in activated immune cells (for example, dendritic cells, macrophages, and T cells), being its expression regulated by a variety of signals, transcription factors, and binding sites (Sheedy, 2015). The role of miR-21 in neurodegenerative diseases is not fully established. MiR-21 has anti-inflammatory effects that can benefit in neurological diseases characterized by toxic neuroinflammatory cascades, eventually playing a neuroprotective role. Several results indicate that a good regulator, both *in vitro* and *in vivo*, of the expression of this miRNA is the presence of Aβ oligomers (Schonrock *et al.*, 2010).

In rat bone marrow-derived macrophages and human blood monocytes, miR-21 is induced by LPS through TLR4-MyD88-NF-κB signalling. In this pathway, miR-21 targets programmed cell death protein 4 expression, leading to attenuation of NF-κB activity and an increase in production of IL-10, which regulates the inflammatory processes induced by LPS (Sheedy *et al.*, 2010). The late induction of miR-21 during inflammation suggests that it can, in fact, negatively regulate the inflammatory response and be an important contributor to the maintenance of homeostasis (Sheedy, 2015). Furthermore, our group showed that miR-21 is continuously upregulated in SH-SY5Y cells with the Swe mutation (SH_{SWE}) and in CHME3 microglia in response to their derived-secretome and exosomes, indicating its essential role in neuron-microglia interaction in AD (Fernandes *et al.*, 2018).

3.2.2 miR-124

MiR-124 is one of the most expressed miRNAs in the CNS. Besides, it was found that it controls the choice between neuronal and astrocyte differentiation and that its expression is almost entirely found in neurons, including spinal cord neurons (Doeppner *et al.*, 2013; Chen *et al.*, 2020).

Several studies have pointed out miR-124 as one of the main players in the regulation of signalling molecules underlying synaptic plasticity and memory. It has also been documented its

involvement in chronic stress, neurodegeneration, synapse morphology, long-term potentiation of neurotransmission, myeloid neurodevelopment, and haematopoiesis (Parisi *et al.*, 2013). By regulating genes encoding proteins involved in cytoskeletal organization, miR-124 modulates neurite growth. During neuronal differentiation, miR-124 suppresses lysine methyltransferase Ezh2 (enhancer of zeste 2 polycomb repressive complex 2 subunit), an important epigenetic factor that limits the expression of genes promoting the promotion of diversified neurogenesis. In this context, when deregulated by miR-124, Ezh2 levels are decreased and promote the neuronal differentiation route (Neo *et al.*, 2014). Recently it has been reported that miR-124 plays a vital role in microglial activation to a more anti-inflammatory stage, targeting the CCAAT-enhancer-α-binding protein (C / EBPα). The CX3CL1 / CX3CR1 (CX3C chemokine ligand 1 / Fractalkine receptor) axis has been reported to be involved in the delivery of miR-124 from neurons to microglia (Chen *et al.*, 2020; Guo *et al.*, 2019).

3.2.3 miR-125b

MiR-125b is one of the miRNAs whose role in AD is not yet fully established, it appears as a multipurpose molecule that plays a role in regulating gene expression in various types of cells (Huang *et al.*, 2013). MiR-125b is involved in the activation of the macrophage and the deregulation of the H-factor protein in primary human astroglial cells (Parisi *et al.*, 2013). In the brain of AD patients, the levels of miR-125b expression were found upregulated in the brain tissue, particularly in mature neurons (Alexandrov *et al.*, 2012; Le *et al.*, 2009). In contrast, miR-125b in sera from both AD patients and APP / PS1 mouse models was found to be significantly downregulated, when compared to control groups (Hong, Li, & Su, 2017; Tan, Yu, & Tan, 2015).

MiR-125b is also involved in the regulation of synaptic glycoprotein-2, thus causing pathogenic factors associated with AD, such as synaptic and neurotrophic deficits (Basavaraju & De Lencastre, 2016). Concerning Aβ, miR-125b is expected to contribute to amyloid-induced neurotoxicity, repressing the pro-apoptotic genes Bak1 and p53. Increased miR-125b expression disturbs the balance of phosphatase and kinase activity, leading to tau hyperphosphorylation in neurons and triggering learning and memory loss in rats. Regarding tau, it was also reported that increased expression of miR-125b disturbs the balance of phosphatase and kinase activity, leading to the hyperphosphorylation of tau in neurons and triggering learning and memory impairment in mice (Ma *et al.*, 2017).

3.2.4 miR-146a

MiR-146a, like miR-21, is found in elevated levels in active mature immune cells and is also described as having a large regulatory role in the immune system (Boldin & Baltimore, 2012). In response to inflammatory stimuli, such as LPS, IL-1β, and TNF-α, its promoter region contains two consensus NF-κB binding sites (Boldin & Baltimore, 2012). By suppressing the TNF-α of receptor factor 6 (TRAF6) and IL-1 receptor-associated with kinase 1 (IRAK1), miR-146a acts as a negative regulator of the NF-κB pathway and an important regulator of TLR signalling, leading to decreased expression of IL-8, IL-1β and TNF-α (Li, Chen, & Li, 2010; Taganov *et al.*, 2006).

In neurological diseases, such as AD, there is an upregulation of this miR-146a, but the first role associated was a compensatory mechanism to control the pathological inflammation and to restore homeostasis due to its neuroprotective properties (Gaudet *et al.*, 2018). In the regions most affected by

the AD pathology, it is normally found high expression of miR-146a, coinciding with Aβ-42 accumulation (Alexandrov *et al.*, 2014). However, multiple studies also indicate miR-146a as a detrimental miRNA in AD pathology, particularly affecting neuronal function (Ansari *et al.*, 2019; G. Wang *et al.*, 2016). Regarding microglia, human CHME3 microglia revealed an early miR-146a upregulation together with miR-155, after exposure to SH_{SWE} secretome, suggesting an implication in the initial inflammatory phenotype of these microglial cells (Fernandes *et al.*, 2018).

3.2.5 miR-155

From all the aborded miRNAs, miR-155 is probably the one whose functions have been more studied and conclusive about its role on inflammation and innate immunity (Faraoni *et al.*,2009). MiR-155 has been described as a key player in the macrophage inflammatory response upon TLR activation, which presupposes that its regulation depends on the JNK pathway (O'Connell *et al.*, 2007). Later, several studies emerged pointing to IFN-β, polyriboinosinic – polyacidic acid (poly IC), and TNF-α as inflammatory mediators capable of inducing miR-155 expression in macrophages and monocytes (Faraoni *et al.*, 2009). MiR-155 controls the survival, differentiation, proliferation, and activation of T helper cells 1 (Th1), Th2, Th17, T regulatory cells (Treg), and $CD8^+$ cells, by being able to regulate the T cell receptor signal and the inflammatory production of cytokines (Song & Lee, 2015).

The pro-inflammatory function of miR-155 is implicated in chronic inflammatory events that are related to the onset and progression of neurodegenerative diseases. Significant increases in the miR-155 levels were observed in CSF (cerebrospinal fluid) and more specifically related to IκB kinase ε, inositol 5-phosphatase 1, and the suppressor of cytokine signalling 1 (SOCS-1). It can be predicted that the high expression of miR-155 constitutes a contributing factor to inflammatory processes in the CNS. Conversely, the inhibition of miR-155 induces a neuroprotective effect against damage induced by microglia, pointing this miRNA as a promising target for the control of neuroinflammation *(*Cardoso *et al.*, 2012). In microglia activation, miR-155 displays a strong proinflammatory role and is required for the progression of the immune response in these cells. miR-155 directly promotes SOCS-1 inhibition leading to the production of nitric oxide (NO) and cytokines. In a study it was described that the activation of TLR4 receptor by Aβ fibrils induce the enhanced expression of miR-155 in the brain of 3xTg AD mice at 12 months, with a simultaneous activation of astrocytes and microglia. Based on their results, the authors indicate miR-155 and subsequent SOCS-1 downregulation as important contributors to the immune response triggered by excessive production of the Aβ peptide in AD patients (Guedes *et al.*, 2018). A study with *in vitro* co-cultures showed that in response to stress signals from SH_{SWE} cells, CHME3 microglia acquire an initial pro-inflammatory polarization with upregulation of both miR-155 and miR-146a. In the same work, the authors refer that an augmented expression of inflammatory cytokines is detected at later time points, where miR-146a is reduced, but miR-155 is still increased, which validate the dominant function of miR-155 in promoting inflammation overriding miR-146a action (Fernandes *et al.*, 2018).

3.3 Secretome as the mediator of neuroinflammation

The term secretome means "both the components of machinery for protein secretion and the native secreted proteins". This definition evolved including all factors that are secreted by a cell, tissue, or organism to the extracellular space under a defined time and conditions. Beyond soluble factors, the secretome also has the presence of lipids and extracellular vesicles (EVs) carrying important molecules for intercellular signalling (Basu & Ludlow, 2016).

In the past years, therapies with secretome and EVs have gained a major projection and revealed promising results for the treatment of CNS-related diseases, even though the mechanism of action remains to be completely understood. Diverse studies refer to secreted factors and vesicles as agents for the regenerative effect, rather than cellular differentiation. The use of secretome has increased exponentially from 2009 until the present (Pinho *et al.*, 2020). The characterization of the secretome constitution is important to understand intercellular signalling because this fraction represents a class of molecules and factors that control and regulate many biological and physiological processes. The components of cellular secretome are behind the paracrine signalling, mediating a plethora of biological activities, not only in the neighbouring, but also in distant cells. Studies on this form of cellular interaction initiated a therapeutic paradigm, as we can understand more about cellular products derived directly from cells (González *et al.*,2019). Secretome-based approaches present advantages in terms of engineering, storage and handling of cellular products, as well as in their potential as direct therapeutic agents (Teixeira *et al.*, 2016; Sevivas *et al.*, 2017).

The variety of components constituting the secretome can be split into two distinct fractions: the soluble fraction (essentially proteins and soluble factors such as cytokines) and the vesicular fraction. The soluble faction contains several components, such as cytokines, chemokines, growth factors, coding and non-coding RNAs, among many others, that are involved in practically every cellular function. On the other hand, the vesicular fraction is composed of exosomes, microvesicles, and apoptotic bodies. These vesicles are usually rich in nucleic acids (*e.g.*, messenger RNAs and miRNAs), small proteins (in the case of exosomes), misfolded proteins (in the microvesicles), or even cellular components, including organelles (typically in apoptotic bodies) (Pinho *et al.*, 2020).

Exosomes, classically defined as nanosized biological particles (50-150 nm), are secreted by the majority of the cells, being found both in cell culture media and in biological fluids, such as blood, urine, breast milk, CSF, saliva, and amniotic fluid (Malm *et al.*, 2016; Chen *et al.*, 2020). Exosomes are derived from endocytic membranes that are formed in multivesicular bodies (MVBs) (Brites & Fernandes, 2015). According to physiological or pathological conditions and cell source, the exosomal cargo and the surface signature might be different. Due to their endosomal origin, the exosome molecular contents comprise a set of constitutive proteins that participate in membrane transport and fusion (*e.g.*, Annexins, Rab GTPases, and Flotillin), in multivesicular body biogenesis (*e.g.*, Alix and TSG101), in events requiring heat shock proteins (Hsc70 and Hsp90), integrins and tetraspanins (*e.g.*, CD63, CD9, CD81, and CD82) (Simons & Raposo, 2009). There are other components, as lipids, such as cholesterol, ceramide, and sphingolipids, that regulate the sorting of small RNAs and proteins. In addition to these molecules, DNA, messenger RNA (mRNA), circular RNA, ribosomal RNA, miRNA, and long noncoding RNA have also been found in exosomes (Xiao *et al.*, 2017).

The discovery of this communication with exosomes triggered a special attention to exosomal miRNAs. Although the exact processes involved in miRNA sorting into exosomes are still to be

explained, it is considered that this event can occur by four main potential mechanisms, including: (1) the neural sphingomyelinase 2-dependent pathway; (2) the miRNA motif and stimulated heterogeneous nuclear ribonucleoproteins-dependent pathway; (3) the 3' end of the miRNA sequence; and (4) the miRNA induced silencing complex-related pathway (Brites & Fernandes, 2015). Being associated with exosomes, miRNAs expand their target possibilities, traveling not only to the neighbouring, but also to distant recipient cells, modulating their gene expression (Zhang *et al.*, 2016).

4. Models to study Alzheimer´s disease

The human brain is a highly complex structure that cannot be easily interrogated *in vivo* and has proven difficult to model *in vitro*. Many of these models represent key events in human brain development and cell-cell interactions between various cell types (Abreu *et al.*, 2018a). Due to the prevalence of AD, several models were developed to study this pathology, including human and animal cell lines, as well as invertebrate, zebrafish, and rodent animal models.

Studying the functions of human microglia is challenging because good-quality human CNS tissue samples are sparse. The isolation of microglia from *post-mortem* tissue or surgery specimens may introduce technical artefacts, and the impact of confounding factors, such as comorbidities, medication, distress is considerable (Banerjee *et al.*, 2020). *Post-mortem* brain samples are crucial for discovering the cellular and molecular changes associated with neurodegeneration, and for recognizing the end-stage of the disease pathology, but do not allow the study of ways to alter or intervene in the course of the disease (Choi & Tanzi, 2012). To understand the mechanisms involved in the disease development, alternative approaches have been applied using *in vitro* cell cultures. Primary rodent cell cultures and immortalized human cancer-derived cells (*e.g.*, SH-SY5Y neuroblastoma cell line) harbouring disease-associated mutations have been widely used. This culture of human and rodent cells has been useful for examining the disease-related molecules, yet, they are limited on modelling neurodegeneration and many other age-related aspects of AD (Penney *et al.*, 2020).

AD mouse models result from the insertion and overexpression of one or more human genes associated with the pathology (*e.g.*, human APP, PSEN1/2, or TAU) leading to memory loss at a young age (Yang *et al.*, 2016). These mouse models do not completely mimic the disease in its complexity and studies argue for the development of species-specific research tools to investigate the biology of human microglia in healthy and diseased states (Jiang *et al.*, 2020). The animal model 5xFAD that contains 5 mutations associated with early-onset familial AD, and the 3xTgAD that can mimic Aβ plaques and cognitive impairment, rarely develop NFT pathology. Most transgenic mice present an accelerated phenotype, leading to the appearance of symptoms early in life, limiting the potential for studying the impact of aging in the development of the pathology (Kitazawa *et al.*, 2012). However, there is increasing evidence that rodent microglia are not able to faithfully mirror the biology of the human cell. In particular, recent transcriptomic studies have clearly demonstrated that several immune genes, not identified as part of the mouse microglial signature, were abundantly expressed in human microglia (Xu *et al.*, 2020).

So, more advanced and reliable models are needed to explore effective therapeutic strategies for AD. Induced pluripotent stem cells (iPSCs) from human patients certainly constitute a huge improvement in that regard. Differentiation of patient iPSCs into neurons can reproduce both Aβ and TAU pathologies and provide a better background to study the disease.

4.1 Induced Pluripotent stem cells (iPSCs)

The iPSCs are a major discover and provide a new and better way to study AD and other diseases. Mainly derived from fibroblasts, iPSCs were first generated through the retroviral transduction of four transcription factors (OCT4, SOX2, KLF4, and c-MYC). When overexpressed, these factors can completely change the fibroblast phenotype into a pluripotent state that functionally and phenotypically resembles embryonic stem cells (Takahashi & Yamanaka, 2006). Besides fibroblasts, several other kinds of donor cells can generate iPSCs, including neural progenitors, liver and stomach cells, human keratinocytes, mature B lymphocytes, pancreatic-β cells, adipocytes, and human amniotic fluid-derived cells (Yang *et al.*, 2016).

The generation of patient-derived iPSCs has facilitated new opportunities to examine the relationships between genetic risk factors and disease (Abud *et al.*, 2017; Brownjohn *et al.*, 2018). Despite of some apprehensions about epigenetic memory and chromosomal instability, the iPSC technology has proven to be extremely helpful in modelling human genetic disorders (Banerjee *et al.*, 2020). iPSCs have the ability to self-renew, expand unlimitedly in culture and share the capacity to differentiate into all three germ layers, thereby providing the possibility of reconstructing all types of cells, tissue, and even organs (Zhang *et al.*, 2016). Recent cellular models generated from patient cells using iPSC technology have provided promising tools for understanding AD mechanisms (Yang *et al.*, 2016).

Most of the human iPSC-based AD models have focus on hippocampal or cortical neurons so far (Nieweg *et al.*, 2015). iPSCs from AD patients with PSEN1, PSEN2, and APP mutations have already been established. These cells can differentiate into neuronal or glial cells, recapitulating at different extents some features of AD (Liu *et al.*, 2012). Studying human microglia is challenging because of the rarity and difficulty in acquiring primary cells from human foetal or adult CNS tissue. Therefore, there is a pressing need to develop a renewable source of human microglia, such as from iPSCs (Abud *et al.*, 2018). The microglia differentiation from iPSCs (iMicroglia) has some benefits such as high throughput, enhanced experimental control and easily modified via gene editing techniques but it also has limitations, such as transcriptomic deficiencies related to culture environment and lack of peripheral immune cell interactions (Hasselmann, 2020).

There are many methods described for microglia differentiation from iPSCs (Table 3), each of them capable of generating iMicroglia with similar expression of many of genes and proteins as biological microglia (Ryan *et al.*, 2020). Although each protocol is distinctive they are all based on the concept that, in order to produce accurate microglial surrogates *in vitro*, one needed to mimic the cues that naturally drive microglia differentiation *in vivo* (Hasselmann, 2020). In this case, cells were available thanks to a collaborative effort with Tarja Malm, from Finland, under the JPND Consortium, as primitive microglia (day 8-10 of differentiation). This protocol that enables EMPs differentiation followed by microglial maturation, has the advantage of being fast (only 24 days), requiring few reagents when compared to others and being relatively easy (Konttinen *et al.*, 2019; Haenseler *et al.*, 2019).

Commonly, the controls used in these studies are from family members or healthy individuals. However, such controls may have a different genetic background than AD cells and consequently, may noy be the most appropriate. To overcome this problem, isogenic cell lines were created, by using gene editing technologies, such as transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR) / CRISPR-associated protein (Cas). In isogenic controls derived from AD-iPSCs, pathological mutations are corrected (Yang *et al.*, 2016).

Even though the great success that it may represent, the iPSC-based cell therapy is still at its early stage and numerous obstacles to its clinical application still need to be overcome in the future.

Table 3. Overview of published iPSC-derived microglia models.

Article	Protocol overview	Notable findings	Disadvantages
(Almeida *et al.*, 2012)	Not described in publication	First to produce hiPSC microglia	Generated through neuronal rather than myeloid pathway.
(Lancaster & Knoblich, 2014)	Grow hESC or iPSC colonies, next EBs are subjected to neural induction in a minimal medium. The neuroectodermal tissues are then transferred to a floating droplet of Matrigel, which promotes outgrowth of neuroepithelial buds. The tissues are transferred to a spinning bioreactor, which promotes improved nutrient and oxygen exchange to allow more extensive growth and further development into defined brain region	The protocol can be used for a variety of developmental and disease studies. The organoids can be used to examine tissue morphogenesis, early embryonic ectodermal fate determination and neuroepithelial polarity establishment.	The method lacks surrounding embryonic tissues that are important for the interplay of neural and non-neural tissue cross-talk. Organoids show marked variability, particularly between preparations
(Muffat *et al.*, 2016)	Embryoid bodies generated and resuspended in neuroglial differentiation media containing (supplement) with the addition of CSF-1/M-CSF and IL-34.	First published study with similar characteristics of fetal primary human and mouse microglia.	Appears to generate a mixed population of cells and is limited to monoculture experiments.
(Abud *et al.*, 2017)	Microglia differentiation media - neuronal base media (DMEM/F-12 +N2+B27) supplemented with M-CSF, IL- 34, and TGFβ-1. Maturation media containing CSF-1, IL-34, and TGF-β supplemented with CD200 and CX3CL1, which is notably secreted by neurons for the final three days.	Successful transplantation of ramified microglia within AD model mice. Subsequent *in vivo* study shows ability to interact with neurotoxic amyloid β.	Requires an isolation step prior to haematopoiesis differentiation, making it highly complex compared to pure single molecule methods.
(Takata *et al.*, 2017)	Generation of hematopoietic lineage macrophages terminally differentiated with IL-3 and CSF-1/M-CSF. Need for co-culture with mouse iPSC derived neurons to promote microglia phenotype.	Described the requirement for tissue-dependent cues to make cells more microglia-like. Demonstrated potential of modelling infiltrating macrophages during adulthood.	Primary characterization with mouse iPSCs.
(Mcquade *et al.*, 2018)	Proprietary composition of initial hematopoietic differentiation media (STEMdiff hematopoietic kit) for 11-days followed by differentiation with IL-34, TGFβ-1, and M-CSF/CSF-1. Additional maturation step with CX3CL1 (fractalkine) and CD200 to induce ramification.	Successfully ramified cells following transplantation in mouse brain. Suggests IDE1 as a small molecule able to replace TGF-β in protocols for differentiation	Describes itself as resembling developmental microglia but does not separate cited fetal *vs.* adult datasets
(Ormel *et al.*, 2018)	This protocol was adapted from Lancaster & Knoblich, 2014**,** with the only change made in media composition - increased concentration of Heparin (0.1 ug/ml to 1 ug/ml)	Characterize innate development of microglia in hiPSC-derived brain organoids, which exhibit some phagocytic function as synaptic material is present within the cells.	Replication of these findings is lacking in the literature regarding the spontaneous differentiation of microglia in the organoid.

(Haenseler *et al.*, 2019)	Utilizes IL-3 and M-CSF to drive myelopoiesis yielding a pure macrophage precursor population. Microglia differentiation and ramification of these cells is induced using a neuronal base media (DMEM/F-12+N2) supplemented with IL-34 and GM-CSF which is used in the cultivation of monocytes and macrophages. The protocol compares with use of M-CSF to mature macrophages.	Matured microglia can be generated at 2-week intervals for a 5- month period. Functional validation completed in a co-culture system.	Requires a very sensitive 6–7-week period before microglia precursors can be collected.

hiPSC microglia, human iPSC-derived microglia; iPSCs, induced pluripotent stem cells, hESC, human embryonic stem cells; EBs, embryoid bodies; CSF-1, colony-stimulating factor-1; M-CSF, macrophage colony stimulating factor; IL-34, interleukin-34; DMEM, Dulbecco's modified eagle medium; TGFβ-1, Transforming growth factor β-1; CX3CL1, CX3C chemokine ligand 1; IL-3, interleukin-3; GM-CSF, granulocyte macrophage-colony stimulating factor.

5. Motivation and aims

Recently, it became clear that microglia have a crucial role in the clearance of amyloid plaques and secretion of pro-inflammatory factors in AD. Although neuroinflammation has an important role in AD pathophysiology, the exact consequences of neuroinflammation in AD are still not clear. Microglia are responsible for many functions to sustain brain homeostasis, including the reparative response, which seems to fail in all stages of the disease. This indicates that the activated, dysfunctional, or degenerative microglia may differently contribute to the onset and progression of such disorder. So, it becomes crucial to understand the interplay between neurons and microglial cells in the AD disease from its onset. Even so, human brain is a highly complex structure that cannot be easily studied *in vivo* and has proven difficult to model *in vitro*. So, recent cellular models generated from patient cells by using the iPSC technology have provided promising tools for understanding human disease mechanisms, including AD. The differentiation of iPSCs into iMicroglia opens space for promising clinical applications, but the obtained cell population still present a huge heterogeneity, variations in their functional maturation and regional diversity.

The global aim of this thesis was to explore and identify inflammatory-associated markers in a human microglia model described to recapitulate some pathological features of the AD, and to surpass species-specific differences to better understand microglia-induced pathological events in AD.

To address this goal, specific tasks were established:

Task 1 – Maturation and characterization of iMicroglia when differentiated from primitive microglia as well as verification of cell morphological alterations by AD mutations.
iMicroglia derived from AD patients with distinct phenotype(s) (PSEN1 and Swe mutations), were maturated in our laboratory and characterized for the presence of usual microglial markers, as well as for cell morphology, together with inflammatory-associated genes and miRNAs, in comparison with a control iPSC cell line (Ctrl) and a gene-corrected isogenic control iPSC cell line (ISO PSEN1).

Task 2 – Assessment of dysregulated signatures in AD iMicroglia polarized phenotypes when exposed to inflammatory activation.

The same cell lines used in Task 1 will be treated with LPS and IFN-γ, and expression of microglial phenotypic genes and inflammatory-associated miRNAs will be assessed and compared with the control iPSC cell line (Ctrl) and the gene-corrected isogenic control iPSC cell line (ISO PSEN1).

II MATERIALS AND METHODS

1. Materials

1.1 Chemicals

Iscove's Modified Dulbecco's Medium (IMDM), Penicillin/Streptomycin, were obtained from Thermo Fisher Scientific (Waltham, MA, USA). IL-34 and M-CSF were obtained from PeproTech (London, UK). Accutase was from STEMCELL™ Technologies (Grenoble, France). IFN-γ was from Selleckchem (Munich, Germany). Fetal bovine serum was from Biochrom AG (Berlin, Germany). Triton X-100 was purchased from Roche Diagnostics (Mannhein, Germany). Hoechst 33258 dye, bovine serum albumin (BSA) (St. Louis, MO, USA). TripleXtractor, Xpert cDNA Synthesis Mastermix Kit and Xpert Fast SYBR Mastermix (Uni) BLUE were purchased from GRISP (Porto, Portugal). miRNANeasy®Mini Kit was acquired from Qiagen (Hilden, Germany). miRNACURY LNATM Universal RT miRNA PCR kit and PCR primer mix for miR-124, miRNA21, miR-146a, miR-155, miR-125b, U6 and RNAUA1 were obtained from Qiagen (Hilden, Germany). Primers for β-Actin, TGF-β, iNOS, IL-10, IL-6, MFGE-8, CX3CR1, TREM2, Arg1, GADPH, MHCII, P21 and CD68 were from Frilabo (Porto, Portugal). PowerUp™ SYBR™ Green Master Mix was obtained from Applied Biosystems, Life Technologies (Foster City, CA, USA). Paraformaldehyde (PFA) were as purchased from Merck-Millipore (Darmstadt, Germany). All the other common chemicals were purchased either from Sigma-Aldrich or Merck.

1.2 Equipment

Cell cultures were maintained in an Heraeus incubator (Thermo Fisher Scientific, Waltham, MA, USA) to ensure a stable environment for optimal cell growth (37ºC and 5% CO2). All the experimental procedures involving cell handling were performed in sterile conditions using a Holten Lamin Air HVR 2460 (Allerod, Denmark). Fluorescence images were acquired by using a Leica DMi8-CS inverted microscope with Leica LAS X software (Leica, Wetzlar, Germany). For RNA quantification it was used the NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). TPersonal Thermocycler (Biometra®, Göttingen, Germany) was used to synthesize cDNA from RNA samples. For determination of mRNA and miRNA, by Real-Time PCR (RT-PCR), it was used the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Eppendorf 5810R (Eppendorf, Hamburg, Germany) centrifuges were used for experimental procedures when necessary.

2. Methods

2.1 Differentiation of microglia from iPSCs

The iPSCs lines were provided from project collaborations and generated from two types of individuals: one carrying a PSEN1 mutation and other carrying the APP Swe mutation both diagnosed with AD. The controls used are one healthy adult and the isogenic control of the mutation PSEN1 correct using CRISPR / Cas9 technology (Table 4).

Table 4. Demographic information of each iPSC-line (Konttinen *et al.*, 2019).

Alias	Cell line	Sex	Health status	Age at biopsy	Mutation genotype	Sample origin	Reprogramming method	Karyotype
Ctrl	Ctrl3	F	Healthy	44	-	Skin biopsy	SeV 1.0	46XX Normal
Swe	LL116	F	FAD pre-symptomatic	30	KM670/671NL APP Swedish	Skin biopsy	SeV 2.0	46XX Normal
PSEN1	AD3	F	FAD pre-symptomatic	47	PSEN1ΔE9 deletion	Skin biopsy	SeV 2.0	46XX Normal
ISO PSEN1	AD3 iso	F	FAD corrected	47	PSEN1ΔE9 corrected	Skin biopsy	SeV 2.0 CRISPR/Cas9	46XX Normal

Ctrl, control: Swe, Swedish; PSEN1, presenilin 1; ISO PSEN1, PSEN1, isogenic control; fAD, familial Alzheimer´s disease; APP, amyloid precursor protein; PSEN1, presenilin 1.

iPSCs were differentiated at Tarja Malm lab and arrived at our lab in an intermediate state of differentiation, as EMPs. Tarja's laboratory carried the first phase of the process in which the iPSCs emerge into EMPs, then cells were frozen at -80ºC and sent to us for later differentiation and maturation into iMicroglia. For the first part of the differentiation process, confluent iPSCs were washed and incubated in ethylenediaminetetraacetic acid (EDTA). iPSCs were detached by pipetting into medium. After counting the cell number, cell suspension was further resuspended to assure seeding of isolated cells. isolated iPSCs were spread around the dish evenly. After a day, the medium was changed to E8BAC supplemented with ROCK inhibitor and confirmed that the cells remain as single cells or in groups of only few cells (Konttinen *et al.*, 2019).

In the next day (day 2), the mesodermal marker brachyury is expected to reach the highest expression level at 40-48h after starting the differentiation. During this time frame, the medium was changed to FVI medium [DF35 supplemented with, FGF2 (0,4%), SB431542 (0,1%), Insulin (0,05%) and VEGF (0,05%)]. On day 3, there the excess of dead cells were removed together with old medium and fresh FVI-medium was added (Konttinen *et al.*, 2019). On day 4, the medium was changed to HPC medium [DF35 supplemented with, FGF2 (0,2%), Insulin (0,05%), VEGF (0,05%), TPO (0,05%), SCF (0,05%), IL-6 (0,05%) and IL-3 (0,05%)]. The cells were transferred to a humidified normoxic incubator and from now on these culture conditions were maintained until the end of the protocol. Fresh EMP medium was changed daily until day 8. At day 8 of differentiation the cells reach the state of EMPs. In this point the cells were frozen and sent to us.

We received the frozen cells as EMPs and started the final phase of differentiation into iMicroglia. The EMPs were routinely cultured in ultra-low attachment (ULA) 10 cm dishes (Corning, NY, USA) with microglial progenitors (MP) medium [IMDM supplemented with, FBS (10%), P/S (1%), Insulin (0,5%), IL-34 (0,1%) and M-CSF (0,0125%)], in a humidified atmosphere containing 5% CO2 at 37ºC. The total medium volume was changed the day after to a primitive macrophage (PM) [IMDM supplemented with, FBS (10%), P/S (0,5%), IL-34 (0,01%) and M-CSF (0,025%)]. Every 2-3 days the total medium volume was changed by new PM medium. iMicroglia were chemically detached with accutase (Sigma) for 5 min at 37ºC. iMicroglia were plated in 12-well dishes coated with Poly-D-Lysine at a final concentration of 70 000 cells per well in PM medium. After one day, cells were maintained in culture in PM medium without additional factors or were stimulated in PM medium with LPS (100ng/ml) and IFN-γ (20ng/mL) for 24h. After this period, non-stimulated and stimulated cells were collected for RNA extraction or fixed for immunocytochemistry, as described below (Figure 4).

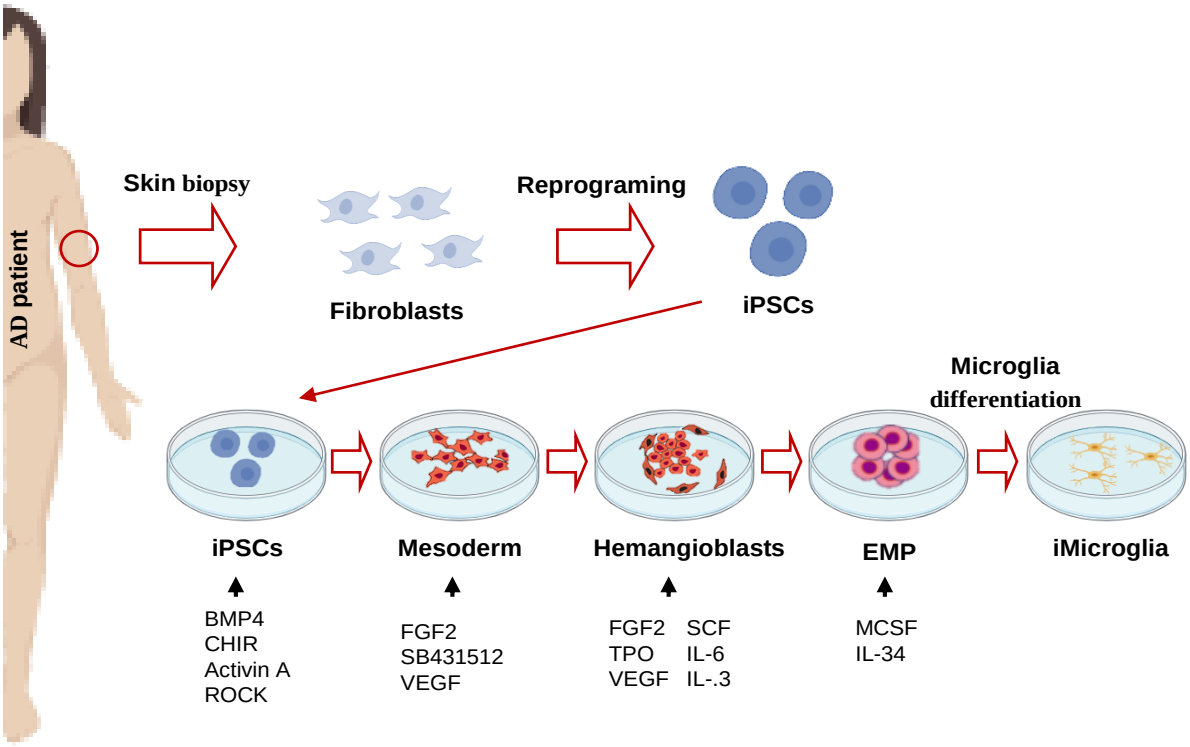

Figure 4. iPSCs Differentiate into iMicroglia through Primitive Haematopoiesis. Schematic protocol. Human iPSCs from a healthy female Ctrl and from 2 female pre-symptomatic individuals carrying Swe and PSEN1 mutations obtained from a skink biopsy and reprograming in iPSCs were differentiated into microglia-like cells. iPSCs were maintained in low oxygen (5% O2 / 5% CO2) in E8BAC media, supplemented with ROCK inhibitor to induce early mesodermal differentiation. Next, media was changed to FVI media to induce hematovascular mesodermal differentiation. In the face of hemangioblasts, cells were subjected to normoxia and cultured in HPC media to induce hemogenic endothelial differentiation. The next days the cells were expanded in HPC media, until they reach the EMP (erythromyeloid progenitor cells) when the cells were suspended to expand myeloid progenitors and maintained in MP media [IMDM supplemented with, FBS (10%), P/S (1%), Insulin (0,5%), IL-34 (0,1%) and M-CSF (0,0125%)] in ULA (ultra-low attachment) plates. Two days later, the first primitive microglia start to attach to the bottom, media is changed to PM [IMDM supplemented with, FBS (10%), P/S (0,5%), IL-34 (0,01%) and M-CSF (0,025%)]. Then, adherent cells are suspended with accutase and plated in PDL (poly-D-lysine) coated plates in PM media.

2.2. Immunocytochemistry

For microglia characterization, cells were fixed for 20min with 4% (w/v) PFA in phosphate-buffer saline (PBS). Cells were permeabilized for 10 min with 0.2% (w/v) Triton-X100 in PBS at RT, and then incubated with blocking solution [3% (w/v) BSA in PBS]. Microglia cells were incubated overnight at 4ºC with 50µL of primary antibodies (Table 5) prepared in an antibody dilution buffer [1% (w/v) BSA in PBS]. Then, cells were washed in PBS and incubated in the dark with the secondary antibodies for 1h at RT (Table 6). Then cells were incubated with Hoechst 33258 dye (1:1000, Sigma-Aldrich) for 2min to cell nuclei staining. At the end, cells were mounted in Flouromount® and observed under a confocal microscope. Multiple representative images of at least 3 independent experiments were captured from random microscopic fields. Number of cells, perimeter, area, fluorescence intensity and Feret´s diameter was measured using Fiji-ImageJ software.

Table 5. List of primary antibodies used in ICC.

Antibody	Host	Source/ reference	Dilution
Anti- Arg1	Goat	Santa Cruz Biotechnology/ sc-18355	1:100
Anti- CD11b	Rat	Biolegend/ 101202	1:50
Anti- CX3CR1	Rabbit	Santa Cruz Biotechnology/ sc-30030	1:100
Anti- TMEM119	Rabbit	Abcam/ ab18533	1:50
Anti-TREM 2	Rabbit	Cell signalling/ 91068S	1:100
Anti-Iba1	Rabbit	Wako/ 019-19741	1:100
Anti-MFGE8	Rabbit	Santa Cruz Biotechnology/ sc-33546	1:100
Anti-iNOS	Mouse	BD Biosciences/ 610329	1:100

Arg1, arginase 1; CD11b, integrin alpha M; CX3CR1, fractalkine receptor; TMEM119, transmembrane protein 119; TREM2, triggering receptor expressed on myeloid cells 2; Iba1, ionized calcium binding adaptor molecule 1; MFGE8, milk fat globule-EGF factor 8 protein; iNOS, inducible nitric oxide synthase.

Table 6. List of secondary antibodies used in ICC.

Antibody	Host	Source/reference	Dilution
Anti-rabbit Alexafluor 405	Goat	Invitrogen/ A31556	1:500
Anti-rabbit Alexafluor 488	Goat	Invitrogen/ A11008	1:500
Anti-rabbit Alexafluor 594	Goat	Invitrogen/ A11012	1:500
Anti-rat Alexafluor 594	Donkey	Invitrogen/ A21209	1:500
Anti-goat Alexafluor 594	Chicken	Invitrogen/ A21468	1:500
Anti-mouse Alexafluor 647	Goat	Invitrogen/ A21236	1:500

2.3. Evaluation of Microglia Phagocytic Ability

The evaluation of the microglial phagocytosis was assessed by analysing the number of CD11b positive iMicroglia that internalized fluorescent beads. The method consisted in incubating the iMicroglia with 0.0025% (w/w) of 1µm of diameter fluorescent latex beads (Sigma) for 75min at 37°C at the end of the stimulation period. Thereafter, cells were fixed with freshly prepared 4% (w/v) PFA in PBS. iMicroglia were stained for CD11b and nuclei counterstained with Hoechst 33258 dye. Fluorescence and image analysis were performed as above mentioned in 2.2 Immunocytochemistry.

2.4. Total RNA extraction, cDNA and Real-Time PCR of genes

Determination of gene expression was performed by quantitative real time-PCR (qRealTime-PCR). Total RNA was extracted from 12-well culture plates using TripleXtractor reagent (GRISP) and according to the manufacturer instructions. RNA concentration was measured using NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies).

For mRNA expression, aliquots of 1000 ng of total RNA were reversely transcribed into cDNA in a TPersonal Thermocycler (Biometra®) using the Xpert cDNA Synthesis Mastermix Kit (GRISP) under the recommended conditions (10min at 25ºC, 15min at 50ºC, 5min at 85ºC, hold at 4ºC). qRealTime-PCR was performed on a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems), using the Xpert Fast SYBR (Blue) Kit (GRISP) and the set of primers indicated in Table 7. For qRealTime-PCR, it was used 384-well plates, with each sample performed in duplicate, and under optimized conditions (2min at 50ºC, 2min at 95ºC, followed by 40 amplification cycles at 95ºC for 5s and 62ºC for 30s). To verify the specificity of the amplification, a melt-curve analysis was executed immediately after the amplification protocol (15s at 95ºC, followed by 60ºC for 30s and 95ºC for 15s). A non-template control was included for each amplification and the genes GAPDH and β-actin were used as endogenous control to normalize each gene expression level. The fold change was determined by the $2^{-\Delta\Delta CT}$ method. This method is based on two assumptions (1) The reaction occurs with 100% efficiency and is one of the reasons for using a low cycle number when the reaction is still in the exponential phase. (2) A reference gene must be included to normalize the target gene. mRNA transcript levels are calculated as follows: the Ct value for the reference gene is subtracted from the Ct value of the target gene, which gives the ΔCt. The ΔCt of the control sample is then subtracted from a treated sample, giving the ΔΔCt. The negative value of this sub-traction ($^{-\Delta\Delta CT}$) is used as the exponent of 2 in the equation and represents the fold change of the treated target gene relative to the control (Does & Differently, 2012). CT represents the cycle number at which fluorescence passes the threshold level of detection.

Table 7. List of primer sequences used for gene expression.

Gene	Sequence (5'-3')
CD68	Fwr: TCA GCT TTG GAT TCA TGC AG Rev: AGG TGG ACA GCT GGT GAA AG
MHCII	Fwr: AGG GAT TGC GCA AAA GCA Rev: TCA CCT CCA TGT GCC TTA CAG A
Arg1	Fwr: TGG AAA CTT GCA TGG ACA Rev: AAG TCC GAA ACA AGC CAA
TREM2	Fwr: ATG ATG CGG GTC TCT ACC AGT G Rev: GCA TCC TCG AAG CTC TCA GAC T
CX3CR1	Fwr: GTG GTG TGC TGA CAA AGC TTG GAA Rev: TCA TGG GTG CCA TCG TAA GAA
MFGE8	Fwr: GCC CTG GAT ATC TGT TCC AA Rev: GCT CGA CAC ATT TCG TCT CA
IL-6	Fwr: GAC AGC CAC TCA CCC TTC A Rev: TTC ACC AGG CAA GTT GTC TCC TC
IL-10	Fwr: CCT GCA GGA GGT GAT GCC CCA Rev: TTC TGC TTG AGA GGT GCT GA
iNOS	Fwr: GGG TTG GGG GTG TGG TGA TGT Rev: TCC GAG GCA AAC AGC ACA TTC A

TGF-β	Fwr: TGC GCT TGA GAT CTT CAA A Rev: GGG CTA GTC GCA CAG AAC
GADPH	FWR: CGC TCT CTG CTC CTC CTGT REV: CCA TGG TGT CTGA GCG ATGT
β-Actin	Fwr: CAG AGC CTG GCC TTT GCC GA Rev: ATC CAT GCT GAG CTG GCG GC

MFGE8, milk fat globule-EGF factor 8 protein; CX3CR1, Fractalkine receptor; CD68, Cluster of Differentiation 68; TREM2, triggering receptor expressed on myeloid cells 2; Arg1, arginase 1; TGF-β, transforming growth factor beta; IL-10, interleukin-10; MHCII, major histocompatibility complex; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

2.5. Total RNA extraction, cDNA and Real-Time PCR of miRNAs

For miRNAs analysis, the extraction was performed exactly at the same way, but conversion of cDNA was achieved with the miRNACURY LNA™ Universal RT miRNA PCR Kit, using 10ng/μL of total RNA and 0,5μL of mRNAUniSp6 and following manufactures recommendations: 60min at 42ºC followed by heat inactivation of the reverse transcriptase for 5min at 95ºC. For miRNA quantification, the PowerUp™ SYBR™ Green Master Mix was used in combination with pre-designed primers listed in Table 8. The reaction conditions consisted of polymerase activation/denaturation at 95ºC for 10min, followed by 50 amplification cycles at 95ºC for 10s and 60ºC for 1min. Quantification of target miRNAs was made in comparison to the reference gene (RNAUA1) and spike-in UniSp6, and fold change was determined by the $2^{-\Delta\Delta CT}$ method.

Table 8. List of primer sequences used for miRNA expression.

miRNA	Sequence (5'-3')
hsa-miR-124-3p	5'-UAAGGCACGCGGUGAAUGCC-3'
hsa-miR-155-5p	5'- UUAAUGCUAAUCGUGAUAGGGGU-3'
hsa-miR-21-5p	5'- UAGCUUAUCAGACUGAUGUUGA-3'
hsa-miR-146a-5p	5'-UGAGAACUGAAUUCCAUGGGUU-3'
hsa-miR-125b-5p	5'- UCCCUGAGACCCUAACUUGUGA-3'
RNAUA1	Reference gene
UniSp6	Spike in quality control gene

3. Statistical analysis

The statistical analysis was performed using *GraphPad Prism 7.0*. The significance of pair-wise comparisons was determined by two-tailed Student's *t*-test and ANOVA test. The results presented in graphs and tables along the work are represented as mean values obtained for each condition and standard error of the mean (SEM). p values less than 0.05 were considered statistically significant.

III RESULTS

1. Expression of iPSCs-derived iMicroglia differentiated markers and cell morphological alterations by AD mutations

1.1. Differentiation and characterization of iMicroglia

We were able to successfully differentiate iMicroglia from the frozen EMP cells received from Tarja Malm under the collaboration established by the JPND project. In Figure 5A, it is depicted the evolution of the cells along the timeline process of differentiation, days 8, 10, 16 and 24. To ensure that the differentiated cells present a microglia-like expression profile, we checked their classic markers. Immunostaining of Day 24 iMicroglia confirmed ubiquitous expression of TMEM119 (transmembrane Protein 119), Iba1 (ionized calcium binding adaptor molecule 1), CX3CR1 (fractalkine receptor), MFGE8 (milk fat globule-EGF factor 8 protein), CD11b (integrin alpha M), TREM2 (triggering receptor expressed on myeloid cells 2) and Arg1 (arginase 1) (Figure 5B). Thus, the iMicroglia generated through induction of primitive EMPs show a typical microglia-like protein expression. Besides, we confirmed the functional behaviour of microglia, through the evaluation of the phagocytic capacity, measured by the ability to phagocyte fluorescent beads (Figure 5C). We observed that about 40% (data not showed) of iMicroglia (CD11b - red) co-localized with the fluorescent beads (green), demonstrating spontaneous and functional phagocytic capacity.

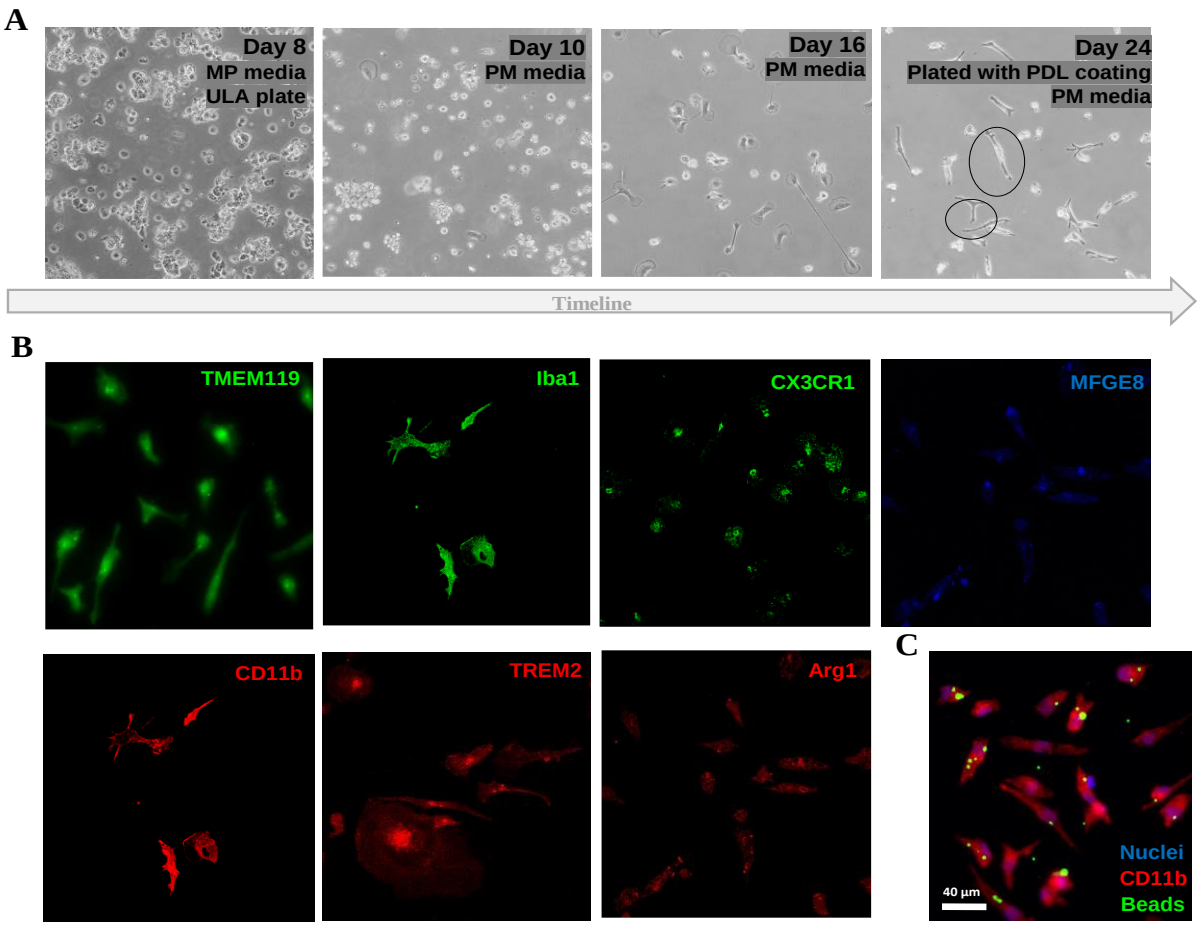

Figure 5. Differentiation steps, immunostaining characterization and phagocytic ability of iPSC-derived induced microglia (iMicroglia) (A) Representative timeline of microglia differentiation and expression of their characteristic markers. iPSC-derived microglia from healthy and isogenic control and from AD patients were plated on ULA (ultra-low attachment) coated dishes, in microglial progenitor (MP) medium [IMDM supplemented with, FBS (10%), P/S (1%), Insulin (0,5%), IL-34 (0,1%) and M-CSF (0,0125%)] at day 8. At day 10, the medium was changed to primitive macrophage (PM) medium [IMDM supplemented with FBS (10%), P/S (0,5%), IL-34 (0,01%) and M-CSF (0,025%)] and microglia were maintained in this medium until day 24. At this day microglia were maintained in PM without additional factors. (B) Immunostainings of day 24 control iMicroglia (C) Representative image of phagocytosed fluorescent beads (green) in $CD11b^{+}$ (red) iMicroglia. TMEM119, transmembrane Protein 119; Iba1, ionized calcium binding adaptor molecule 1; CX3CR1, fractalkine receptor; MFGE8, milk fat globule-EGF factor 8 protein; CD11b, integrin alpha M; TREM2, triggering receptor expressed on myeloid cells 2; and Arg1, arginase 1.

1.2. Morphological alterations in iMicroglia

Next, we investigated morphological alterations between the different iMicroglia lines. Results demonstrated no differences between the Control and PSEN1 or Swe mutation in relation to their perimeter or Feret's diameter (Figure 6A, 6C). Regarding the area, while Swe mutated cells showed a noticeable, but not significant increase in cell area (Figure 6B), the correction in the PSEN1 mutation led to a significant cell area reduction in ISO PSEN1 iMicroglia ($p<0.01$) (Figure 6B). Such alterations were corroborated by the measurement of the transformation index, calculated by the formula [perimeter of cell (μm)] ^{2} / 4π [cell area (μm^2)] (Figure 6D), where Swe cells appear a reduction (given the preliminary data of only one experiment), while ISO PSEN1 demonstrated significantly higher values when compared to PSEN1 cells ($p<0.001$). Overall, the differences observed between Control and Swe cells, although not significant, may indicate that Swe mutation might compromise these iMicroglia leading them to a more amoeboid morphology (Figure 6D), though this should be further clarified. On the other hand, the mutation correction in ISO PSEN1 cells may be influencing microglial function, by altering its morphology comparing to PSEN1. The more ramified morphology observed in ISO PSEN1 cells, comparing to PSEN1 counterpart ones, may suggest that the mutation correction is shifting these cells into a less amoeboid and closer to the normal steady state morphology.

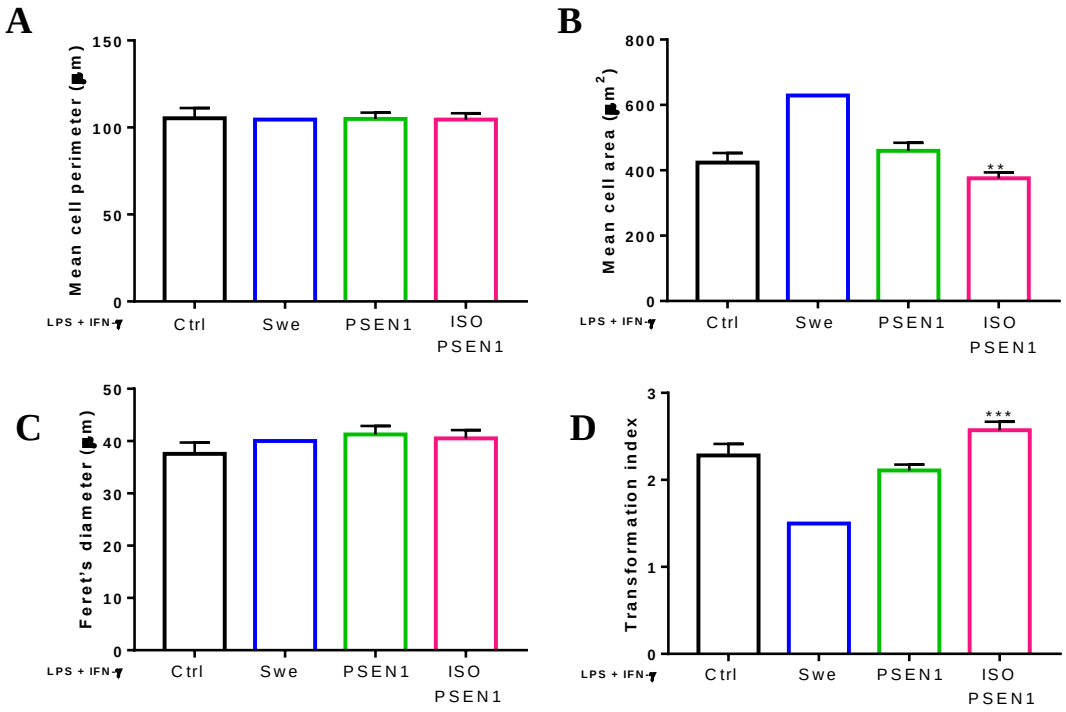

Figure 6. **Alteration in iMicroglia morphology.** iMicroglia were obtained as previously mentioned and allowed to differentiate until day 24. Then, cells were fixed and immunostained for TMEM119 and morphology evaluated. (A) Microglia perimeter, (B) Area, and (C) Feret's diameter were measured using the computer software Fiji-ImageJ. (D) Transformation index values calculated as [perimeter of cell (μm)]^2/4π [cell area (μm^2)]). n= 3 except Swe (n =1). Data presented as mean ± SEM. *t*-test analysis: **p<0.01 and ***p<0.001 *vs.* PSEN1. PSEN1, presenilin 1; Swe, Swedish; ISO PSEN1, PSEN1 isogenic control; Ctrl, Control.

2. Inflammatory profile of iMicroglia and dependence from mutations

We analysed the expression of cellular markers in iMicroglia that have currently been linked with neuroinflammation and related with cell behaviour in AD. As previously described, it is hypothesized that neuroinflammation plays an important role in AD development and that cytokines are mediators of the inflammatory process while miRNAs are one of the primary players in genetic regulation and cell-to-cell interaction. Therefore, we evaluated the inflammatory status of the obtained iMicroglia to determine whether such inflammatory signature is affected by the specific mutations and if the mutations affect the way microglia may respond to inflammatory stimuli. To do so, we assessed the expression of multiple cytokines and miRNAs in the iMicroglia lines.

2.1. Inflammatory signature of iMicroglia

We selected some genes related to microglia inflammatory status, which somehow are associated to AD pathology. The mRNA levels were evaluated by quantitative Real-Time PCR. Heatmap of overall gene expression is presented in Figure 7A. We observed that Swe iMicroglia appears to present some differences when compared to the Control, such as an elevation in TGF-β (transforming growth factor beta) IL-10, IL-6, MHCII (major histocompatibility complex) and CX3CR1 gene expression, with no changes in TREM2 and iNOS levels and a slight decrease of CD68 (cluster of differentiation 68). CD68 is localized in the lysosomal membrane and is present independently of cell polarization. Data on the heatmap for PSEN1 iMicroglia revealed slightly alterations when compared to the Control iMicroglia, which included a mild reduction in TGF-β, IL-10, IL-6 and CX3CR1 and a minor increase in TREM2 and iNOS gene expression. In respect to the correction of the mutation (ISO PSEN1), there was a decrease in the expression of TGF-β, MHCII, CD68, TREM2 and iNOS, suggesting that the cell partially returned to a phenotype closer to the Control one. The increase in both IL-10 and IL-6, relatively to the PSEN1 iMicroglia, may suggest the acquisition of a less inflammatory phenotype, if we consider that they may act as anti-inflammatory cytokines (Figure 7A).

To confirm this response, we evaluated besides the gene (Figure 7A), the protein expression of Arg1, a natural competitive enzyme for iNOS being described mostly as an anti-inflammatory mediator (Figure 7B). We observed a significant reduction of Arg1 gene expression in PSEN1 iMicroglia ($p<0.0001$) that was reverted in ISO PSEN1 iMicroglia. Regarding MFGE8, a protein involved in microglia-mediated phagocytosis (P. Wang, 2014), analysis of protein expression by immunostaining revealed a significant decrease in PSEN1 and ISO PSEN1 cells ($p<0.01$, Figure 7C) when compared to Control, suggesting an impaired phagocytic function not restored by the mutation correction. It was not possible to get data from Swe iMicroglia for this marker. For the iNOS expression (Figure 7D), we observed a marked increase in PSEN1 ($p<0.0001$) mutated cells in comparison to the Control, which was also evident in the ISO PSEN iMicroglia ($p<0.0001$), indicating that the correction of the mutation was not able to restore the microglia steady state. Thus, we may hypothesize that additional epigenetic mechanisms mat be involved in the deregulation of both MFGE8 and iNOS in the PSEN1 iMicroglia.

In respect to TREM2 (Figure 7E), which is reported as involved in microglia-mediated pathogenicity in neurodegenerative context (Y. Wang *et al.*, 2016) when decreased, this feature was suggested in Swe mutation iMicroglia and significant in the PSEN1 one ($p<0.0001$). Remarkably, in this case the correction adopted in ISO PSEN1 iMicroglia showed not only to revert but even to increase TREM2 expression to higher levels than Control ($p<0.0001$), thus ameliorating the cell phagocytic ability for Aβ, and probably accounting to restrain the gene expression (Figure 7A). Finally, the analysis

of the fractalkine receptor CX3CR1 (Figure 7F) that mediates a critical neuron-glia crosstalk in health and disease (Chen *et al.*, 2020) was found significant downregulated in ISO PSEN1 when compared to PSEN1 ($p<0.001$). Since, its detection was not accomplished in the other cell lines, only further studies may add on these data relevance.

In sum, these results demonstrate an overall deficiency of the phagocytic and inflammatory machinery, especially in PSEN1 iMicroglia with decreased expression of MFGE8 and TREM2. From these two markers, only TREM2 was significantly reverted when the mutation was corrected (ISO PSEN1). In general, ISO PSEN results demonstrated that the correction of the mutation is only capable to promote a partial recovery to control levels, reinforcing the need for other studies focusing on epigenetic factors.

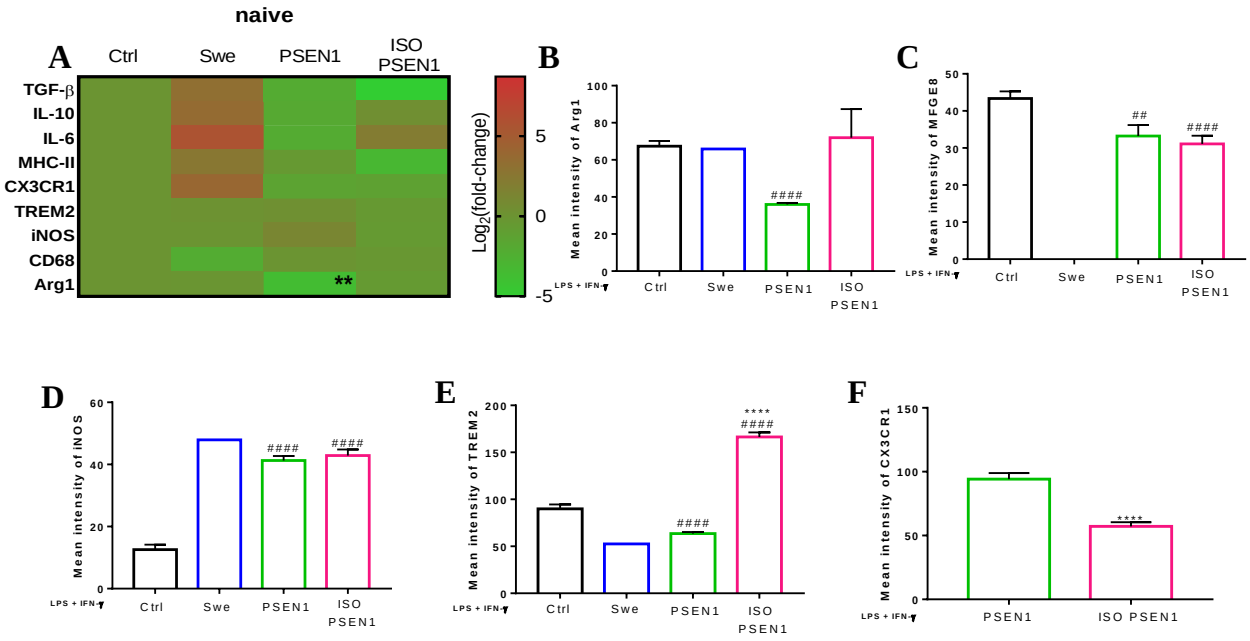

Figure 7. **Inflammatory signature of iMicroglia lines.** iMicroglia were obtained as previously mentioned and allowed to differentiate until day 24. Then, mRNA was isolated to assess specific microglia and inflammatory gene markers (A) by quantitative Real Time PCR. Data is expressed as $\log_2$. Cells were also fixed and immunostained to assess the expression of Arg1(B), MFGE8 (C), TREM2 (D), iNOS (E) and CX3CR1 (F). Mean intensity was measured using the computer software Fiji-ImageJ. n= 3 except Swe (n =1). Data presented as mean ± SEM. *t*-test analysis: $^{\#\#}p<0.01$ and $^{\#\#\#\#}p<0.0001$, *vs.* Ctrl; ****$p<0.0001$ *vs.* PSEN1. MFGE8, milk fat globule-EGF factor 8 protein; CX3CR1, fractalkine receptor; CD68, cluster of Differentiation 68; TREM2, triggering receptor expressed on myeloid cells 2; Arg1, arginase 1; TGF-β, transforming growth factor beta; IL-10, interleukin-10; MHCII, major histocompatibility complex; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; PSEN1, presenilin 1; Swe, Swedish; ISO PSEN1, PSEN1 isogenic control; Ctrl, Control.

2.2. Inflammatory signature of iMicroglia simulated with LPS+IFN-γ

Either in health or in disease, microglia are not alone in its environment, being surrounded by other CNS cells, such as neurons and astrocytes that release factors that may stimulate microglia activation/polarization (Colonna & Butovsky, 2017; Salter & Stevens, 2017). Besides, microglia are excellent "detectives" able to identify foreign invaders, such as bacteria using specific receptors, as TLR4 (Caldeira *et al.*, 2017). Neuroinflammation has been described to be present in the onset of AD and even to impact in the progression of the disease. Therefore, to mimic this complex environment in disease context, we stimulated the iMicroglia with LPS and IFN-γ as described in methods. Our objective was to turn more evident any defects that the diseased cells could have by demanding an additional effort to respond to inflammatory stimuli mimicking and inflammatory milieu. Hence, we can investigate whether the mutations are affecting the activation process in these cells, and how they impact microglia function in a situation more similar to what should happen in the AD patient's brain. The results obtained are presented in Figure 8.

To better assess the differences, we evaluated the same parameters considered for the non-stimulated iMicroglia, in order to further establish comparisons, represented as a heatmap for the gene expression (Figure 8A). As expected, the stimulation with LPS+IFN-γ favoured general iMicroglia inflammatory signature, including in Control cells. Interestingly, Swe iMicroglia show the most distinctive pattern with an increased expression of TGF-β, IL-10, MHCII, TREM2 and iNOS, in comparison to stimulated Control iMicroglia, although not significant. In opposite, minor decreases were observed for IL-6, CX3CR1 and CD68 (Figure 8A). PSEN1 iMicroglia showed a mild depression of IL-10, MHCII, CX3CR1 and CD68, and increased IL-6 and iNOS, though not significant. Data suggest that the cells sustain a small ability to respond to the inflammatory stimulus. TGF-β and TREM2 gene values were similar to Control. Of note, Arg1 gene expression was markedly suppressed following the stimulation with LPS+IFN-γ, except for PSEN1 ($p<0.001$) and ISO PSEN1 (Figure 8A). In summary, lower IL-10 and higher iNOS in the PSEN1 iMicroglia coexist with higher Arg1 and lower MHC-II, indicating the acquisition of a mixed disease phenotype by the inflammatory LPS+IFN-γ stimuli.

Moreover, when PSEN1 was corrected, despite a subtle increase in the expression of all genes, excepting iNOS, the overall pattern was very similar to the PSEN1, meaning that mutation correction did not provided major benefits in the response of the cell to neuroinflammation.

Intriguingly, as seen before, Arg1 immunostaining revealed an opposite pattern comparing to its gene expression data. A small decrease was noticed for Arg1 in Swe, but markedly in PSEN1 iMicroglia ($p<0.001$, Figure 8B). In this case, the ISO PSEN1 stimulated iMicroglia responded by overexpressing Arg1 to abrogate the inflammatory stimuli.

Remarkably, the immunostimmulation led to an upregulated MFGE8 immunofluorescence in both PSEN1 and ISO PSEN1 iMicroglia, as compared to the Control one ($p<0.0001$, Figure 8C), much more accentuated in ISO PSEN cells, suggesting that the cells try to counteract the harmful effects of LPS+IFN-γ stimuli. No data were obtained for the Swe iMicroglia.

In the case of TREM2, we detected different patterns for distinct mutations, with a trend to decrease in Swe iMicroglia, unaltered in PSEN1 and reduced in ISO PSEN1 ($p<0.05$, Figure 8E). This finding may derive from the genetic manipulation caused by the gene editing CRISPR / Cas9 intervention.

For iNOS intensity, in accordance with gene expression, we observed a trend to increase in Swe cells that was however potentiated in PSEN1 stimulated iMicroglia ($p<0.0001$, Figure 8D). The same effect, but with lower intensity, was noticed in ISO PSEN1 cells ($p<0.0001$), as well.

Concerning CX3CR1 expression (Figure 8F), as in the absence of LPS+IFN-γ stimuli, it was evident that the correction of the mutation triggered a decreased protein expression ($p<0.0001$) by the immunostimmulation, again only detected for ISO PSEN1 iMicroglia. The receptor was not identified in the other cell lines.

When focusing only in PSEN1 *vs*. ISO PSEN1 in stimulated iMicroglia, we could see that Arg1 mRNA increases, but the protein decreases, that the MFGE8 (though increased by LPS+IFN-γ stimuli) show lower levels (Figure 8C; $p<0.0001$), TREM2 do not change *vs.* Control but is enhanced (Figure 8E; $p<0.05$), as well as both iNOS and CX3CR1 that are also higher (Figures 8E,F; $p<0.0001$) by immunostaining. Hypothetical benefits by the correction in ISO PSEN1 iMicroglia only include the parameters Arg1 and iNOS that differed from the PSEN1 iMicroglia (Figures 8B,8D).

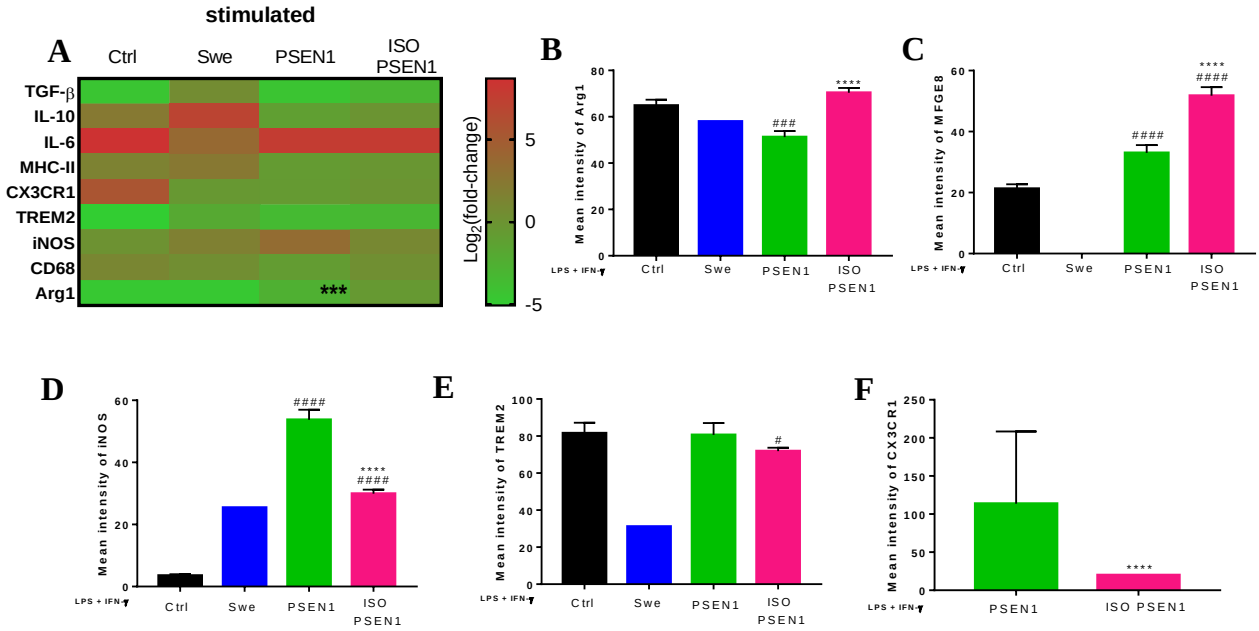

Figure 8. Inflammatory signature of simulated microglia iMicroglia were obtained as previously mentioned and allowed to differentiate until day 24. Then, mRNA was isolated to assess specific microglia and inflammatory markers (A) by quantitative Real Time PCR. Data is expressed as $\log_2$, Cells were also fixed and immunostained to assess the expression of Arg1(B), MFGE8 (C), iNOS (D), TREM2 (E) and CX3CR1 (F). Mean intensity was measured using the computer software Fiji-ImageJ. n= 3 except Swe (n =1). Data presented as mean ± SEM. *t*-test analysis: $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ and $^{\#\#\#\#}p<0.0001$, *vs.* Ctrl; ****$p<0.0001$ *vs.* PSEN1. MFGE8, milk fat globule-EGF factor 8 protein; CX3CR1, fractalkine receptor; CD68, cluster of Differentiation 68; TREM2, triggering receptor expressed on myeloid cells 2; Arg1, arginase 1; TGF-β, transforming growth factor beta; IL-10, interleukin 10; MHCII, major histocompatibility complex; IL-6, interleukin 6; iNOS, inducible nitric oxide synthase; PSEN1, presenilin 1; Swe, Swedish; ISO PSEN1, PSEN1 isogenic control; Ctrl, Control.

2.3. Stimulated *vs.* non-stimulated iMicroglia: a direct comparison

As expected, we detected significant differences between non-stimulated and stimulated microglia within the same iMicroglia line. Such data also deserved our attention, being therefore summarized as a ratio between the fold value of stimulated cells and the fold value of non-stimulated cells in the tables below, where alterations with a significant value are shown.

The most notorious data concerning Control iMicroglia refers to the IL-6 accentuated increase ($p<0.01$), followed by the moderate IL-10 elevation ($p<0.05$) in stimulated *vs.* non-stimulated cells. Also, a significant decrease in Arg1 ($p<0.0001$) and TREM2 ($p<0.001$) was found. Regarding the average intensity of the immunostaining markers, there was a decrease of MFGE8 ($p<0.0001$) and iNOS ($p<0.05$), suggesting loss of in the phagocytic capacity and in the innate immune response in Control iMicroglia upon stimulation (Table 9).

Table 9. Inflammatory status of Control iMicroglia based on the gene expression and mean intensity of specific markers, before and after LPS+IFN-γ-stimulation

	FOLD DIFFERENCES (**Control stimulated *vs.* Control non-stimulated ratio**) MEAN ± SEM	p VALUES
GENE EXPRESSION		
IL-6	336.4	<0.01
Arg1	0.05	<0.0001
TREM2	0.06	<0.001
IL-10	6.3	<0.05
IMMUNOSTAINING INTENSITY		
MFGE8	0.49	<0.0001
iNOS	0.28	<0.05

Comparisons were made between non-stimulated Control iMicroglia and LPS+IFN-γ-stimulated Control iMicroglia. mRNA expression levels were evaluated by Real-Time quantitative PCR (RT-qPCR) and data are expressed as the ratio of the fold value of stimulated cells to fold value of non-stimulated cells; p values were calculated by Student's *t* test, considering at least 3 independent experiments. IL-6, interleukin 6; Arg1, arginase 1, TREM2, triggering receptor expressed on myeloid cells 2; IL-10, interleukin 10; iNOS, inducible nitric oxide synthase; MFGE8, milk fat globule-EGF factor 8.

Concerning PSEN1 mutant iMicroglia (Table 10), a significant decrease in Arg1 gene expression was observed upon stimulation ($p<0.01$). However, the average fluorescence intensity of Arg1 was increased ($p<0.0001$), together with TREM2 ($p<0.01$) and iNOS ($p<0.0001$). Under normal circumstances, the elevation in these markers is expected, but intriguingly they contrasted with the results on stimulated Control iMicroglia, suggesting at some point influence of PSEN1 mutation in the iMicroglia response to the stimuli.

Table 10. Inflammatory status of PSEN1 iMicroglia based on the gene expression and mean intensity of specific markers, before and after LPS+IFN-γ-stimulation

	FOLD DIFFERENCES (**PSEN1 stimulated to PSEN1 non-stimulated ratio**) MEAN ± SEM	P VALUES
GENE EXPRESSION		
Arg1	0.41	<0.01
IMMUNOSTAINING INTENSITY		
Arg1	1.42	<0.0001
TREM2	1.27	<0.01
iNOS	1.30	<0.0001

Comparisons were made between non-stimulated Control iMicroglia and LPS+IFN-γ-stimulated Control iMicroglia. mRNA expression levels were evaluated by Real-Time quantitative PCR (RT-qPCR) and data are expressed as the ratio of fold value of stimulated cells to fold value of non-stimulated cells. p values were calculated by Student's *t* test, considering at least 3 independent experiments. Arg1, arginase 1, TREM2, triggering receptor expressed on myeloid cells 2; iNOS, inducible nitric oxide synthase.

Finally, when we analysed the effects of the LPS+IFN-γ-stimulation in ISO PSEN1 iMicroglia (Table 11), a significant increase in MFGE8 immunostaining ($p<0.0001$) together with significant decreases in TREM2 ($p<0.0001$), iNOS ($p<0.0001$) and CX3CR1 immunostainings ($p<0.0001$) were clearly observed (Table 11). When comparing to the PSEN1 counterparts, such differences in the stimulated profile confirm the influence of the PSEN1 correction, by restraining the pro-inflammatory polarization of such iMicroglia upon LPS+IFN-γ-stimulation.

Table 11. Inflammatory status of ISO PSEN1 iMicroglia based on the mean intensity of specific markers, before and after LPS+IFN-γ-stimulation.

	FOLD DIFFERENCES (**ISO PSEN1 stimulated to ISO PSEN1 non-stimulated ratio**) MEAN ± SEM	P VALUES
IMMUNOSTAINING INTENSITY		
MFGE8	1.67	<0.0001
TREM2	0.43	<0.0001
iNOS	0.70	<0.0001
CX3CR1	0.35	<0.0001

Comparisons were made between non-stimulated Control iMicroglia and LPS+IFN-γ-stimulated Control iMicroglia. mRNA expression levels were evaluated by Real-Time quantitative PCR (RT-qPCR) and data are expressed as the ratio of fold value of stimulated cells to fold value of non-stimulated cells. p values were calculated by Student's *t* test, considering at least 3 independent experiments. TREM2, triggering receptor expressed on myeloid cells 2; iNOS, inducible nitric oxide synthase; MFGE8, milk fat globule-EGF factor 8; CX3CR1, fractalkine receptor.

2.4. Inflamma-miRNA signature in iMicroglia

Deregulation of miRNAs is on the one hand associated with microglia activation, deactivation and senescence but, on the other hand can be a gain in function - rejuvenation or revitalization. To address this issue in this study, five different miRNAs were selected and assessed based on their association with inflammation, as it is the case of miR-21, miR-124, miR-125b, miR-146a and miR-155 (Brites, 2020). Each of these miRNAs plays multiple roles in AD progression, mainly by regulating neuroinflammation. The expression data obtained is presented in Figure 9, for non-stimulated (A) and LPS+IFN-γ-stimulated (B) iMicroglia.

Results showed that both Swe and PSEN1 iMicroglia exhibit an intensified miRNA expression when compared to the Control, except for miR-124 in the Swe cells, what it is not a surprise given its association to anti-inflammatory effects. However, mutations seem to differently influence the signature toward a deregulated activation state. The most pronounced were miR-146a, miR-155 and miR-21 in Swe cells, which are known to contribute to pro- and anti-inflammatory effects, suggesting the attempt of the cell to regulate miRNA dysregulation. In PSEN1 iMicroglia, we may highlight the upregulation of miR-125b (associated to Tau hyperphosphorylation in AD), as well as miR-146a and miR-21. Again, the profile indicate that the stimulation induced opposite mechanisms, whose consequences require further studies in terms of paracrine signalling, mainly over AD neurons. To note, that such distinctive miRNA signatures are in line with the profiles found associated to AD in other studies (X. H. Wang & Wang, 2018; Freilich *et al.*, 2013b). We may emphasize that the inflamma-miRNA signature obtained in the mutated iMicroglia is in conformity with a stressed cell. What was a surprise was to verify that iMicroglia subjected to CRISPR/Cas9 editing for the correction of PSEN1 mutation (ISO PSEN1), become increasingly stressed exhibiting the overexpression of all the assessed pro- and anti-miRNAs (Figure 9A).

These data made us questioned about the cell response to an extra inflammatory stimulus. Therefore, we evaluated how the same panel of miRNAs was modified in LPS+IFN-γ-stimulated iMicroglia (Figure 9B). As expected, Control iMicroglia showed an overexpression in all miRNAs upon stimulation, though more notoriously for miR-146a and miR-155, the first a regulator and the later a mainly inflammatory associated miRNA. Interestingly, when comparing to Control the Swe iMicroglia showed a decreased level in all miRNAs (miR-124, miR-146a, miR-155 and miR-21), except for miR-125b. Results show that the cells are not as able as iMicroglia Control to mount a response to the LPS+IFN-γ-stimulation, while also acquire a more pathological phenotype by overexpressing miR-125b, which we already mentioned to be linked to tau hyperphosphorylation. This miR-125b was also upregulated in PSEN1 iMicroglia after stimulation reinforcing the acquisition of pathological phenotype that, however, disappeared in cells corrected for the mutation. These cells also evidenced like the Swe ones to be less able to respond to the immune stimulation, revealing lower levels of activation (decreased miR-124, miR-146a and miR-155 *vs.* Control) and some compensatory mechanisms (increased miR-21 *vs.* Control). In sum, these data highlight a deficient response of Swe and PSEN iMicroglia to adequately react to LPS+IFN-γ stimuli when considering the inflamma-miRNA profiling.

Relatively to iMicroglia, ISO PSEN1 cells it was surprising to see that despite showing a stressed profile in terms of inflamma-miRNAs they seem to deficiently counteract LPS+IFN-γ stimulation by showing a general decrease of inflamma-miRNAs relatively to Control iMicroglia, except miR-21. If compared to PSEN1 iMicroglia, we emphasize the decrease in miR-125b (already indicated above), miR-146a and miR-21, as well as the increase in miR-155. It seems that epigenetic factors are controlling

this signature. As before, to better understanding these differences we need to access the paracrine influence of these cells on the neighbouring neurons, either performing iMicroglia/neuron cocultures or testing iMicroglia secretome on neurons.

If we compare the non-stimulated cells with the stimulated ones, we see that Control cells are the only ones that seem to be responding to the stimulus provided, since the Swe and PSEN1 non-stimulated do not present different values from the stimulated ones.

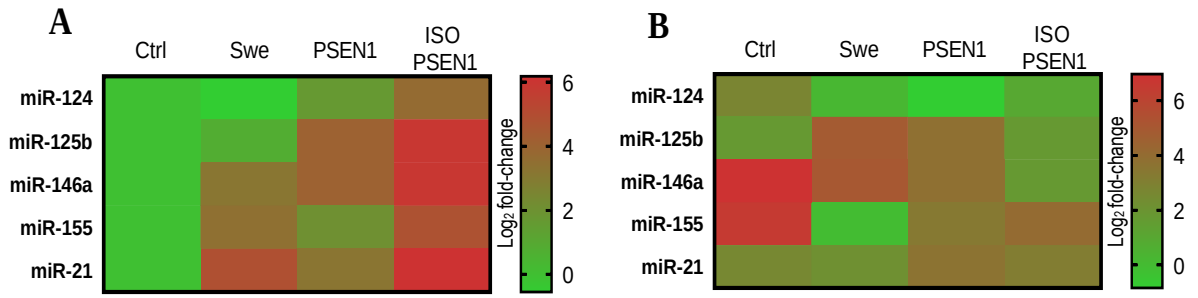

Figure 9. Inflammatory miRNA signature in iMicroglia, before and after stimulation with LPS+IFN-γ. iMicroglia were obtained as previously mentioned and allowed to differentiate until day 24. Then, miRNA expression was evaluated by Real-Time quantitative PCR (RT-qPCR). RNAUA1 was used as the reference gene. Data is expressed as $\log_2$ fold-change. (A) non-stimulated microglia; (B) stimulated microglia; PSEN1, presenilin 1; Swe, Swedish; ISO PSEN1, PSEN1 isogenic control; Ctrl, Control.

.

IV DISCUSSION

Due to the increase in average life expectancy over the past few decades, AD has become a major public health problem with high socioeconomic and political impact worldwide. Due to the increase of these problems, scientists have challenged themselves every day to acquire new insights to unveil the pathogenesis of AD. For this, researchers have conducted several studies using *in vitro* and *in vivo* models of AD, where neuroinflammation emerged as a promising therapeutic target and glial cells as critical regulators of chronic inflammation (Heneka *et al.*, 2015). In AD, activated microglia and astrocytes accumulate in the surrounding area of senile plaques and contribute to the severity of the disease by exacerbating the inflammatory response (Minter *et al.*, 2016).

Despite its fundamental role in the progression of AD, the microglial population remains surprisingly under-investigated in studies related to AD, mainly due to the lack of reliable experimental biological platforms to study the function of glial cells in a disease context (Strooper & Karran, 2016). In fact, though animal models and human immortalized cell lines derived from cancer tissue have been crucial to investigate AD pathophysiology (Yang *et al.*, 2016), they do not completely reflect all aspects of the disease, turning extrapolation of data in animals to human cells a difficult task (Choi & Tanzi, 2012). Recently, the development of human iPSCs technology opened the opportunity to study cellular disease phenotypes in patient-derived cell models, thus surpassing species specific differences (Abreu *et al.*, 2018b). There are still few methodologies for obtaining microglia from iPSCs, but our collaborator Tarja Malm, based on existing methods, has developed a relatively simple protocol, as mentioned above, in which the differentiated microglia produced, the iMicroglia, is able to respond to a stimulus (Konttinen *et al.*, 2019).

In the present work we demonstrated that iMicroglia can be efficiently generated from iPSCs following a defined protocol. It is important to note that iMicroglia showed the usual microglia markers based on the gene expression and immunofluorescence protein data, while also revealed phagocytic ability. Apart from microglia signature, we tested the phagocytic capacity of the generated iMicroglia, since one of the problems pointed out to the microglia in the case of AD is its inefficiency in clearing the Aβ plaques. As expected due to their structural characteristics, the iMicroglia spontaneously phagocytosed the fluorescent latex beads, confirming this important function as other studies have shown for iMicroglia (Konttinen *et al.*, 2019; Douvaras *et al.*, 2017).

Within the various markers used for iMicroglia, we chose TREM2, TMEM119, Iba1, CD11b, Arg1, CX3CR1 and MFGE8 as referred by several authors (Abud *et al.*, 2017, Douvaras *et al.*, 2017 and Konttinen *et al.*, 2019). These markers are involved in several processes linked to microglia: MFGE8 promotes the phagocytosis of apoptotic cells by microglia (Ying-ying & Jing-hui, 2020) and TREM2 is a transmembrane protein associated to AD development and believed to interfere with the brain's ability to prevent plaque accumulation (Lue *et al.*, 2015); Arg1 main function is to break down the amino acid arginine, competing with iNOS for such substrate (Cherry *et al.*, 2014); CD11b is a membrane marker that mediates inflammation processes by regulating leukocyte adhesion and migration (Jeong *et al.*, 2013); Iba1 is a cytoplasmic calcium-binding protein specifically expressed by microglia / macrophages (Ohsawa *et al.*, 2004); TMEM119 is another trans-membrane protein, which is expressed specifically by microglia and not by other macrophages and/or neuronal cells (Bohnert *et al.*, 2020); and CX3CR1 which is a receptor for fractalkine (CX3CL1) produced by neurons, together constituting an important axis in neuron-glia communication (Wolf *et al.*, 2013).

We had the opportunity to differentiate and test multiple iMicroglia lines obtained from: a Control person (44 years-old) without any type of mutation associated with AD (Control); a familial pre-symptomatic AD patient (30 years-old) carrying the APP Swedish double mutation KM670 / 671NL (Swe); another familial pre-symptomatic AD patient (47 years-old) carrying a familial deletion in exon 9 (ΔE9) of the PSEN1 gene (PSEN1), and the same individual with the correction of PSEN1ΔE9 deletion, mediated by CRISPR / Cas9 technology, to be used as isogenic control (ISO PSEN1). This last control is relevant to understand if correction of the mutation *per se* is able to recover any parameter, as previously supported (Konttinen *et al.*, 2019). Interestingly, all three individuals (Control, Swe and PSEN1 / ISO PSEN1) were females. Due to operational limitations, we only managed to differentiate one batch of Swe cells. Thus, we only have data from one preliminary set of parameters. However, we decided to show these results due to the unique genotype of these cells, even considering the lack of statistical analysis. Besides, the use of these different genetic backgrounds, offered a unique chance to investigate and compare the impact of each of these genetic variants in iMicroglia dynamics under non-stimulated and stimulated conditions.

Cytokine levels are reported as altered in AD patients and considering that cytokines are key components of neuroinflammation, several studies have explored the association between AD and cytokine expression (Su *et al.*, 2016). In this study, we started by evaluating the expression of multiple cytokines in the different iMicroglia, under non-stimulated conditions. The Swe iMicroglia showed a mixed polarization pattern, combining pro-regenerative and disease-associated gene expression levels. Besides the increase in anti-inflammatory cytokines TGF-β, IL-10, together with increased Arg1 marker, we also observed increases in IL-6, MHC-II and CX3CR1 markers. The last one indicates that iMicroglia are functionally favourable to communicate with neurons via the fractalkine axis, which should be interesting to test under a co-culture system. Differently, PSEN1 iMicroglia show a more disease-associated polarization, accentuated by the reduced pro-regenerative markers TGF-β and IL-10 as well as Arg1 (by immunostaining). The increases in MHC-II, iNOS and TREM2, together with decreases in CX3CR1 and MFGE8 are highly suggestive of a disrupted communicative phenotype and loss of phagocytic capacity, both substituted by an increased pro-inflammatory activity. Together, such signature constitutes a deviation from the typical disease-associated microglia, by showing reduced phagocytic capacity (Deczkowska *et al.*, 2018). To elucidate this issue, further studies should be performed to validate these preliminary data.

Regarding the correction of the mutation, we verified that, in comparison to its homologous, PSEN1, ISO PSEN shows a decreased gene expression of TGF-β, Arg1 MHC-II, followed by TREM2 and iNOS. Although these iMicroglia show reduced expression of several pre-regenerative and pro-inflammatory markers, they still demonstrate increased expression of functional homeostatic IL-6 and IL-10. The controversial results on Arg1 gene expression and immunostaining may suggest a shift in the iMicroglia polarization, which can be still in progress. On the contrary, the huge increase in TREM2 immunofluorescence suggests a previous mRNA peak in this marker expression, probably occurring prior to our assessment. As in the PSEN iMicroglia, there is a significant decrease in MFGE8, which suggests that ISO PSEN cells, despite having the mutation corrected, preserve, and further accentuate the phagocytic impairments of PSEN1. Even so, when compared to the Control, iMicroglia, the ISO PSEN1 still show similar expression values of IL-10, CD68, as well as iNOS and TREM2 (despite the differences in these last two observed in the immunostaining). Thus, in spite of the correction of the mutation, phagocytic impairment was not prevented, though it induced a partial recovery in the expression of some AD pathological-associated markers.

The exposure to a large array of sterile and exogenous stimuli is reported to induce microglia activation, which constitutes a deviation from their homeostasis aiming to orchestrate a defensive response in the context of health and disease, including phagocytosis, cytokine expression and production of reactive oxygen / nitrogen species. Different proinflammatory stimulants can, however, produce common and distinct phenotypes *in vitro* in such assays and, therefore, the choice of stimulation paradigm or testing multiple stimulation contexts is essential to generate meaningful results. Several factors are normally used to stimulate microglia, namely IL-1β, TNF-α and LPS and, in the case of human cells, the use of IFN-γ is also recurrent. Recent studies using cells similar to human microglia differentiated from iPSCs demonstrate that these cells have a clear activation response after stimulation with LPS and IFN-γ (Hanger *et al.*, 2020). Thus, for a better understanding of the inflammatory capabilities of our iMicroglia lines, we subjected the cells to this mixed (sterile / infectious) stimulation with LPS and IFN-γ, as previously reported (Konttinen *et al.*, 2019).

A functional assessment of our differentiated iMicroglia revealed that they are able to secrete cytokines in response to inflammatory stimuli in a similar profile as previously described for other iMicroglia (Abud *et al.*, 2017). Indeed, we observed that Control iMicroglia showed elevated expression of almost all cytokines in response to LPS + IFN-γ, including IL-10, IL-6, but also other inflammatory markers, as CD68, MHC-II, CX3CR1 and iNOS. Such results clearly show that, under normal circumstances (absence of disease-associated genetic background), iMicroglia orchestrate a typical activation signature. When we evaluated Swe iMicroglia, a different pro-regenerative profile was observed, with an increase in the expression of anti-inflammatory markers such as TGF-β and IL-10. Even so, Swe iMicroglia also preserved most of the proinflammatory signature of the Control, and further accentuated iNOS increase (evaluated either by gene expression and immunofluorescence). Such iNOS increase could potentially antagonize Arg1 since they compete for the same substrate (arginine) (Cherry *et al.*, 2014). However, we must keep in mind that all Swe cell results are preliminary, being representative of a unique differentiation batch.

In the case of the PSEN1 mutation, the gene expression profile was like what was observed in Control iMicroglia, with a predominant IL-6 expression. Yet, we observed a decreased gene expression of IL-10, MHC-II and CX3CR1, suggesting a reduction in the capacity to respond to stimulus, comparing to Control iMicroglia. Even so, the iNOS increase (both in gene expression and immunocytochemistry) revealed that this inflammatory enzyme may be especially favoured by PSEN1 mutations. One interesting point is the CX3CR1 reduced expression in both mutated iMicroglia, which suggests that these iMicroglia are somehow less receptive to an eventual CX3CR1-CX3CL1 crosstalk with neurons. The exploration of such disrupted communication should be accomplished in futured studies, using neuron-microglia co-cultures.

Regarding the ISO PSEN1 iMicroglia, we noticed a very similar stimulated profile comparing to the PSEN1 counterparts, anticipating a limited effect of the mutation correction. Besides, ISO PSEN1 preserved most of the alterations that PSEN1 had relatively to Control iMicroglia. However, ISO PSEN1 showed a reduction in iNOS signature, comparing to PSEN1, that was further corroborated by the significant reduction in iNOS immunofluorescence. As consequence, Arg1 was increased (by immunocytochemistry) and near the transcriptional levels of the Control. Finally, TREM2 and CX3CR1 also showed to be significantly reduced with the PSEN1 correction. In sum, differences are observed between all cell types, either in non-stimulated or stimulated condition. Besides, the mutation correction only partially restored the Control values, indicating the influence of other epigenetic factors that are taking place in these iMicroglia (Veremeyko *et al.*, 2019).

All this exhaustive description of iMicroglia including mixed signatures, supporting phagocytosis, innate or adaptative immunity responses, anti- or pro-inflammatory cytokine expression and disease-associated markers, clearly demonstrates the full spectrum of microglia polarizations, totally different from the old M1 / M2 perspective abandoned long time ago (Hanger *et al.*, 2020). Currently, many microglial subtypes are being described in literature (Stratoulias *et al.*, 2019). In an elaborated review, Stratoulias and colleagues demonstrated the existence of 6 major subtypes of functionally and regionally different microglia, and further described other deviations from these subtypes. One of these deviations is TREM2 microglia. Not all microglia express the cell surface receptor TREM2 and loss-of-function variants in the TREM2 protein increase the risk of developing late-onset AD among other forms of dementia. However, despite the apparent essential function of TREM2 in microglia, its expression is far from being ubiquitous and homogenous in those cells throughout the brain. The number of TREM2-expressing cells are highest in the cingulate cortex and lateral entorhinal cortex, and much lower in the hypothalamus and habenula, while some regions, such as the circumventricular organs, completely lack TREM2 expression. This regional heterogeneity in TREM2 expression could point toward a specific subtype (Stratoulias *et al.*, 2019).

Considering the crescent investigation on miRNAs, we selected a set of disease-associated miRNAs involved in neuron-glia communication and inflammation in AD and evaluated their profile in iMicroglia, before and after pro-inflammatory stimulation. Differences in the expression of these miRNAs are notorious even, without stimulation, with every miRNA overexpressed either in Swe or PSEN1 mutant iMicroglia (excepting miR-124 in Swe). Such general miRNA increase demonstrates the implication of these miRNAs in the pathological mechanisms derived from each mutation. Interestingly, different profiles are distinguishable between each mutant iMicroglia, with predominance of miR-155 and miR-21 in the Swe and miR-125b and miR-146a in the PSEN1. Focusing on Swe iMicroglia, the up-regulation of miR-155 is considered a disease-associated phenotype. Indeed, miR-155 was previously associated with neuroinflammation, preceding appearance of extracellular Aβ aggregates (Brites, 2020). In the case of miR-21, such increase in Swe cells reveals an immune cell activation, but most interestingly, it might be acting as pro-survival miRNA by attenuating amyloid-induced apoptosis, as other studies suggested (Feng *et al.*, 2018). In the case of non-stimulated PSEN1 iMicroglia, the increase in miR-125b might indicate an induction of increased astrogliosis mediated by microglia (Pogue *et al.*, 2010) and a pro-inflammatory disease-associated phenotype (X. H. Wang & Wang, 2018), which is explained by a strong inhibition of STAT3 pro-regenerative pathway, together with IL-6, that were previously described for this miRNA in microglia (Parisi *et al.*, 2013). Besides miR-125b, also miR-146a increase was very pronounced in PSEN1 cells. Its enhancement in several neurological diseases has been considered a compensatory mechanism to control disease-associated inflammation and restore homeostasis (Gaudet *et al.*, 2018).

When we further evaluated the same panel of miRNAs in iMicroglia following LPS + IFN-γ stimulation, we observed a huge increase in every miRNA in the Control cells, as expected. However, the stimulation was not so manifest in the pathological cell lines revealing either that the cells are unable to mount the proinflammatory response or are able to exert an-auto control of their stimulation. The stimulation induced Swe iMicroglia to overexpress miR-125b relatively to Control, while the lower levels of miR-146a were similar to that found in non-stimulated. PSEN1 signature was again similar to the cells non-stimulated, but with more miR-155, though it was much lower than Control. Intriguingly, both miR-155 and miR-146a, which are deeply associated with inflammatory regulation (Alexandrov *et al.*, 2014) were not much affected by the stimulation in the mutated iMicroglia, suggesting that these cells somehow are not fully responsive to the stimulus, comparing to Control iMicroglia or acquired defensive mechanisms. Also, miR-124 was not one of the most responsive miRNAs, corroborating the

data of non-stimulated cells. MiR-124 has proven to be of high importance, regulating microglia steady state, while its knockdown cause activation (Brites, 2020). However, since miR-124 is essentially a neuronal expressed miRNA (Makeyev *et al.*, 2007), perhaps it could be of higher relevance if evaluated in a co-culture system to assess the neuron-microglia paracrine communication.

Finally, the ISO PSEN1 results suggest that the mutation correction may be effective in preventing miR-125b upregulation, but without significant modifications for the other miRNAs. To note that the genetic manipulation by stressing the cells may have contributed for their exhaustion and absent response to LPS + IFN-γ. Also, to consider that these mechanisms by being exacerbated in the non-stimulated cells may undergo autocrine regulation of the stimuli.

We must never fail to keep in mind and emphasize the use of these iPSCs-derived microglia which are stimulus-responsive and represent an important tool to assess neuroinflammatory microglia dysfunction in neurodegenerative diseases, such as AD. We also have to recall that in the brain these cells are not alone, having a 3D complex environment, so our study symbolizes a simplified microglia model of AD. iPSCs have revolutionized biological models by allowing them to mimic normal and pathological human tissue *in vitro*, thus allowing a better understanding of the pathophysiology of the disease and new patient-specific therapeutic approaches (Zhang *et al.*, 2016). Recent 3-dimensional (3D) culture and human-mouse chimeric brain modelling systems developed using human pluripotent stem cells helped us to understand the complex properties of human microglia. In the 3D cultures, microglia can survey their neuronal environment and respond to localized cellular damage. The microglia also respond to inflammatory stimulation (LPS) and can release inflammatory cytokines. In the chimeric mouse brains, xenografted microglia rapidly respond to laser-induced focal brain injury and phagocytize cell debris after repeated mild closed head injury. Upon treatment with LPS, xenografted microglia adopt a more activated state, displaying ameboid morphology (Jiang *et al.*, 2020).

Though it is a process that offers many improvements in relation to those previously established (*e.g.*, immortalized cell lines that have already been extensively studied), it is not yet defined whether different studies can be comparable with each other due to differences in the protocols for obtaining these cells, especially iMicroglia (*e.g.*, origin of tissue donor, differentiation protocol or even cell reprogramming method). Obtaining these cells is a time-consuming and costly process as well as a requiring a very strict differentiation protocol (Potdar, 2017). In our work, we obtained many heterogeneous results in terms of markers and miRNAs, also observed in other studies (Konttinen et al., 2019), reinforcing that patients with AD may have specific inflammatory signatures, thus probably benefiting from precision medicine. In this case, the stratification of patients by these advanced methodologies may indicate their specific needs in terms of therapeutic strategies. Nevertheless, further studies are required to better characterize iPSCs-derived iMicroglia potentialities and limitations. Only then, it will be possible to obtain more conclusive/reproducible/understandable results in the same amount of time, if not in less.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As previously mentioned, the differentiation of iMicroglia can have very promising clinical applications. Although studies on iPSC-based modelling of AD aren´t reproducible or show opposite results. This cannot be bad and simply indicate that each patient has its own inflammatory fingerprint. However, we should not disregard that a better standardization of differentiation protocols is necessary because currently most labs use their own protocols. Improved characterization of the obtained cell

cultures and use of more defined media and coatings will reduce the variability among labs and increase reproducibility. Another concern, which is related to poor reproducibility, is the heterogeneity among different individuals, as mentioned. Some studies refer that higher donor numbers, patients as well as controls, will be necessary (De Leeuw & Tackenberg, 2019). Our data elucidates the presence of morphological alterations and changes in functional microglia gene expression markers (iNOS, CX3CR1, TREM2, MFGE8 and Arg1) in the PSEN1 microglia, some of them corrected in the ISO PSEN1. A few markers were more notorious after the activation of cells with the IFN-γ + LPS.

Regarding the expression of miRNAs, both mutant iMicroglia lines revealed specific miRNA signatures, which were altered upon stimulation. We noticed that almost all were increased in the isogenic cells, with miR-146a and miR-21 overexpression in all the cell lines relatively to control. In the IFN-γ + LPS activated microglia miR-125b was found elevated in Swe and PSEN1 cells and like control in the ISO PSEN1. Though, in some cases the differences were not significant, given the reduced number of samples that were analysed. Yet, our data supports that the presence of Swe and PSEN1 mutations increase the expression of almost all markers for both non-stimulated and IFN-γ + LPS-stimulated cells. In addition, ISO PSEN1 cells showed some recovery benefits for the increased expression of Arg1 and decreased iNOS (Figure 10). When activated with IFN-γ+LPS these cells additionally sustained the low levels of miR-125b and miR-146a. The isogenic correction is not enough to fully restore the healthy microglia phenotype, while also exacerbates inflamma-miRNA expression levels, probably by the stressed genetic manipulation. Another important finding was to observe that Swe, PSEN1 and ISO PSEN1 cells are defective in responding to activation by not raising miR-146a and miR-155 levels, what can be due to cell exhaustion.

In conclusion, while promising, iPSCs-derived microglia deserve to be further studied to push forward our efforts to combat AD. Indications on the revitalization of such exhausted microglia may be more important that simply propose anti-inflammatory compounds that have systematically failed or even worse the patient AD progression in this regard, and with the aim of increase cellular maturity of iPSC-derived cells, 3D cell cultures and organoid technologies represent a major step forward. Taken together, iPSCs – either patient-derived or isogenic – constitute an improvement in modelling human diseases and the epigenetic influence.

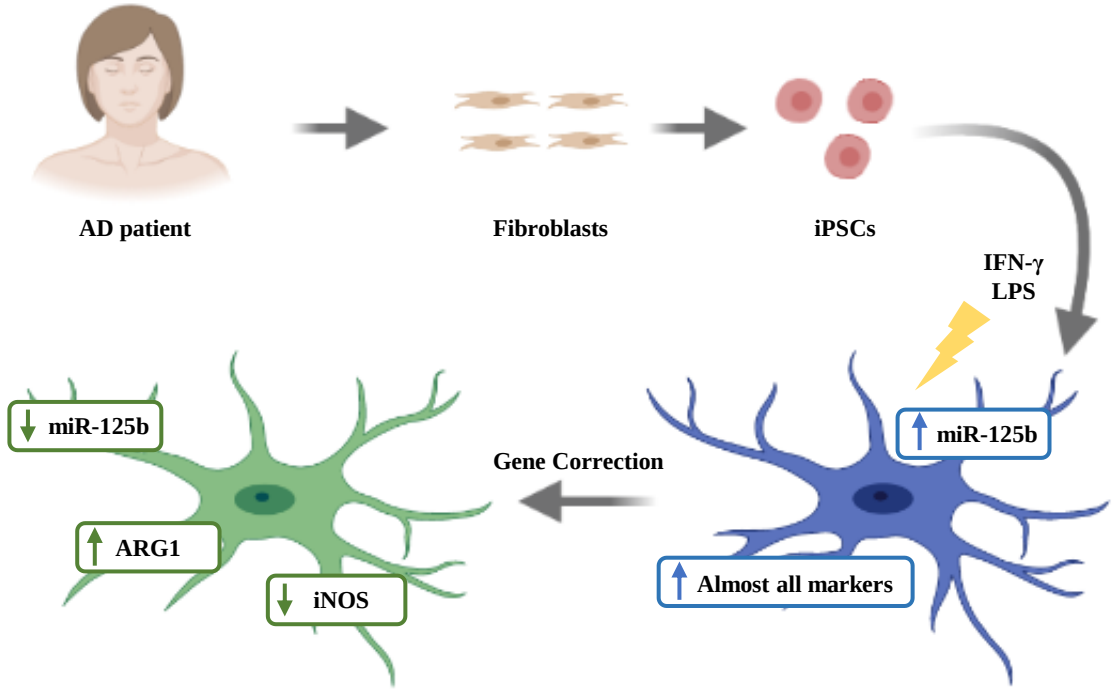

Figure 10. Schematic representation of the main research findings of this thesis. iPSCs-derived microglia from AD patients with PSEN1 mutation and the ISO PSEN1 with the mutation corrected were obtained after a process of differentiation, and posteriorly activated with IFN-γ + LPS. Upon activation, microglia secrete high amounts of miR-125b and increase the expression of almost all markers tested. ISO PSEN1 cells showed some recovery benefits for the increased expression of Arg1 and decreased iNOS. When activated with IFN-γ these cells additionally sustained the low levels of miR-125b. AD, Alzheimer's disease; Arg1, Arginase 1; IFN-γ, Interferon-γ; iNOS, inducible nitric oxide synthase; iPSCs, induced pluripotent stem cells; LPS, lipopolysaccharide; miR, microRNA; PSEN1, presenilin1; ISO PSEN1, PSEN1 isogenic control.

Future work should include an assessment of the secretory signature associated with inflammatory signalling and EV load when derived from non-stimulated and stimulated iMicroglia since paracrine communication is critical for neuron-glia function. Another very promising work would be to study the effects of the iMicroglia-derived secretome in the human neuronal models of AD, enabling the evaluation of the microglial pro-regenerative *vs.* pro-detrimental consequences. Ideally, the use of iPSC-derived neurons (with the same genetic background of iMicroglia) would be essential to better understand the effects of a specific mutation in the neuron-glia communication. Even considering the pandemic time it was possible to collect and process many of these samples that are reserved for future studies. Following the line of this work, it would be equally interesting to study the response of Control iMicroglia in the presence of patient iPSC-derived neurons subjected to stressed conditions, complementing this study, and providing new insights into neuron-microglia communication in AD.

V BIBLIOGRAPHY

Abreu, C. M., Gama, L., Krasemann, S., Chesnut, M., Odwin-Dacosta, S., Hogberg, H. T., Hartung, T., & Pamies, D. (2018a). Microglia Increase Inflammatory Responses in iPSC-Derived Human BrainSpheres. *Frontiers in Microbiology*, *9*, 1–12.

Abud, E. M., Ramirez, R. N., Martinez, E. S., Carson, M. J., Poon, W. W., Blurton-jones, M., Abud, E. M., Ramirez, R. N., Martinez, E. S., Healy, L. M., Nguyen, C. H. H., & Newman, S. A. (2017). iPSC-Derived Human Microglia-like Cells to Study Neurological Diseases. *Neuron*, *94*, 278-293

Alexandrov, P. N., Dua, P., Hill, J. M., Bhattacharjee, S., Zhao, Y., & Lukiw, W. J. (2012). MiRNA (miRNA) speciation in Alzheimer's disease (AD) cerebrospinal fluid (CSF) and extracellular fluid (ECF). *International Journal of Biochemistry and Molecular Biology*, *3*, 365–373.

Alexandrov, P. N., Dua, P., & Lukiw, W. J. (2014). Up-regulation of miR-146a in progressive, age-related inflammatory neurodegenerative disorders of the human CNS. *Frontiers in Neurology*, *5*, 1–6.

Almeida, S., Zhang, Z., Coppola, G., Mao, W., Futai, K., Karydas, A., Geschwind, M. D., Tartaglia, M. C., Gao, F., Gianni, D., Sena-Esteves, M., Geschwind, D. H., Miller, B. L., Farese, R. V., & Gao, F. B. (2012). Induced Pluripotent Stem Cell Models of Progranulin-Deficient Frontotemporal Dementia Uncover Specific Reversible Neuronal Defects. *Cell Reports*, *2*, 789–798.

Ansari, A., Maffioletti, E., Milanesi, E., Marizzoni, M., Frisoni, G. B., Blin, O., Richardson, J. C., Bordet, R., Forloni, G., Gennarelli, M., & Bocchio-Chiavetto, L. (2019). miR-146a and miR-181a are involved in the progression of mild cognitive impairment to Alzheimer's disease. *Neurobiology of Aging*, *82*, 102–109.

Ascencio González, D., Hernández Pando, R., Ángel Gómez Lim, M., Ayala Fraustro, S., & Torres Garcia, A. (2019). Therapeutic Strategies of Secretome of Mesenchymal Stem Cell. In M. Valarmathi (Ed.) *Stromal Cells - Structure, Function, and Therapeutic Implications.* (185-207) IntechOpen, London.

Ballatore, C., Lee, V. M. Y., & Trojanowski, J. Q. (2007). Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nature Reviews Neuroscience*, *8*, 663–672.

Banerjee, P., Paza, E., Perkins, E. M., James, O. G., Kenkhuis, B., Lloyd, A. F., Burr, K., Story, D., Yusuf, D., He, X., Backofen, R., Dando, O., Chandran, S., & Priller, J. (2020). Generation of pure monocultures of human microglia-like cells from induced pluripotent stem cells. *Stem Cell Research*, *49*, 1-13.

Basavaraju, M., & De Lencastre, A. (2016). Alzheimer's disease: Presence and role of miRNAs. *Biomolecular Concepts*, *7*, 241–252.

Basu, J., & Ludlow, J. W. (2016). Exosomes for repair, regeneration and rejuvenation. *Expert Opinion on Biological Therapy*, *16*, 489–506.

Bekris, L. M., & Leverenz, J. B. (2015). The biomarker and therapeutic potential of miRNA in Alzheimer's disease. *Neurodegenerative Disease Management*, *5*, 61–74.

Bird, T. D. (2018). Alzheimer Disease Overview 1 . Clinical Characteristics of Alzheimer Disease. *Gene Reviews*, *8*, 1–12.

Bohnert, S., Seiffert, A., Trella, S., Bohnert, M., Distel, L., Ondruschka, B., & Monoranu, C. (2020). TMEM119 as a specific marker of microglia reaction in traumatic brain injury in postmortem examination. *International Journal of Legal Medicine*, *134*, 2167–2176

Brites D. Regulatory function of microRNAs in microglia. (2020). *Glia*.1–12.

Brites, D., & Fernandes, A. (2015). Neuroinflammation and depression: Microglia activation, extracellular microvesicles and miRNA dysregulation. *Frontiers in Cellular Neuroscience*, *9*, 1–20.

Brownjohn, P. W., Smith, J., Solanki, R., Lohmann, E., Houlden, H., Hardy, J., Dietmann, S., & Livesey, F. J. (2018). Functional Studies of Missense TREM2 Mutations in Human Stem Cell-Derived Microglia. *Stem Cell Reports*, *10*, 1294–1307.

Caldeira, C., Cunha, C., Vaz, A. R., Falcão, A. S., Barateiro, A., Seixas, E., Fernandes, A., & Brites, D. (2017). Key aging-associated alterations in primary microglia response to beta-amyloid stimulation. *Frontiers in Aging Neuroscience*, *9*, 1–23.

Cardoso, A. L., Guedes, J. R., Pereira de Almeida, L., & Pedroso de Lima, M. C. (2012). miR-155 modulates microglia-mediated immune response by down-regulating SOCS-1 and promoting cytokine and nitric oxide production. *Immunology*, *135*, 73–88.

Carlyle, W. C., Mcclain, J. B., Tzafriri, A. R., Bailey, L., Brett, G., Markham, P. M., Stanley, J. R. L., Edelman, E. R., Sciences, C. B., Technologies, E., & Park, L. T. (2015). TREM2 maintains microglial metabolic fitness in Alzheimer's disease. *Cell*, *162*, 561–567.

Chen, X., Jiang, M., Li, H., Wang, Y., Shen, H., Li, X., Zhang, Y., Wu, J., Yu, Z., & Chen, G. (2020). CX3CL1/CX3CR1 axis attenuates early brain injury via promoting the delivery of exosomal miR-124 from neuron to microglia after subarachnoid hemorrhage. *Journal of Neuroinflammation*, *17*, 1–15.

Cherry, J. D., Olschowka, J. A., & O'Banion, M. K. (2014). Neuroinflammation and M2 microglia: The good, the bad, and the inflamed. *Journal of Neuroinflammation*, *11*, 1–15.

Choi, S. H., & Tanzi, R. E. (2012). IPSCs to the rescue in Alzheimer's research. *Cell Stem Cell*, *10*, 235–236.

Colonna, M., & Butovsky, O. (2017). Microglia Function in the Central Nervous System During Health and Neurodegeneration. *Annual Review of Immunology*, *35*, 441–468.

Connell, J. W., Gibb, G. M., Betts, J. C., Blackstock, W. P., Gallo, J. M., Lovestone, S., Hutton, M., & Anderton, B. H. (2001). Effects of FTDP-17 mutations on the in vitro phosphorylation of tau by glycogen synthase kinase 3β identified by mass spectrometry demonstrate certain mutations exert long-range conformational changes. *FEBS Letters*, *493*, 40–44.

Dá Mesquita, S., Ferreira, A. C., Sousa, J. C., Correia-Neves, M., Sousa, N., & Marques, F. (2016). Insights on the pathophysiology of Alzheimer's disease: The crosstalk between amyloid pathology, neuroinflammation and the peripheral immune system. *Neuroscience and Biobehavioral Reviews*, *68*, 547–562.

Davidson, Y., Gibbons, L., Pritchard, A., Hardicre, J., Wren, J., Stopford, C., Julien, C., Thompson, J., Payton, A., Pickering-Brown, S. M., Pendleton, N., Horan, M. A., Burns, A., Purandare, N., Lendon, C. L., Neary, D., Snowden, J. S., & Mann, D. M. A. (2006). Apolipoprotein E ε4 allele frequency and age at onset of Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders*, *23*, 60–66.

De Leeuw, S., & Tackenberg, C. (2019). Alzheimer's in a dish - Induced pluripotent stem cell-based disease modeling. *Translational Neurodegeneration*, *8*, 1–13.

Deczkowska, A., Keren-Shaul, H., Weiner, A., Colonna, M., Schwartz, M., & Amit, I. (2018). Disease-Associated Microglia: A Universal Immune Sensor of Neurodegeneration. *Cell*, *173*, 1073–1081.

Doeppner, T. R., Doehring, M., Bretschneider, E., Zechariah, A., Kaltwasser, B., Müller, B., Koch, J. C., Bähr, M., Hermann, D. M., & Michel, U. (2013). MiR-124 protects against focal cerebral ischemia via mechanisms involving Usp14-dependent REST degradation. *Acta Neuropathologica*, *126*, 251–265.

Does, E., & Differently, I. (2012). *Real-Time Reverse Transcription Polymerase Chain Reaction to Measure mRNA : Use , Limitations , and Presentation of Results*. *3*, 1–3.

Douvaras, P., Sun, B., Wang, M., Kruglikov, I., Lallos, G., Zimmer, M., Terrenoire, C., Zhang, B., Gandy, S., Schadt, E., Freytes, D. O., Noggle, S., & Fossati, V. (2017). Directed Differentiation of Human Pluripotent Stem Cells to Microglia. *Stem Cell Reports*, *8*, 1516–1524.

Faraoni, I., Antonetti, F. R., Cardone, J., & Bonmassar, E. (2009). miR-155 gene: A typical multifunctional miRNA. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1792*, 497–505.

Feng, M. G., Liu, C. F., Chen, L., Feng, W. B., Liu, M., Hai, H., & Lu, J. M. (2018). MiR-21 attenuates apoptosis-triggered by amyloid-β via modulating PDCD4/ PI3K/AKT/GSK-3β pathway in SH-SY5Y cells. *Biomedicine and Pharmacotherapy*, *101*, 1003–1007.

Fernandes, A., Ribeiro, A. R., Monteiro, M., Garcia, G., Vaz, A. R., & Brites, D. (2018). Secretome from SH-SY5Y APPSwe cells trigger time-dependent CHME3 microglia activation phenotypes, ultimately leading to miR-21 exosome shuttling. *Biochimie*, *155*, 67–82.

Freilich, R. W., Woodbury, M. E., & Ikezu, T. (2013). Integrated expression profiles of mRNA and miRNA in polarized primary murine microglia. *PLOS ONE*, *8*, 1-15 .

Fujita, Y., Maeda, T., Sato, C., Sato, M., Hatakeyama, H., Ota, Y., Iwabuchi, N., Tatesawa, K., Nomura, A., Zou, K., & Komano, H. (2020). Engulfment of Toxic Amyloid β-protein in Neurons and Astrocytes Mediated by MEGF10. *Neuroscience*, *443*, 1–7.

Gaudet, A. D., Fonken, L. K., Watkins, L. R., Nelson, R. J., & Popovich, P. G. (2018). MiRNAs: Roles in Regulating Neuroinflammation. *Neuroscientist*, *24*, 221–245.

Graves, P., & Zeng, Y. (2012). Biogenesis of Mammalian MiRNAs: A Global View. *Genomics, Proteomics and Bioinformatics*, *10*, 239–245.

Gu, L., & Guo, Z. (2013). Alzheimer's Aβ42 and Aβ40 peptides form interlaced amyloid fibrils. *Journal of Neurochemistry*, *126*, 305–311.

Guedes, J. R., Lao, T., Cardoso, A. L., & El Khoury, J. (2018). Roles of microglial and monocyte chemokines and their receptors in regulating Alzheimer's disease-associated amyloid-β and tau pathologies. *Frontiers in Neurology*, *9*, 1–8.

Guo, Y., Hong, W., Wang, X., Zhang, P., Körner, H., Tu, J., & Wei, W. (2019). MiRNAs in microglia: How do MiRNAs affect activation, inflammation, polarization of microglia and mediate the interaction between microglia and glioma? *Frontiers in Molecular Neuroscience*, *12*, 1–14.

Hanger, B., Couch, A., Rajendran, L., Srivastava, D. P., & Vernon, A. C. (2020). Emerging

Developments in Human Induced Pluripotent Stem Cell-Derived Microglia: Implications for Modelling Psychiatric Disorders With a Neurodevelopmental Origin. *Frontiers in Psychiatry*, *11*, 1–15.

Hansen, D. V, Hanson, J. E., & Sheng, M. (2017). 311. Microglia in Alzheimer ' s disease. *Journal of Cell Biology*, *217*, 459–472.

Hasselmann, J. (2020). Human iPSC-derived microglia : A growing toolset to study the brain ' s innate immune cells *Glia*. *9*, 1–19.

Heneka, M. T., Carson, M. J., Khoury, J. El, Landreth, G. E., Brosseron, F., Feinstein, D. L., Jacobs, A. H., Wyss-Coray, T., Vitorica, J., Ransohoff, R. M., Herrup, K., Frautschy, S. A., Finsen, B., Brown, G. C., Verkhratsky, A., Yamanaka, K., Koistinaho, J., Latz, E., Halle, A., … Heneka, M. (2015). Neuroinflammation in Alzheimer's Disease HHS Public Access. *Lancet Neurol*, *14*, 388–405.

Heneka, M. T., Carson, M. J., Khoury, J. El, Landreth, G. E., Brosseron, F., Feinstein, D. L., Jacobs, A. H., Wyss-Coray, T., Vitorica, J., Ransohoff, R. M., Herrup, K., Frautschy, S. A., Finsen, B., Brown, G. C., Verkhratsky, A., Yamanaka, K., Koistinaho, J., Latz, E., Halle, A., Kummer, M. P. (2015). Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, *14*, 388–405.

Heppner, F. L., Ransohoff, R. M., & Becher, B. (2015). Immune attack: The role of inflammation in Alzheimer disease. *Nature Reviews Neuroscience*, *16*, 358–372.

Hickman, S. E., Allison, E. K., & El Khoury, J. (2008). Microglial dysfunction and defective β-amyloid clearance pathways in aging alzheimer's disease mice. *Journal of Neuroscience*, *28*, 8354–8360.

Hong, H., Li, Y., & Su, B. (2017). Identification of circulating miR-125b as a potential biomarker of Alzheimer's disease in APP/PS1 transgenic mouse. *Journal of Alzheimer's Disease*, *59*, 1449–1458.

Hsieh, C. L., Koike, M., Spusta, S. C., Niemi, E. C., Yenari, M., Nakamura, M. C., & Seaman, W. E. (2009). A role for TREM2 ligands in the phagocytosis of apoptotic neuronal cells by microglia. *Journal of Neurochemistry*, *109*, 1144–1156.

Huang, K., Dong, S., Li, W., & Xie, Z. (2013). The expression and regulation of miR-125b in cancers Expression of miR-125b in Cancers miR-125b and Its Biological Functions. A*cta Biochim Biophys Sin*, *45*, 803–805.

Jeong, H., Ji, K., Min, K., & Joe, E. (2013). Brain Inflammation and Microglia : Facts and Misconceptions. *Experimental Neurobiology*, *22*, 59–67.

Jiang, P., Turkalj, L., & Xu, R. (2020). High-Fidelity Modeling of Human Microglia with Pluripotent Stem Cells. *Cell Stem Cell*, 26: 629–63

Kitazawa, M., Medeiros, R., & M. LaFerla, F. (2012). Transgenic Mouse Models of Alzheimer Disease: Developing a Better Model as a Tool for Therapeutic Interventions. *Current Pharmaceutical Design*, *18*, 1131–1147.

Kober, D. L., Alexander-Brett, J. M., Karch, C. M., Cruchaga, C., Colonna, M., Holtzman, M. J., & Brett, T. J. (2016). Neurodegenerative disease mutations in TREM2 reveal a functional surface and distinct loss-of-function mechanisms. *ELife*, *5*, 1–24.

Konttinen, H., Cabral-da-Silva, M. e. C., Ohtonen, S., Wojciechowski, S., Shakirzyanova, A., Caligola, S., Giugno, R., Ishchenko, Y., Hernández, D., Fazaludeen, M. F., Eamen, S., Budia, M. G., Fagerlund, I., Scoyni, F., Korhonen, P., Huber, N., Haapasalo, A., Hewitt, A. W., Vickers, J.,

Malm, T. (2019). PSEN1ΔE9, APPSwe, and APOE4 Confer Disparate Phenotypes in Human iPSC-Derived Microglia. *Stem Cell Reports*, *13*, 669–683.

Kosik, K. S., Orecchio, L. D., Bakalis, S., & Neve, R. L. (1989). Developmentally regulated expression of specific tau sequences. *Neuron*, *2*, 1389–1397.

Kowalska, A., Wender, M., Florczak, J., Pruchnik-Wolinska, D., Modestowicz, R., Szczech, J., Rossa, G., & Kozubski, W. (2003). Molecular genetics of Alzheimer's disease: Presenilin 1 gene analysis in a cohort of patients from the Poznań region. *Journal of Applied Genetics*, *44*, 231–234.

Kumar, A., Singh, A., & Ekavali. (2015). A review on Alzheimer's disease pathophysiology and its management: An update. *Pharmacological Reports*, *67*, 195–203.

Lancaster, M. A., & Knoblich, J. A. (2014). Generation of cerebral organoids from human pluripotent stem cells. *Nature Protocols*, *9*, 2329–2340.

Le, M. T. N., Xie, H., Zhou, B., Chia, P. H., Rizk, P., Um, M., Udolph, G., Yang, H., Lim, B., & Lodish, H. F. (2009). MiR-125b Promotes Neuronal Differentiation in Human Cells by Repressing Multiple Targets. *Molecular and Cellular Biology*, *29*, 5290–5305.

Li, L., Chen, X. P., & Li, Y. J. (2010). MiR-146a and human disease. *Scandinavian Journal of Immunology*, *71*, 227–231.

Liddelow, S. A., Marsh, S. E., Bennett, F. C., Liddelow, S., & Marsh, S. (2020). Microglia and astrocyte interactions in health and disease: Dynamic Duo or Partners in. *Trends in Immunology*, *41*, 1–16.

Lih-Fen Lue, T. G. B. and D. G. W. (2019). Alzheimer's disease research using human microglia. *Cells*, *8*, 1–19.

Liu, G. H., Ding, Z., & Izpisua Belmonte, J. C. (2012). IPSC technology to study human aging and aging-related disorders. *Current Opinion in Cell Biology*, *24*, 765–774.

Liu, P.P., Xie, Y., Meng, X., Kang, J., Ad, M., Ad, M., & Ad, M. (2019). History and progress of hypotheses and clinical trials for Alzheimer ' s disease. *Signal Transduction and Targeted Therapy*, **4**, 208-230.

Long, J. M., Ray, B., & Lahiri, D. K. (2014). MiR-339-5p down-regulates protein expression of β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) in human primary brain cultures and is reduced in brain tissue specimens of alzheimer disease subjects. *Journal of Biological Chemistry*, *289*, 5184–5198.

Lopera, F., Ardilla, A., Martínez, A., Madrigal, L., Arango-Viana, J. C., Lemere, C. A., Arango-Lasprilla, J. C., Hincapié, L., Arcos-Burgos, M., Ossa, J. E., Behrens, I. M., Norton, J., Lendon, C., Goate, A. M., Ruiz-Linares, A., Rosselli, M., & Kosik, K. S. (1997). Clinical features of early-onset Alzheimer disease in a large kindred with an E280A presenilin-1 mutation. *Journal of the American Medical Association*, *277*, 793–799.

Lue, L. F., Schmitz, C., & Walker, D. G. (2015). What happens to microglial TREM2 in Alzheimer's disease: Immunoregulatory turned into immunopathogenic? *Neuroscience*, *302*, 138–150.

Ma, X., Liu, L., & Meng, J. (2017). MiR-125b promotes neurons cell apoptosis and Tau phosphorylation in Alzheimer's disease. *Neuroscience Letters*, *661*, 57–62.

Maertzdorf, J., Van Lelij, A. Der, Seerp Baarsma, G., Osterhaus, A. (2001). Herpes simplex virus type 1 (HSV-1)-induced retinitis following herpes simplex encephalitis: Indications for brain-to-eye

transmission of HSV-1. *Annals of Neurology*, *49*, 104–106.

Majolo, F., Marinowic, D. R., Machado, D. C., Costa, J., & Costa, D. (2019). Important advances in Alzheimer ' s disease from the use of induced pluripotent stem cells. *Journal of Biomedical Science*. *5*, 1–19.

Makeyev, E. V., Zhang, J., Carrasco, M. A., & Maniatis, T. (2007). The MiRNA miR-124 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA Splicing. *Molecular Cell*, *27*, 435–448.

Malm, T., Loppi, S., & Kanninen, K. M. (2016). Exosomes in Alzheimer's disease. *Neurochemistry International*, *97*, 193–199.

McQuade, A., Coburn, M., Tu, C. H., Hasselmann, J., Davtyan, H., & Blurton-Jones, M. (2018). Development and validation of a simplified method to generate human microglia from pluripotent stem cells. *Molecular Neurodegeneration*, *13*, 1–13.

Minter, M. R., Taylor, J. M., & Crack, P. J. (2016). The contribution of neuroinflammation to amyloid toxicity in Alzheimer's disease. *Journal of Neurochemistry*, *136*, 457–474.

Mishra, P. J. (2014). MiRNAs as promising biomarkers in cancer diagnostics. *Biomarker Research*, *2*, 1–4.

Moehlmann, T., Winkler, E., Xia, X., Edbauer, D., Murrell, J., Capell, A., Kaether, C., Zheng, H., Ghetti, B., Haass, C., & Steiner, H. (2002). Presenilin-1 mutations of leucine 166 equally affect the generation of the Notch and APP intracellular domains independent of their effect on Aβ42 production. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 8025–8030.

Muffat, J., Li, Y., Yuan, B., Mitalipova, M., Omer, A., Corcoran, S., Bakiasi, G., Tsai, L. H., Aubourg, P., Ransohoff, R. M., & Jaenisch, R. (2016). Efficient derivation of microglia-like cells from human pluripotent stem cells. *Nature Medicine*, *22*, 1358–1367.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., & Lannfelt, L. (1992). A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N–terminus of β–amyloid. *Nature Genetics*, *1*, 345–347.

Nalivaeva, N. N., & Turner, A. J. (2013). The amyloid precursor protein: A biochemical enigma in brain development, function and disease. *FEBS Letters*, *587*, 2046–2054.

Neo, W. H., Yap, K., Lee, S. H., Looi, L. S., Khandelia, P., Neo, S. X., Makeyev, E. V., & Su, I. H. (2014). MiRNA miR-124 controls the choice between neuronal and astrocyte differentiation by fine-tuning Ezh2 expression. *Journal of Biological Chemistry*, *289*, 20788–20801.

Nieweg, K., Andreyeva, A., Van Stegen, B., Tanriöver, G., & Gottmann, K. (2015). Alzheimer's disease-related amyloid-β induces synaptotoxicity in human iPS cell-derived neurons. *Cell Death and Disease*, *6*, 1–13.

Nilsberth, C., Westlind-Danielsson, A., Eckman, C. B., Condron, M. M., Axelman, K., Forsell, C., Stenh, C., Luthman, J., Teplow, D. B., Younkin, S. G., Näslund, J., & Lannfelt, L. (2001). The "Arctic" APP mutation (E693G) causes Alzheimer's disease by enhanced Aβ protofibril formation. *Nature Neuroscience*, *4*, 887–893.

O'Connell, R. M., Taganov, K. D., Boldin, M. P., Cheng, G., & Baltimore, D. (2007). MiR-155 is induced during the macrophage inflammatory response. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 1604–1609.

Ohsawa, K., Imai, Y., Sasaki, Y., & Kohsaka, S. (2004). Microglia / macrophage-specific protein Iba1 binds to fimbrin and enhances its actin-bundling activity. *Journal of Neurochemistry*, *88*, 844–856.

Ophir, G., Amariglio, N., Jacob-Hirsch, J., Elkon, R., Rechavi, G., & Michaelson, D. M. (2005). Apolipoprotein E4 enhances brain inflammation by modulation of the NF-κB signaling cascade. *Neurobiology of Disease*, *20*, 709–718.

Ormel, P. R., Vieira de Sá, R., van Bodegraven, E. J., Karst, H., Harschnitz, O., Sneeboer, M. A. M., Johansen, L. E., van Dijk, R. E., Scheefhals, N., Berdenis van Berlekom, A., Ribes Martínez, E., Kling, S., MacGillavry, H. D., van den Berg, L. H., Kahn, R. S., Hol, E. M., de Witte, L. D., & Pasterkamp, R. J. (2018). Microglia innately develop within cerebral organoids. *Nature Communications*, *9*, 4167.

Parisi, C., Arisi, I., D'Ambrosi, N., Storti, A. E., Brandi, R., D'Onofrio, M., & Volonté, C. (2013). Dysregulated miRNAs in amyotrophic lateral sclerosis microglia modulate genes linked to neuroinflammation. *Cell Death and Disease*, *4*, 1–10.

Penney, J., Ralvenius, W. T., & Tsai, L. H. (2020). Modeling Alzheimer's disease with iPSC-derived brain cells. *Molecular Psychiatry*, *25*, 148–167.

Pimplikar, S. W. (2014). Neuroinflammation in Alzheimer's disease: From pathogenesis to a therapeutic target. *Journal of Clinical Immunology*, 34, 64-69.

Pinho, A. G., Cibrão, J. R., Silva, N. A., Monteiro, S., & Salgado, A. J. (2020). Cell secretome: Basic insights and therapeutic opportunities for CNS disorders. *Pharmaceuticals*, *13*, 1–18.

Pocock, J. M., & Piers, T. M. (2018). Modelling microglial function with. *Nature Reviews Neuroscience*, *19*, 445–452.

Pogue, A. I., Cui, J. G., Li, Y. Y., Zhao, Y., Culicchia, F., & Lukiw, W. J. (2010). Micro RNA-125b (miR-125b) function in astrogliosis and glial cell proliferation. *Neuroscience Letters*, *476*, 18–22.

Posner, H., Curiel, R., Edgar, C., Hendrix, S., Liu, E., Loewenstein, D. A., Morrison, G., Shinobu, L., & Wesnes, K. (2017). Outcomes assessment in clinical trials of alzheimer's disease and its precursors: Readying for short-term and long-term clinical trial needs. *Innovations in Clinical Neuroscience*, *14*, 22–29.

Potdar, P. (2017). Current challenges in the therapeutic use of induced pluripotent stem cells (iPSCs) in cancer therapy. *Applied Cancer Research*, *37*, 1–8.

Querfurth, H. W., & Laferla, F. M. (2010). Mecanisms of disease: Alzheimer's Disease. *New engl and journal o f medicine*, *2*, 329–344.

Rademakers, R., Dermaut, B., Peeters, K., Cruts, M., Heutink, P., Goate, A., & Van Broeckhoven, C. (2003). Tau (MAPT) Mutation Arg406Trp Presenting Clinically with Alzheimer Disease Does Not Share a Common Founder in Western Europe. *Human Mutation*, *22*, 409–411.

Rippon, G. A., Crook, R., Baker, M., Halvorsen, E., Chin, S., Hutton, M., Houlden, H., Hardy, J., & Lynch, T. (2003). Presenilin 1 mutation in an African American family presenting with atypical Alzheimer dementia. *Archives of Neurology*, *60*, 884–888.

Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., Chi, H., Lin, C., Holman, K., Tsuda, T., Mar, L., Sorbi, S., Nacmias, B., Piacentini, S., Amaducci, L., Chumakov, I., Cohen, D., Lannfelt, L., Fraser, P. E., George-Hyslop, P. H. S. (1995). Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the

Alzheimer's disease type 3 gene. *Nature*, *376*, 775–778.

Ryan, S. K., Gonzalez, M. V., Garifallou, J. P., Bennett, F. C., Williams, K. S., Sotuyo, N. P., Mironets, E., Cook, K., Hakonarson, H., Anderson, S. A., & Jordan-Sciutto, K. L. (2020). Neuroinflammation and EIF2 Signaling Persist despite Antiretroviral Treatment in an hiPSC Tri-culture Model of HIV Infection. *Stem Cell Reports*, *14*, 703–716.

Salter, M. W., & Stevens, B. (2017). Microglia emerge as central players in brain disease. *Nature Medicine*, *23*, 1018–1027.

Schonrock, N., Ke, Y. D., Humphreys, D., Staufenbiel, M., Ittner, L. M., Preiss, T., & Götz, J. (2010). Neuronal miRNA deregulation in response to Alzheimer's disease amyloid-β. *PLoS ONE*, *5*, 6-13.

Sevivas, N., Teixeira, F. G., Portugal, R., Araújo, L., Carriço, L. F., Ferreira, N., Vieira Da Silva, M., Espregueira-Mendes, J., Anjo, S., Manadas, B., Sousa, N., Salgado, A. J., & Serra, S. C. (2017). Mesenchymal Stem Cell Secretome. *American Journal of Sports Medicine*, *45*, 179–188.

Sheedy, F. J. (2015). Turning 21: Induction of miR-21 as a key switch in the inflammatory response. *Frontiers in Immunology*, *6*, 1–10.

Sheedy, F. J., Palsson-Mcdermott, E., Hennessy, E. J., Martin, C., O'Leary, J. J., Ruan, Q., Johnson, D. S., Chen, Y., & O'Neill, L. A. J. (2010). Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the miRNA miR-21. *Nature Immunology*, *11*, 141–147.

Sherrington, R., Bruni, A. C. (1998). Cloning of a gene bearing missense mutations in early onset familial Alzheimer's disease: A Calabrian study. In *Functional Neurology*, *13*, 257–261.

Simons, M., & Raposo, G. (2009). Exosomes - vesicular carriers for intercellular communication. *Current Opinion in Cell Biology*, *21*, 575–581.

Song, J., & Lee, J. E. (2015). MiR-155 is involved in Alzheimer's disease by regulating T lymphocyte function. *Frontiers in Aging Neuroscience*, *7*, 1–8.

Stratoulias, V., Venero, J. L., Tremblay, M., & Joseph, B. (2019). Microglial subtypes: diversity within the microglial community. *The EMBO Journal*, *38*, 1–18.

Strooper, B. De, & Karran, E. (2016). Review The Cellular Phase of Alzheimer ' s Disease. *Cell*, *164*, 603–615.

Su, F., Bai, F., & Zhang, Z. (2016). Inflammatory Cytokines and Alzheimer's Disease: A Review from the Perspective of Genetic Polymorphisms. *Neuroscience Bulletin*, *32*, 469–480.

Sun, L., Zhou, R., Yang, G., & Shi, Y. (2017). Analysis of 138 pathogenic mutations in presenilin-1 on the in vitro production of Aβ42 and Aβ40 peptides by γ-secretase. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 476–485.

Sweeney, M. D., Sagare, A. P., & Zlokovic, B. V. (2018). Blood-brain barrier breakdown in Alzheimer disease and other neurodegenerative disorders. *Nature Reviews Neurology*, *14*, 133-150.

Taganov, K. D., Boldin, M. P., Chang, K. J., & Baltimore, D. (2006). NF-κB-dependent induction of miRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, *103*, 12481–12486.

Tai, L. M., Ghura, S., Koster, K. P., Liakaite, V., Maienschein-Cline, M., Kanabar, P., Collins, N., Ben-Aissa, M., Lei, A. Z., Bahroos, N., Green, S. J., Hendrickson, B., Van Eldik, L. J., & LaDu, M. J. (2014). APOE-modulated Aβ-induced neuroinflammation in Alzheimer's disease: Current landscape, novel data, and future perspective. *Journal of Neurochemistry*, *133*, 465–488.

Takahashi, K., & Yamanaka, S. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell*, *126*, 663–676.

Takata, K., Kozaki, T., Zhe, C., Lee, W., Garel, S., & Pouladi, M. A. (2017). Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Resource Induced-Pluripotent-Stem-Cell-Derived Primitive Macrophages Provide a Platform for Modeling Tissue-Resident Macrophage Differentiation and Function. *Immunity*, *47*, 183–198.

Tan, L., Yu, J. T., & Tan, L. (2015). Causes and Consequences of MiRNA Dysregulation in Neurodegenerative Diseases. *Molecular Neurobiology*, *51*, 1249–1262.

Teixeira, F. G., Panchalingam, K. M., Assunção-Silva, R., Serra, S. C., Mendes-Pinheiro, B., Patrício, P., Jung, S., Anjo, S. I., Manadas, B., Pinto, L., Sousa, N., Behie, L. A., & Salgado, A. J. (2016). Modulation of the Mesenchymal Stem Cell Secretome Using Computer-Controlled Bioreactors: Impact on Neuronal Cell Proliferation, Survival and Differentiation. *Scientific Reports*, *6*, 1–14.

Theuns, J., & Van Broeckhoven, C. (2000). Transcriptional regulation of Alzheimer's disease genes: Implications for susceptibility. *Human Molecular Genetics*, *9*, 2383–2394.

Thinakaran, G., Borchelt, D. R., Lee, M. K., Slunt, H. H., Spitzer, L., Kim, G., Ratovitsky, T., Davenport, F., Nordstedt, C., Seeger, M., Hardy, J., Levey, A. I., Gandy, S. E., Jenkins, N. A., Copeland, N. G., Price, D. L., & Sisodia, S. S. (1996). Endoproteolysis of presenilin 1 and accumulation of processed derivatives in vivo. *Neuron*, *17*, 181–190.

Tomita, T., Maruyama, K., Saido, T. C., Kume, H., Shinozaki, K., Tokuhiro, S., Capell, A., Walter, J., Grünberg, J., Haass, C., Iwatsubo, T., & Obata, K. (1997). The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid β protein ending at the 42nd (or 43rd) residue. *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 2025–2030.

Veremeyko, T., Yung, A. W. Y., Dukhinova, M., Strekalova, T., & Ponomarev, E. D. (2019). The Role of Neuronal Factors in the Epigenetic Reprogramming of Microglia in the Normal and Diseased Central Nervous System. *Frontiers in Cellular Neuroscience*, *13*, 1–9.

Verkkoniemi, A., Somer, M., Rinne, J. O., Myllykangas, L., Crook, R., Hardy, J., Viitanen, M., Kalimo, H., & Haltia, M. (2000). Variant Alzheimer's disease with spastic paraparesis: Clinical characterization. *Neurology*, *54*, 1103–1109.

Wakutani, Y., Watanabe, K., Adachi, Y., Wada-Isoe, K., Urakami, K., Ninomiya, H., Saido, T. C., Hashimoto, T., Iwatsubo, T., & Nakashima, K. (2004). Novel amyloid precursor protein gene missense mutation (D678N) in probable familial Alzheimer's disease. *Journal of Neurology, Neurosurgery and Psychiatry*, *75*, 1039–1042.

Walther Haenseler, R. (2019). Pluripotent stem cells Concise Review : Modeling Neurodegenerative Diseases with Human Pluripotent Stem. *Stem cells*, *37*, 724–730.

Wang, G., Huang, Y., Wang, L. L., Zhang, Y. F., Xu, J., Zhou, Y., Lourenco, G. F., Zhang, B., Wang, Y., Ren, R. J., Halliday, G. M., & Chen, S. Di. (2016). MiR-146a suppresses ROCK1 allowing hyperphosphorylation of tau in Alzheimer's disease. *Scientific Reports*, *6*, 1–12.

Oshima, K., Takehiko, Y., Nishio, S. and Matsuda P. (2014). MFG-E8: Origin, Structure, Expression,

Functions and Regulation. In Wang, P. (Ed.), *MFG-E8 and Inflammation* (1-31). Springer, Dordrecht.

Wang, X. H., & Wang, T. L. (2018). MiRNAs of microglia: Wrestling with central nervous system disease. *Neural Regeneration Research*, *13*, 2067–2072.

Wang, Y., Cella, M., Mallinson, K., Ulrich, J. D., Young, K. L., Robinette, M. L., Gilfillan, S., Krishnan, G. M., Sudhakar, S., Zinselmeyer, B. H., Holtzman, D. M., Cirrito, J. R., & Colonna, M. (2015). TREM2 lipid sensing sustains the microglial response in an Alzheimer's disease model. *Cell*, *160*, 1061–1071.

Wang, Y., Ulland, T. K., Ulrich, J. D., Song, W., Tzaferis, J. A., Hole, J. T., Yuan, P., Mahan, T. E., Shi, Y., Gilfillan, S., Cella, M., Grutzendler, J., DeMattos, R. B., Cirrito, J. R., Holtzman, D. M., & Colonna, M. (2016). TREM2-mediated early microglial response limits diffusion and toxicity of amyloid plaques. *Journal of Experimental Medicine*, *213*, 667–675.

Wolf, Y., Yona, S., Kim, K., & Jung, S. (2013). Microglia , seen from the CX 3 CR1 angle. *Frontiers in Cellular Neuroscience. 7*, 1–9.

Xiao, T., Zhang, W., Jiao, B., Pan, C. Z., Liu, X., & Shen, L. (2017). The role of exosomes in the pathogenesis of Alzheimer' disease. *Translational Neurodegeneration*, *6*, 1–6.

Xu, R., Li, X., Boreland, A. J., Posyton, A., Kwan, K., Hart, R. P., & Jiang, P. (2020). Human iPSC-derived mature microglia retain their identity and functionally integrate in the chimeric mouse brain. *Nature Communications*, *11*, 1577.

Y. Saito, MD, PhD; A. Geyer, MD; R. Sasaki, MD, PhD; S. Kuzuhara, MD, PhD; E. Nanba, MD, PhD; T. Miyasaka, PhD; K. Suzuki, MD; and S. Murayama, MD, P. (2002). Progressive familial tauopathy with R406W. *Neurobiology*, 58, 811–813.

Yang, J., Li, S., He, X. B., Cheng, C., & Le, W. (2016). Induced pluripotent stem cells in Alzheimer's disease: Applications for disease modeling and cell-replacement therapy. *Molecular Neurodegeneration*, *11*, 1–11.

Yiannopoulou, K. G., & Papageorgiou, S. G. (2013). Current and future treatments for Alzheimer's disease. *Therapeutic Advances in Neurological Disorders*, *6*, 19–33.

Zhan, X., Stamova, B., & Sharp, F. R. (2018). Lipopolysaccharide associates with amyloid plaques, neurons and oligodendrocytes in Alzheimer's disease brain: A review. *Frontiers in Aging Neuroscience*, *10*, 1–14.

Zhang, W., Jiao, B., Zhou, M., Zhou, T., & Shen, L. (2016). Modeling Alzheimer's Disease with Induced Pluripotent Stem Cells: Current Challenges and Future Concerns. *Stem Cells International*, *5,* 1-12.

Zhao, J., Bi, W., Xiao, S., Lan, X., Cheng, X., Zhang, J., Lu, D., Wei, W., Wang, Y., Li, H., Fu, Y., & Zhu, L. (2019). Neuroinflammation induced by lipopolysaccharide causes cognitive impairment in mice. *Scientific Reports*, *9*, 1–12.