UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

The role of secondary modification of S100B in protein aggregation and its influence on Alzheimer's disease pathology

" Documento Definitivo"

Doutoramento em Biologia
Especialidade de Biologia de Sistemas

Romina José Carlotta Lopes Coelho

Tese orientada por:
Prof. Cláudio M. Gomes
Prof. Andreas M. Grabrucker

Documento especialmente elaborado para a obtenção do grau de doutor

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Júri:
Presidente:

- Rui Manuel dos Santos Malhó, Professor Catedrático e Membro do Conselho Científico da Faculdade de Ciências da Universidade de Lisboa

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- Doutora Adelaide Maria Afonso Fernandes Borralho, Professora Associada da Faculdade de Farmácia da Universidade de Lisboa
- Doutor Cláudio Emanuel Moreira Gomes, Professor Catedrático da Faculdade de Ciências da Universidade de Lisboa
- Doutor Federico Herrera Y Garcia, Professor Auxiliar da Faculdade de Ciências da Universidade de Lisboa.

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Thesis Outputs

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Publications not included in this dissertation

Pederzoli F, Ruozi B, Duskey J, Hagmeyer S, Sauer AK, Grabrucker S, Coelho R, Oddone N, Ottonelli Ilaria, Daini E, Zoli M, Vandelli MA, Tosi G and Grabrucker AM (2019) Nanomedicine Against Aβ Aggregation by β-Sheet Breaker Peptide Delivery: In Vitro Evidence. Pharmaceutics 11, 572. https://www.mdpi.com/1999-4923/11/11/572

Communications at scientific conferences

Coelho R, Cordeiro C, Gomes CM (2019) Post-translational oxidative modifications of S100B modulate its novel chaperone activity against amyloid β aggregation. Poster presented at the Workshop on Integrative Approaches to Protein Folding & Aggregation. Lisbon (Portugal). 11-12 Jun. Abs no. P5 p 49

Coelho R, Cordeiro C, Gomes CM (2019) Post-translational oxidative modifications of S100B modulate its novel chaperone activity against amyloid β aggregation. Poster presented at the BioISI PhD Students Symposium. Lisbon (Portugal). 28 Jun. Abs no. P2 p. 25

Coelho R, Cordeiro C, Gomes CM (2019) Post-translational oxidative modifications of S100B modulate its novel chaperone activity against amyloid β aggregation. Poster presented at the 5th Annual Meeting of Mind-Brain College of the University of Lisbon. Lisbon (Portugal). 12-13 Nov.

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Abbreviations

Aβ – Amyloid beta

ACH – Acetylcholine

ACHI – Acetylcholine inhibitor

AD – Alzheimer's Disease

ANS – Bis-ANS 4,4-Anilino-1,1-Binaphthyl -5,5- disulfonic acid

APOE – Apoliprotein E

APP – Amyloid precursor protein

BBB – Blood-brain barrier

BBBM – Blood based biomarkers

BDNF – Brain-derived neurotrophic factor

CD – Circular dichroism spectroscopy

CNS – Central nervous system

CSF – Cerebrospinal fluid

DAMP – Damage associated molecular pattern

DI TNC1 – (Astrocytic cell line)

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

ENO1 – Enolase 1

ER – Endoplasmic reticulum

FDG-PET – Fluorodeoxyglucose-positron emission tomography

FTIR – Fourier transform infrared spectroscopy

GABA – Gamma-aminobutyric acid

GAPDH – Glyceraldehyde-3-posphate dehydrogenase

GFAP – Glial fibrillary acidic protein

GSNO – S-Nitrosoglutathione

HEPES – 4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid

HMGB1 – High mobility group box 1 protein

HOCl – Hypochlorite

HSP – Heat shock protein

IL – Interleukin

IFN – Interferon

iNOS – inducible nitric oxide synthase

JAK – Janus kinase

kn – primary nucleation rate

k2 – secondary nucleation rate

k+ – Elongation rate

LPS – Lipopolysaccharide

MCI – Mild cognitive impairments

MCP1 – Monocyte chemoattractant Protein-1

MPO – Myeloperoxidase

MRI – Magnetic resonance imaging

MS – Mass spectrometry

NADPH – Nicotinamide adenine dinucleotide phosphate

NF-κB – Nuclear Factor κB

NfL – Neurofilament light

NMDA – N-methyl D-aspartate

NMJ – Neuromuscular junction

NO – Nitric oxide

p-tau – Phosphorylated tau

PARP-1 – Poly (adenosine diphosphate-ribose) polymerase 1

PET –Positron emission tomography

PP5 – Protein phosphatase 5

PS – Presenilin protein

PSEN – Presenilin gene

qRT-PCR – Quantitative Real-time polymerase chain reaction

RAGE – Receptor for advanced glycation endproducts

RNS – Reactive nitrogen species

ROS – reactive oxygen species

$S100B_{ox}$ – Oxidized S100B

SEC – Size exclusion chromatography

SOD1 – Superoxide dismutase

STAT – Signal transducer and activator of transcription

TGN – Trans Glogi-network

ThT – Thioflavin T

TNF-α – Tumor necrosis factor α

TTR – Transtyretin

UPS – Ubiquitin proteosome system

Dissertation Abstract

S100 proteins are implicated in Alzheimer's Disease (AD), and recent findings demonstrated a novel chaperone function for the astrocytic S100B protein, a secreted neuroinflammatory mediator associated with senile plaques capable of mitigating Aβ aggregation and toxicity. These findings suggest a protective role for S100B in the extracellular suppression of Aβ aggregation from the earliest stages of AD, which, however, takes place in a biochemically complex milieu capable of promoting oxidative damage. Herein we report an investigation in which extracellular oxidative conditions are mimicked to test if the susceptibility of S100B to oxidation influences its chaperone activity. Resorting to mild in vitro chemical oxidation of S100B, we observed considerable methionine oxidation inferred from mass spectrometry analysis and no cysteine-mediated disulfide crosslinking. Fourier-transform infrared (FTIR) analysis evidenced vibrational bands characteristic of methionine oxidation, providing confirmatory evidence. Spectroscopic analysis showed that the folding, structure, and stability of oxidized S100B were not affected, nor was its dimeric quaternary structure. However, chemical kinetics and mechanistic analysis revealed that oxidized S100B is a more effective anti-Aβ aggregation chaperone, delaying nucleation and fibril elongation rates more effectively and decreasing the amount of formed toxic Aβ oligomers. Structural analysis suggests that this enhancement of chaperone activity is tied with oxidation of Met-74 and Met-79, favouring interactions with the Aβ client, as these residues are located within the regulatory binding cleft on the S100B chaperone. In line with these findings, cell studies reveal that oxidized S100B is significantly more potent in reducing Aβ-induced astrocytic inflammatory cytokine expression than non-oxidized S100B and reduces cellular Aβ-toxicity more effectively. Overall, this study reveals the underlying mechanisms by which oxidation of S100B, which is likely to occur in the AD brain, functionally contributes to its neuroprotective role against Aβ-induced toxicity.

Key words: Alzheimer´s Disease; oxidized S100B; Aβ; cytokines

Sumário da dissertação

A doença de Alzheimer (DA) foi descrita pela primeira vez por Alois Alzheimer em 1906. É uma doença neurodegenerativa irreversível e, em mais de 95% casos, esporádica, ou seja, não herdada. Não se lhe conhece a origem. Sem cura, o desenvolvimento da doença, que pode levar mais do que duas décadas, torna-se devastador para o indivíduo e desolador para os que o rodeiam. Não só a nível humano como também a nível dos recursos económicos, a doença tornou-se uma das principais preocupações das políticas de saúde. No meio clínico, os médicos recorrem à ajuda de biomarcadores para reconhecer, diferenciar e diagnosticar a DA. Os estudos epidemiológicos lançaram luz sobre os fatores de risco da patologia. Em primeiro lugar, muitos especialistas argumentaram ser uma doença de idade, tornando as pessoas incapazes de escapar a esse destino. Nesta tese, será também dado voz à argumentação segundo a qual a idade é apenas uma precondição. Fatores de risco apresentados são o género feminino, fatores alimentares e ambientais, fatores genéticos, que incluem mutações em genes relevantes para a DA. A nível bioquímico, os fatores proteicos que se revelaram cruciais na DA são o β amiloide (Aβ) e a proteína tau. O Aβ é um produto de clivagem da Proteína Precursora Amiloide (APP) extracelular, e a tau uma proteína intracelular. Estes sustentam as principais hipóteses de explicação de progressão da doença. As terapias aprovadas pela FDA até à data, serão sumariamente apresentadas. Contudo não curam, tendo como consequência que o mecanismo subjacente ainda apresenta muitas questões por resolver a nível celular e proteico. Proteínas desempenham um papel crucial na manutenção da estrutura e função celular. No entanto, em determinadas condições, como no caso da DA, as proteínas podem agregar, levando à formação de agregados proteicos insolúveis no cérebro. A agregação de proteínas é um fenómeno complexo que pode ter implicações profundas, como no caso da DA. A manutenção da homeostase proteica é controlada pelo sistema de controlo de qualidade que inclui os chaperões moleculares. No entanto, na DA, quando a concentração de monómeros amyloids malformados é suficientemente elevada, formam-se oligómeros tóxicos e espécies amilóides de ordem superior. O processo de

agregação macroscópica é normalmente representado por uma forma sigmoide: Com uma fase de desfasamento, uma fase de crescimento/alongamento e uma fase de planalto. No contexto deste trabalho, são principalmente importantes duas vias de nucleação catalítica: nucleação primária, aggregação de monómeros e nucleação secundária, agregação de oligómeros na superfície de fibrilhas já formadas. Os chaperões moleculares no espaço extracelular podem impedir a agregação ao ligarem-se a regiões hidrofóbicas expostas por proteínas incorretamente formadas. Um desses chaperões moleculares é certamente a S100B.

Na DA, o stress oxidativo aumenta (detalhes são apresentados na tese). Os radicais ou produtos secundários podem levar à oxidação de proteínas, e à diminuição de agentes antioxidantes. *No entanto, põe-se a questão se proteínas uma vez oxidadas, não podem ter ações antioxidantes?* Esta questão será tratada no capítulo 3. Aí é demonstrado que a S100B, uma proteína da família das proteínas S100, desempenha um papel crucial na patologia de Alzheimer. Envolvida na agregação de tau e Aβ, a S100B também demonstrou desempenhar um papel na neuroinflamação. Suspeita-se que a mudança entre a sua ação pró-inflamatória e a sua possível ação anti-inflamatória resida no facto de ela estar ou não oxidada. Além disso, um aumento da produção de oxidantes devido a uma reação neuroinflamatória, a agregação de proteínas, pode aumentar a atividade antiagregante da S100B. Por conseguinte, a S100B oxidada ($S100B_{ox}$) / S100B pode ser um elo de ligação entre a agregação e a neuroinflamação na DA. Como se vai ver no capítulo 3, esta funcionalidade redox não esta limitada à S100B. De facto, as proteínas S100A1, S100A6, S100A7, S100A8, S100A9, S100A12 e a S100B, mostraram desempenhar um papel na DA. Excluindo a S100A12, todas elas podem sofrer alterações oxidativas. O estudo destas proteínas oxidadas em geral ainda está num estado embrionário. Contudo, tem sido mostrado que o stress oxidativo no cérebro dos doentes de Alzheimer é elevado, sugerindo oxidação. Por isso, não vão ser só discutidos as S100s relevantes na DA, mas também essas S100 no seu estado oxidativo. Como visto anteriormente, os agregados proteicos acumulam-se em doenças neurodegenerativas como na DA, desencadeando defesas celulares e alterando o equilíbrio redox. A família S100 de citocinas pró-inflamatórias, particularmente a S100B, é ativada durante a DA. Estudos recentes revelam que a S100B atua como um chaperão molecular, inibindo a agregação e a toxicidade do Aβ, sugerindo um papel

protetor nas fases inicias da proteotoxicidade num ambiente de stress oxidativo. Neste estudo, investigamos o impacto das condições oxidativas na função protectora da S100B. S100B ligeiramente oxidada, em resíduos de metionina, ocorrendo muito provavelmente na região de ligação ao $A\beta_{42}$. Estruturalmente, a $S100B_{ox}$ não apresenta alterações significativas em relação à sua homóloga (S100B não oxidada). A S100B retarda a agregação de $A\beta_{42}$, *in vitro*, mas a $S100B_{ox}$ retarda a agregação *ainda mais*, possivelmente modulando mesmo a agregação de $A\beta_{42}$ de forma substoquiométrica. Além disso, será demonstrado que esta oxidação não interfere com o mecanismo de nucleação da agregação secundária.

Em seguida, a relação entre a resposta imunitária, a resposta inflamatória e os mediadores inflamatórios será apresentada. Neste quadro, será discutida a forma de como as funções das células da glia são alteradas após estímulos patológicos, levando ao stress oxidativo e à expressão alterada de citocinas. Será ainda abordado o modo como uma citocina, no seu estado oxidativo, pode ser estudada num modelo especifico de DA. Seguem-se os métodos e resultados do nosso estudo, no qual testamos o efeito da S100B oxidada num modelo astrocítico da DA, utilizando um método dinâmico, em tempo real e sem corantes. A adição de espécies de $A\beta_{42}$ de baixo peso molecular, às células astrocíticas em conjunto com S100B oxidada revelou-se prometedora em termos de saúde celular. A expressão de citocinas neste modelo de DA foi também testada, nomeadamente para IL-17, IFN-α e S100B. Estes resultados corroboram ainda mais o papel benéfico da $S100B_{ox}$, abrindo a possibilidade de um ciclo de feedback loop negativo da expressão da *S100b*.

Concluindo e discutindo, os chaperões moleculares desempenham um papel fundamental nas doenças neurodegenerativas onde podem ser neuroprotectores. A S100B demonstrou ter uma atividade do tipo "holdase", que reduz a toxicidade do $A\beta_{42}$. A oxidação com hipoclorito (HOCl) mostrou potenciar esta atividade do chaperão *in vitro* e acrescentar uma possível atividade anti-inflamatória à proteína, reduzindo a expressão de citocinas entre elas a própria *S100b*. Os dois resíduos de metionina cruciais, identificados para esta atividade de chaperão, são conservados nas diferentes espécies, sublinhando a sua importância *in vivo*. Foi demonstrado que doses baixas de S100B conduzem à proliferação de células astrocíticas. Este dado possivelmente elucida ainda mais o mecanismo subjacente aos resultados, ao mostrar um aumento da saúde celular quando tratados com $A\beta_{42}$ e $S100B_{ox}$, em comparação com células tratadas com

$A\beta_{42}$ e S100B. Dado que o stress oxidativo é elevado no cérebro de pacientes de Alzheimer e os fármacos anti-inflamatórios mostraram efeitos apenas nas fases iniciais (antes do comprometimento cognitivo), a S100B oxidada por hipoclorito pode ser um acontecimento posterior (após o comprometimento cognitivo), servindo possivelmente (em combinação com outros biomarcadores específicos da DA) como biomarcador da progressão da DA, podendo contribuir para caraterizar melhor este "ponto de viragem".

Palavras-chave: Doença de Alzheimer; S100B oxidada; Aβ; citocinas

Thesis outline

Chapter 1:

Alzheimer's disease (AD) is an irreversible neurodegenerative disease without cure. A top concern on a personal and a financial level. The numbers speak for themselves. Hallmarks of AD are amyloid β (Aβ) and hyperphosphorylated tau protein. Three main hypotheses have attempted to explain the disease progression. Risk factors have been identified by epidemiological studies. Biomarkers and how everything integrates is key in the first chapter. Awareness and progress is crucial to prevent AD.

Chapter 2:

When protein homeostasis becomes aberrant, as it is the case in AD, this has profound implications for the cell. From fundamental contributions to the cells quality control system, with focus on molecular chaperones, to how in AD, protein aggregation despite all occurs, is discussed. On a molecular level, protein aggregation in the context of AD will be taken into focus and what inspires translational medicine will be addressed.

Chapter 3:

In Alzheimer's Disease, oxidative stress is increased and may oxidize proteins. S100B is a potentially crucial molecular chaperone in AD. Involved in tau and Aβ aggregation and neuroinflammation, the idea about S100B to switch between pro-inflammatory effects to anti-inflammatory effects, may lay in it being oxidized or not. Therefore, $S100B_{ox}$/S100B may be a link between aggregation and neuroinflammation in AD. In this chapter, proteins of the S100 protein family involved in AD and how they interact once oxidized are discussed, with special emphasis on S100B.

Chapter 4:

In the context of AD, there are several oxidative modifications a protein may undergo. Our study, shows the chaperone S100B, can be oxidized at probably the Aβ binding

site. Structurally with no significant alterations to S100B, but with a potentiated chaperone activity at various concentrations tested.

Chapter 5:

The relationship between the immune response, the inflammatory response and inflammatory mediators is presented. How a molecular chaperone can be studied in an AD model, will be addressed. Followed by our study, in which we test the effect of the cytokine, S100B, oxidized, in an astrocytic AD model using a xCELLigence system. $S100B_{ox}$ (in comparison to S100B) and $A\beta_{42}$ added to DITNC1 astrocytes showed to increased cell health. Cytokine expression tested was downregulated, noticeably among them *S100b*.

Chapter 6:

Molecular chaperones, like S100B, may be neuroprotective in the inicial phases of AD. S100B oxidation has shown to potentiate its chaperone activity and reduce cytokine expression tested, among them *S100b* itself, which is new to this thesis. $S100B_{ox}$ and $A\beta_{42}$ treated cells showed an increase in cell health possibly partly due to low levels of S100B promoting astrocytic cell proliferation. $S100B_{ox}$ possibly at later stages a more recurrent event, may possibly serve as an additional blood inflammatory biomarker for AD progress.

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Chapter I

Alzheimer's Disease – Overview

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3. *Alzheimer's Disease Biomarkers*
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Alzheimer's disease is an irreversible neurodegenerative disease without known cure. It is devastating for the individual and their surroundings, which makes it also one of the top concerns in terms of disease healthcare costs and a research priority. This chapter will address the state of the art in numbers, symptoms, risk factors and biomarkers. This will be followed by discussing the two main proteinaceous actors that have been reported as crucial: amyloid β (Aβ), the extracellular Amyloid Precursor Protein (APP) cleavage product, and the intracellular tau protein, though tau will be addressed more briefly since it plays a limited role for this thesis. Further, the main hypotheses to attempt to explain disease progression are presented. In addition, this chapter will briefly mention the currently FDA approved drugs for AD.

1. Facts and Figures

According to the literature, Alzheimer's Disease (AD) is devastating and irreversible [1]. AD is characterized by neurodegeneration and impairments in synaptic plasticity [2]. It is mainly not hereditary (over 90%) [3], making it difficult to foresee and take appropriate measures. AD shows an effect on memory, thinking, and social abilities. For those over 65 years old, it is the most common cause of dementia, the risk further correlating with increasing age [4]. Not only on a human level but also in terms of costs, over 1.3 trillion for AD (and related dementias) are the expenses worldwide in terms of healthcare costs alone. At the same time, the economic burden is expected to rise with the aging population [5] (Fig. 1).

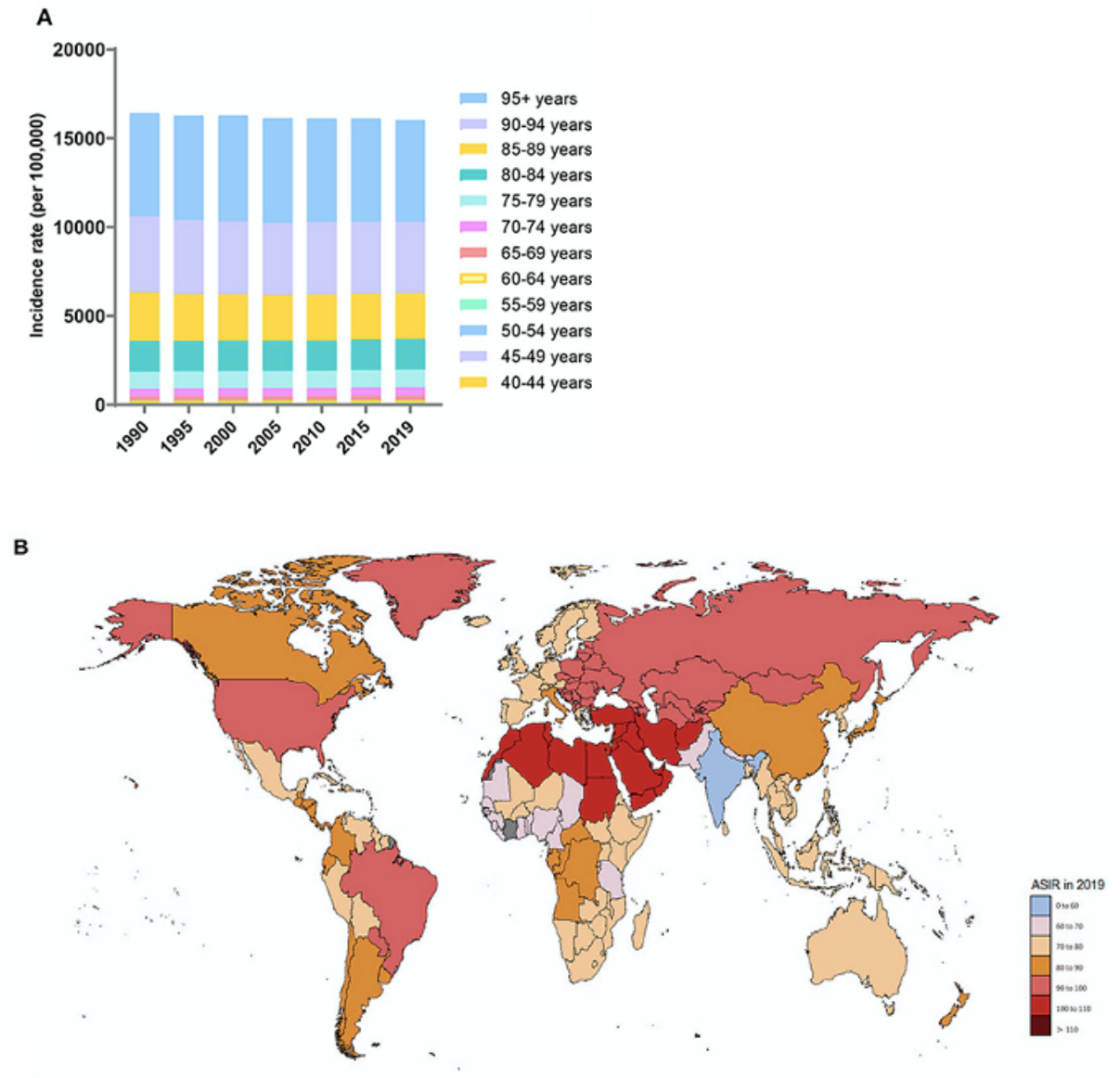

Fig. 1: Distribution of the incidence of Alzheimer's Disease and other dementias across 204 countries and territories (A) Incident rate by age group (B) Correlations between the (regarding gender) age standardized incident rate of Alzheimer's Disease and other dementias and sociodemographic index regions in 2019 (Image modified from [6])

2. Symptoms and main hallmarks of AD

Early clinical symptoms include difficulty of remembering recent events, apathy and depression. Later symptoms show a worsening in memory, impaired judgement, disorientation, confusion difficulty speaking and walking, to name some. The three clinical

phases AD patients are divided in, are: presymptomatic, the symptomatic prodromal phase, and dementia. Each phase is characterized by molecular changes. Presymptomatic AD patients show amyloid deposition, although without cognitive impairments. The symptomatic prodromal patients show amyloid deposition, mild cognitive impairments and slightly more pronounced neurodegeneration [7]. Upon dementia [8], cognitive impairment worsens and interferes with activities of the persons daily life [7].

The main neuropathological hallmarks of the AD brain are Aβ and Aβ plaques cerebral dystrophy and hyperphosphorylated tau tangles (aggregates). In addition, neuropathological changes include: neuronal loss, gliosis, degenerative changes in the white matter and cerebral amyloid angiopathy to name some [7] (Fig. 2). How AD neuropathological hallmarks can be used as biomarkers, will be further explored in the following section.

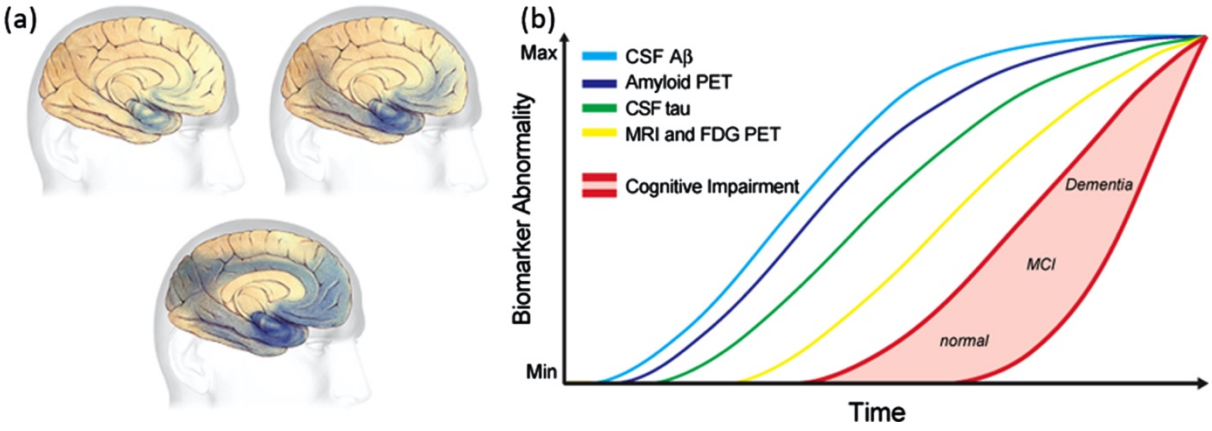

Fig. 2: (a) amyloid distribution throughout the brain (b) biomarker abnormality along time in an AD patient. Abbreviations: CSF, cerebral spinal fluid, Aβ, amyloid β, PET, positron emission tomography, MRI, magnetic resonance imaging, FDG-PET, fludeoxyglucose PET, MCI, mild cognitive impairment. (Image from [9])

3. Alzheimer's Disease Biomarkers

In the clinical setting, to recognize, differentiate and diagnose AD, clinicians use the help of pathophysiological and topographic biomarkers. Later being related to AD regional consequences [10].

Pathophysiological biomarkers:

Amyloid positron emission tomography (PET) is used to visualize fibrillar or insoluble Aβ plaques [10].

Cerebral spinal fluid (CSF) biomarkers proceed in detectability neuroimaging biomarkers. CSF biomarkers can be used to detect Aβ and tau. Elevated CSF tau levels are an indicator for neuronal death, p-Tau being more specific for AD. Moreover, the amount of neurodegeneration and neurofibrillary tangles can be followed by total tau and phosphorylated tau (p-tau). In terms of Aβ, a decrease in CSF $A\beta_{42}/A\beta_{40}$ ratio correlates in an inversed manner with the accumulation of insoluble Aβ plaques in the AD brain [10].

Blood based biomarkers (BBBM) are also very important biomarkers in the context of AD. For instance, reduction of the ratio of $A\beta_{42}/A\beta_{40}$ during the continuum of AD gives an indication of cerebral amyloid pathology, and is a predictor for an amyloid-positive PET status. Challenge hereby are the small fold-changes between Aβ positive and negative individuals and the possible interference of common drugs in older adults, which in a clinical setting puts the risk of misclassificating individuals [11].

Plasma phosphorylated tau protein (namely p-tau181, p-tau217 and p-tau231) increases after a drop in CSF $A\beta_{42}/A\beta_{40}$ ratio and before Aβ-PET positivity is gained, mainly p-tau217 is highly discriminative in distinguishing between AD and non-AD disorders.

Total tau levels suffer from the interference of peripheral (for AD unspecific) tau levels in the plasma [11].

Increase Neurofilament light (NfL) is a used marker for neuroaxonal injury however not AD specific, which can make it of limited use in this context. Glial fibrillary acidic protein (GFAP) is a marker for plasma astrocytic activation related to Aβ pathology, giving better results in comparison to cerebral spinal fluid GFAP [11].

S100B has been considered as a potential biomarker, however considering the complexity of its expression, the utility is still uncertain following Arslan *et al.*. Early on MCI-AD biomarkers that show to be promising are also chitinase 3-like 1 for instance [11].

Topographic biomarkers: Topographic biomarkers are Fludeoxyglucose (FDG)-PET for regional hypometabolism, tau PET to visualize neurofibrillary tangles and regional/local atrophy made visible by structural magnetic resonance imaging (MRI) [10].

While 78% of AD patients show the typical amnestic syndrome phenotype, there are slight differences in-between AD phenotypes concerning biomarkers [10] (Fig. 3).

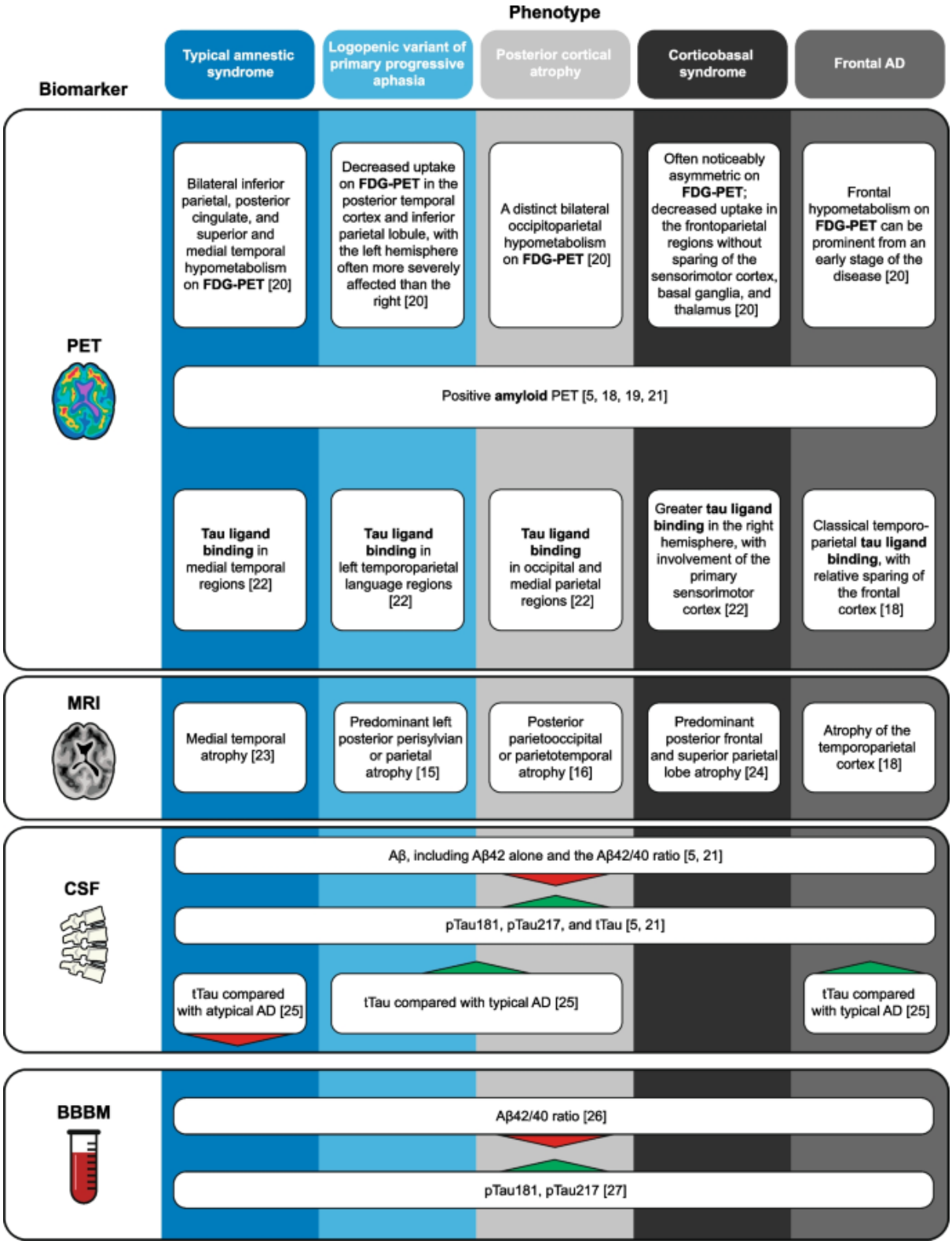

Fig. 3: Overview of anticipated biomarker results for clinical divers AD phenotypes.

Abbreviations: Aβ, amyloid beta, BBBM, blood-based biomarker; CSF, cerebrospinal fluid; FDG, fluorodeoxyglucose; FTD, frontotemporal dementia; MRI, magnetic resonance imaging; PET, positron emission tomography; pTau, phosphorylated tau; tTau, total tau (Image from [10])

Risk factors that may lead to the development of Alzheimer's disease will be discussed in the following section.

4. Risk factors for Alzheimer's Disease

Age, the main risk factor for AD?

It is almost a consensus in the scientific AD community that advanced age is the leading risk factor for AD [12]. However, several authors have challenged this idea. Therefore, we will briefly mention some of the later.

Even though age correlates in many epidemiological studies with AD [13], its causative role is debatable. The aged brain may solely be a required precondition for AD to develop, based on the following studies. Decade-early insulin resistance measurements by PET have become an additional biomarker of high sensitivity for AD. In societies with high life expectancies, like in some areas of Japan, lifestyle and not age (or ethnicity) have prevailed as a factor explaining AD occurrence [14] [15]. The influence of additional environmental factors like diet cannot be excluded [16], [17], [18].

Genetics and other AD risk factors

Alzheimer's Disease is neither a pure genetic nor a pure environmental-caused disease. While most cases are sporadic, some show dominant mendelian trait inheritance of rare mutations or increased risk due to genetic predisposition [3]. More specifically, point mutations or duplication/triplication of the gene APP (Chromosome 21) are dominantly inherited but rare. Multiple gamma secretase mutations that favor the production of $A\beta_{42}$ fragment, namely in PSEN1/2 (Chromosome 14, 1 respectively), are also dominantly inherited but rare. APOE4, which plays a role in cholesterol metabolism, is 'only' a risk factor for late-onset AD. However, those alleles are common predispositions [3]. Thus, with genetic mutations in APP,

Presenilin 1 (PS1), and Presenilin 2 (PS2), the probability of developing familiar or sporadic AD is close to 100%. In contrast, upon genetic modification like APOE4, the risk is increased several times for late-onset AD [3].

Other genetic mutations associated with AD play a role in cholesterol mechanisms [19], immune system [20] dysfunction, and synaptic or membrane dysfunction [21]. AD is a genetically complex disease because a phenotype can be generated by different genetic loci, alleles, mutations, or polymorphisms [22]. Another risk factor for developing AD is (female) gender [23].

Other risk factors are environmental factors like aluminium, pesticides, diabetes, high blood pressure, head trauma, and low educational attainment [24].

Means of dealing with potential AD patients

When AD is suspected, AD risk factors are assessed and screened for, diagnosis and staging are performed, and treatment and its monitorization follow. With tests, people with AD are then categorized mainly into having symptomatic or asymptomatic AD. Interestingly, changes in cerebrospinal fluid (CSF) tau, characteristic of AD pathology, occur ca. 15 years before the onset of clinical AD, while for $A\beta_{42,}$ it is an estimate of up to 20 years, that it appears, dependent on the AD form a patient suffers from. There is also the "at risk" stage, characterized by either an amyloidopathy or tauopathy outside the medial temporal lobe, both not being enough to establish an AD diagnosis [25].

FDA approved AD drugs

Tacrine, Donepezil, Carbalatine and Galanthamine are four drugs approved by the FDA to this date for AD, that function as acetylcholinesterase inhibitors (AChEIs). They can inhibit acetylcholinesterase, the enzyme that degrades acetylcholine in the synaptic cleft. This increases the cholinergic effect which improves the abilities to form memories and to learn. Memantine is an receptor agonist for N-methyl-D-aspartate (NMDA), with an reducing effect on neuronal apoptosis.

These drugs are there to ameliorate symptoms but do not cure AD. More recently, Lacanemab was also approved. However its safety and efficacy will be more precisely known with time [26].

On a molecular level AD is a complex disease and in the following sections an overview is given of main events occurring in Alzheimer's Disease.

5. Protein Misfolding in AD

Protein folding is a fundamental process in cells, and their dysfunction has significant deleterious implications. Alzheimer's is a protein-folding disease that is part of amyloid diseases, with the affected proteins being Aβ peptide and tau. Central to these diseases is that proteins lose their native structure and form fibrillar aggregates, structures rich in β-sheets. These aggregates agglomerate and can then be stained postmortem, for instance, in the brains of AD patients. These Aβ aggregates are usually referred to as Aβ (or senile/neuritic) plaques and for tau as neurofibrillary tangles [27]. Protein misfolding in AD will be further elaborated in chapter 2.

5.1 Amyloid β plaques

Aβ plaques are one of the main hallmarks of the disease. Alois Alzheimer described them for the first time in 1906. Aβ plaques consist mainly of Aβ peptides [27]. Additional proteins and metal ions have been found near plaque lesions in the brain [28].
The spreading of Aβ plaques has been analyzed in human brains, showing trajectories go from the neocortex and allocortex to the diencephalic nuclei, striatum, and the cholinergic nuclei of the basal forebrain to brain stem nuclei (Fig. 4) and the cerebellum. [29]

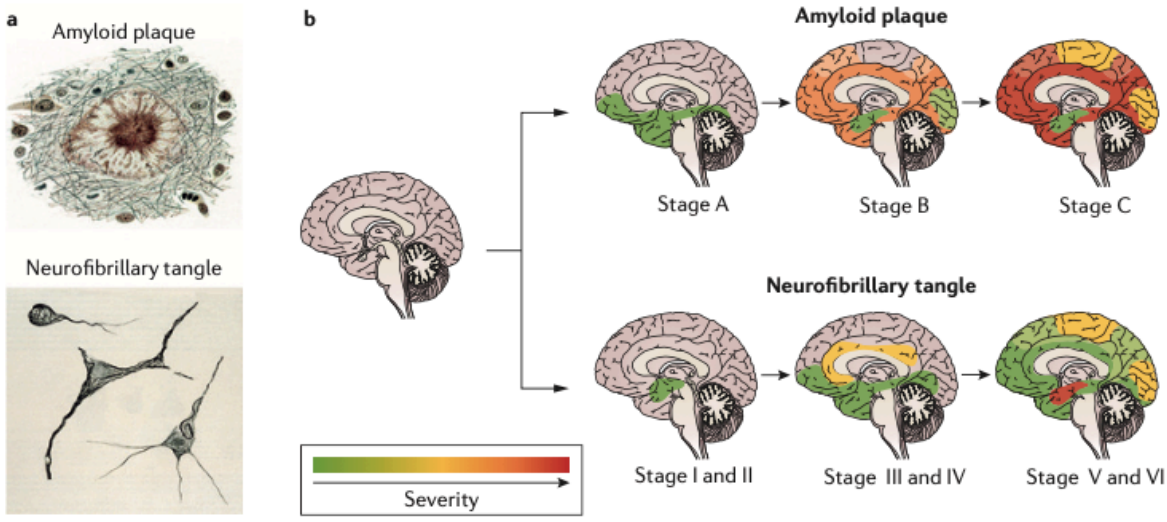

Fig. 4: Progression of Alzheimer's Disease. **a** Schematic representation of Aβ plaques and neurofibrillary tangles. **b** Aβ plaque and neurofibrillary tangle distribution upon AD pathology progression. (From [30])

5.2 Amyloid β and the Amyloid precursor protein

APP is central to AD as the transmembrane protein, giving rise to Aβ upon cleavage. APP can be cleaved either following the non-amyloidogenic pathway or the amyloidogenic pathway.

APP is important in cellular functions, particularly in the nervous system. In humans, the APP gene is located on chromosome 21 [31] and can be spliced into 695 to 751 amino acid-long isoforms. The primary form in human neurons is APP695, while 751 and 770 amino acid-long APP isoforms are characteristic of more peripheral cells [32]. APP is a type I transmembrane protein. In the cell, the trans-Golgi-network (TGN), between the endoplasmic reticulum and the cell membrane, is part of the APP-generating process in neurons. The TGN is where APP levels can be found to be the highest [31]. The APP ectodomain mass represents about 88% of the APP protein [33]. APP has no enzymatic activity. However, it is involved in various biological activities, and APP has been reported to play a role in neurodevelopment, endocytosis, and the neuromuscular junction (NMJ), among others [34].

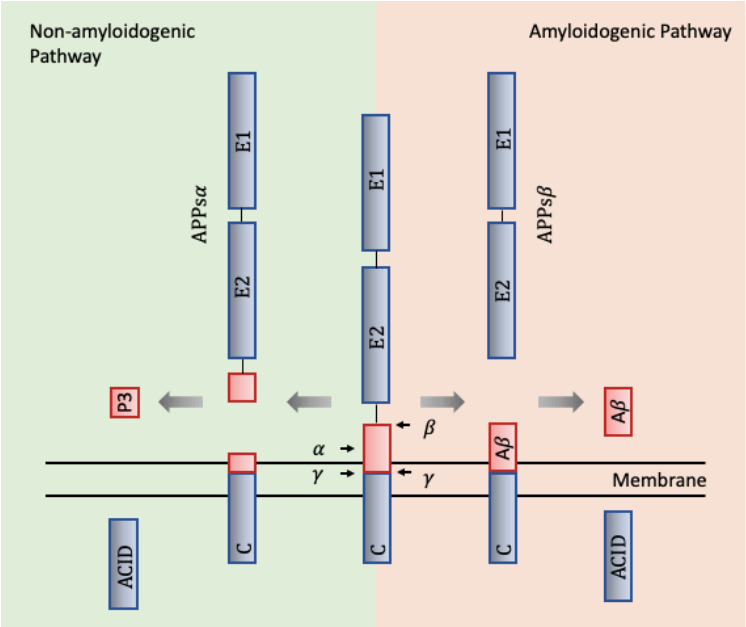

Fig. 5: Schematic non-amyloidogenic and amyloidogenic processing of APP (adapted from [35])

APP can be cleaved following the pathological and non-pathological pathways. In a healthy human brain, the synaptic transmembrane protein APP is mainly cleaved by two proteolytic secretase complexes, namely α-secretase and β-secretase, into the ectodomain sAPPα [31] a growth factor [36] and a small p3 peptide also suggested to be involved in neuroprotective functions [37]. This pathway is considered the non-amyloidogenic pathway (Fig. 5). In more detail, the alpha-secretase cleaves the Aβ sequence at residue 16, generating an 83 amino acid-long fragment (C83). The subsequent cleavage of the short p3 peptide occurs by gamma-secretase [38]. Research suggests that the generation of soluble APPalpha (sAPPalpha) may have neuroprotective potential in neurite outgrowth LTP modulation and neurogenic and neurotrophic effects, among others [39].

In the healthy brain, the non-amyloidogenic pathway dominates in cleaving APP (Aβ is produced in a controlled manner by clearance mechanisms of the brain) [40]. The distribution in cellular compartments plays a role in how APP is processed. Excluding the non-canonical pathways, APP accumulation on the cell surface compared to acidic compartments does not/ does promote amyloidogenic processing, respectively [34]. Moreover, Aβ neurotoxicity may be concentration and structure-dependent. Low quantities have been suggested to be involved in neurodevelopment [40]. Aβ's role in neurotoxicity is that Aβ aggregates have been shown to interfere with synaptic transmission, new synapses, and spine formation, reducing synapse and spine numbers, for instance [41].

In the brain of an AD patient, APP undergoes mainly the amyloidogenic pathway (Fig. 5) and is cleaved by the β-secretase [31] on the N-terminal region [38] of APP, cleaving off a sAPPβ peptide. Then, the γ-secretase cleaves off an Aβ peptide [31] at the C terminal end of APP [38] of chain length varying between 37-49 amino acids [32]. These show affinity to homologous structures like themselves, cling to synapses, and form oligomers and fibrils, eventually leading to senile plaques [42].

5.3 Tau

Hyperphosphorylated tau is, as mentioned previously, also a main hallmark of AD. In the healthy brain, the microtubule associated protein tau regulates the assembly and structural stability of microtubules in neurons and glia cells [43]. Upon a tauopathie like AD, tau becomes abnormally hyperphosphorylated losing its function of structural supporting and maintaining microtubules. It forms aggregates. These insoluble, helical filaments then accumulate as neurofibrillary tangles [43] (Fig. 6) . To aggregate tau must undergo a shift from a random coil conformation into a compact “tangle” state, which represents a massive conformational change following *Binder et al*. [44].
Interestingly, the morphology of the tau aggregate is characteristic for each tauopathy [45], and in AD hyperphosphorylated tau levels are closely associated to cognitive decline [26].

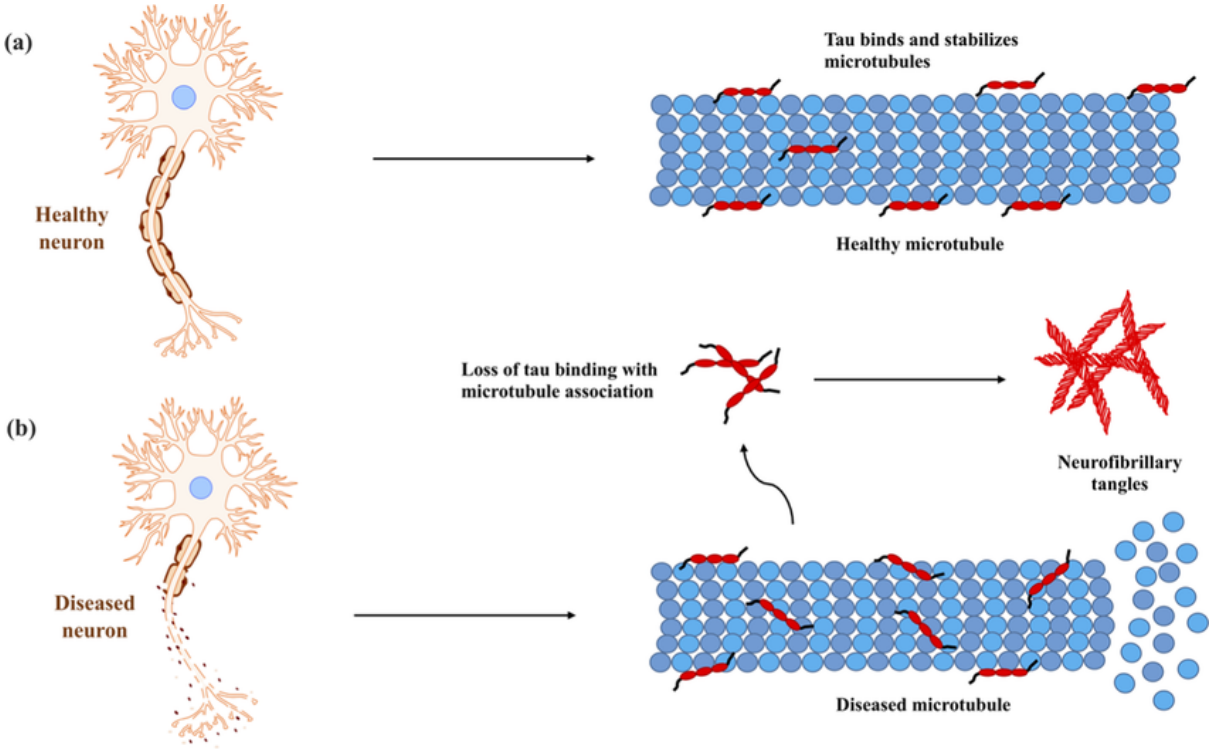

Fig. 6: Tau dependent microtubule stabilization. (a) In a healthy neuron axonal tau binds and stabilizes microtubules. (b) Upon AD tau dissociates, destabilizing microtubules and aggregating into neurofibrillary tangles. Vesicle transport and axonal stability are interrupted in this context. (Image from [46])

To understand the underlying mechanism of such a complex disease like Alzheimer's Disease, hypotheses were proposed and the validity of the main ones today, will be discussed in the following section.

5.4 AD hypotheses and their validity today

There are three main AD hypotheses: the amyloid cascade (Fig. 7), the tau, and the cholinergic hypothesis, which we will further elaborate. The amyloid cascade hypothesis is the most renowned and possibly accepted hypothesis for AD. This is because low molecular weight Aβ aggregates, namely oligomers, are known for their neurotoxicity. Levels of Aβ oligomers correlate more strongly with cognitive decline than plaque number [47]. In short, these oligomeric species may then lead to the induction of tau pathology, leading to the degeneration of neurons and, subsequently, cell death [48].

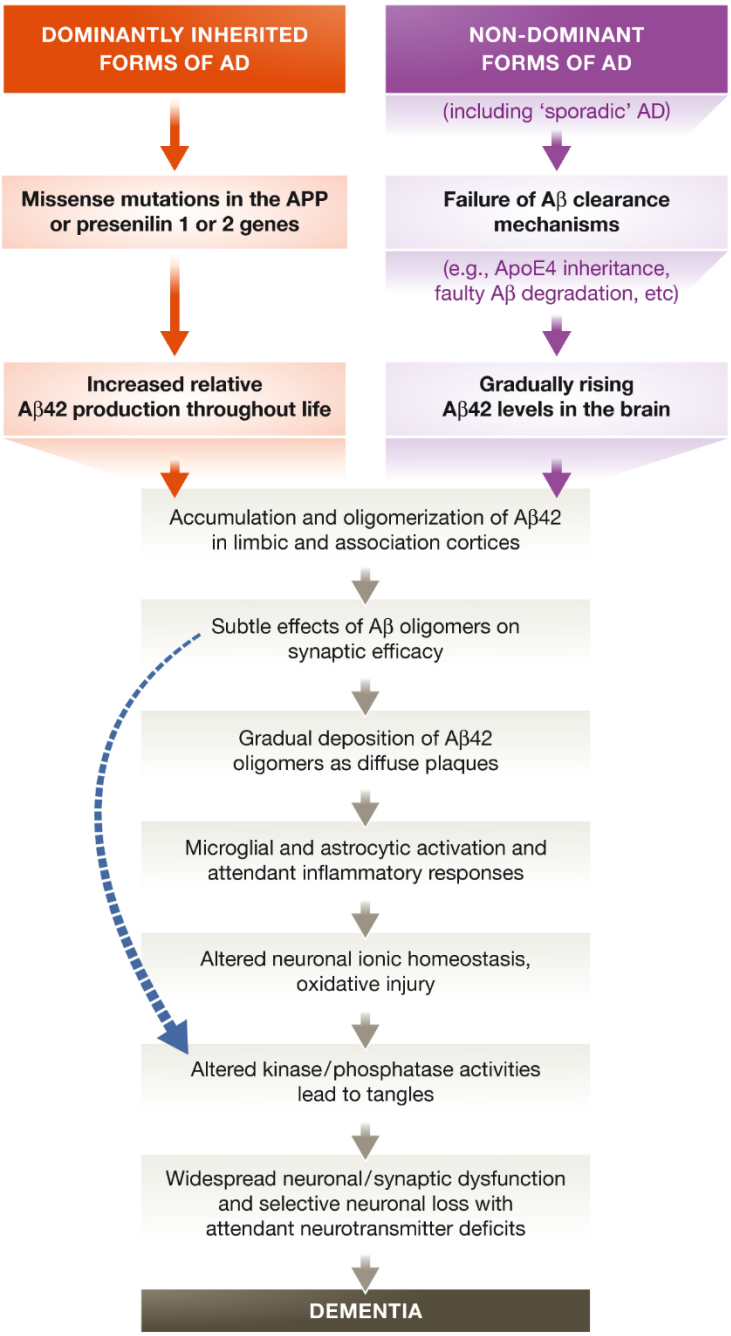

Fig. 7: Amyloid cascade hypothesis. Shown are the main events, in sequence (indicated by grey or black arrow), that follow the amyloid cascade hypothesis and lead to AD. (From [49])

Selkoe and Hardy, defenders of the amyloid cascade hypothesis, argued that amyloid burden is indeed causative and proximate to neuronal dysfunction since it is an early event in the disease. However, findings show Aβ burden in humans with no noticeable dementia. The argument for the hypothesis is that a possibly higher percentage of plaques were diffuse and, therefore, not so easily detectable. Other findings in humans suggest that Aβ plaque burden follows tau tangle formation in terms of events [49]. The tau hypothesis proclaims that AD's

causative "substance" is tau. Hyperphosphorylated tau converts physiological tau into modified (hyperphosphorylated) tau, leading to a prion-like propagation of tau seeds, resulting in neurodegeneration and cell death.

Furthermore, tau distribution correlates with memory deficits [48] more than Aβ distribution [50]. Previous findings, Selkoe and Hardy claim, are the result of a lack of systematic amyloid plaque search since genetically, APP mutations could result in wild-type tau aggregation, but not the other way around. Lastly, findings trying to undermine the amyloid cascade hypothesis also show the failure of targeting Aβ in clinical trials. In favor of the hypothesis is that Aβ lesions, the main constituents of plaque lesions, are the first lesions to appear in AD mice models, which correlates with inflammation, hypermetabolism, and degeneration around these plaques [49]. Problems include that solely Aβ mouse models show no neurofibrillary tangles (accumulated tau) and limited neuron death following the authors Kametani and Hasegawa [48]. Since mice do not live long enough to form Aβ plaques sporadically, probably non-human primates, especially the grey mouse lemur from Madagascar and the Octodon degus, a rodent may be more applicable. Both live around ten years and can spontaneously develop plaques and neurofibrillary tangles [51].

Another obstacle to the Aβ hypothesis is that immunotherapies decreasing Aβ deposition in the brain do not affect actual symptom amelioration [48]. However, at the stage of detecting Aβ deposition, many brain cells have already died.

One has to keep in mind that elderly non-demented individuals exist, who also show a similar extensive distribution of Aβ plaques in the brain as do AD patients [48].

The cholinergic hypothesis

Up to 2017, drugs tested against AD were mainly neurotransmitter-related. Dopamine, synthesized in the midbrain, plays a role in cognition, memory formation, and synaptic plasticity. Dopamine (DA) neurons degenerate in AD during the disease progression, and DA supplements show favorable neuroprotective effects in patients. The excitatory neurotransmitter glutamate can have neuroprotective/neurotoxic effects and is altered in the brains of AD patients. In the case of gamma-aminobutyric acid (GABA), studies show contradictory results on whether it is decreased in AD. What could be shown is that dopamine, glutamate, and GABA play an essential role in memory and neurodegeneration in AD patients and AD models [52].

AD patients show cognitive decline primarily due to a lack of acetylcholine synthesis, making this a cornerstone neurotransmitter hypothesis for the AD [53].

Therefore the last one of the three corner-stone hypotheses for Alzheimer's Disease presented here is the cholinergic hypothesis. Upon cholinergic neuron death, a decrease of the neurotransmitter acetylcholine (ACh) seems to relate to deficits in memory and cognition in AD, and increasing cholinergic tonus with choline agonists may improve memory performance. Alterations of the cholinergic system due to antagonists of cholinergic receptors induce memory deficits in the same tasks as in aged individuals [54].
There are two AD models for the cholinergic hypothesis: the pharmacological (nicotinic muscarinic antagonists) and the lesion models. Latter can be subdivided into non-specific and specific[55].
Non-specific means lesions, e.g., to the fornix. This shows disruption in idiothetic navigation in rodents. However, these lesions are not related to lesions found in AD.
The specific ones consist of damage to the cholinergic basal forebrain. Rats with such damages were exposed and tested in a radial and water maze. However, memory deficits in such rats were limited [56].

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Chapter II

Protein aggregation and quality control in health and disease

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Proteins are critical macromolecules that play vital roles in maintaining cellular structure and function, but their ability to do so relies on correct folding. However, under certain conditions, proteins can misfold and aggregate, forming misfolded forms and/or insoluble protein aggregates. Protein aggregation is a complex phenomenon that can have profound implications for cellular health and disease. Therefore, protein homeostasis, which ensures the proper folding, function, and degradation of proteins, is essential in a complex and crowded cellular environment. This involves molecular chaperones, which play a key role in *in vivo* folding as well as trafficking, disaggregation, and degradation of protein machinery [1, 2]. In numerous human diseases characterized by defective protein folding or aggregation — referred to as proteinopathies or protein folding disorders — dysfunction arises from the accumulation of misfolded proteins. In Alzheimer's disease, for example, these proteins self-assemble into amyloid fibrils and other toxic conformers. This chapter will explore the principles of protein folding and stability, examine the mechanisms of protein misfolding and amyloid aggregation, and provide an overview of the cellular protein quality control systems that counteract these processes. Alzheimer's disease will be highlighted as a case study of a protein-folding disorder characterized by aggregation. The role of chaperone networks in preventing amyloid-beta and tau aggregates will be discussed, with a particular focus on the S100 family of chaperones, especially S100B, which is central to this thesis.

1. Protein Folding and Stability: a brief introduction

The correct folding of most proteins is vital for the cell and, therefore, for the organism [3]. Most proteins require folding into their native state to be biologically functional. Aberrant folding is prone to lead to toxic species formation [4]. Therefore, a tight regulation of protein homeostasis, a balance between folded and unfolded proteins [5], is key for cellular function and tissue maintenance [6]. In this section, we will go from early research, which lays the fundamentals for protein folding, to protein folding and aggregation and finalize with mechanisms of amyloid formation.

1.1 Principles of Protein Folding

In the history of protein folding research, the following experiments from Christian Anfinsen and Cyrus Levinthal crystallized themselves as pivotal for protein aggregation.

In 1961 [7] Anfinsen experiments showed that in a non-crowded environment, proteins contained all the information required to fold to attain the native state (the minimal energy configuration) in the amino acid sequence [8]. This would give a protein enormous conformational "exploration" space. Considering that folding is partly a matter of milliseconds, Levinthal proposed that a slight preference towards the native state would allow rapid, local amino acid interactions, limiting the protein to fold, following a funnel-like energy landscape to attain its most stable conformation state [9] [10] (Fig. 1).

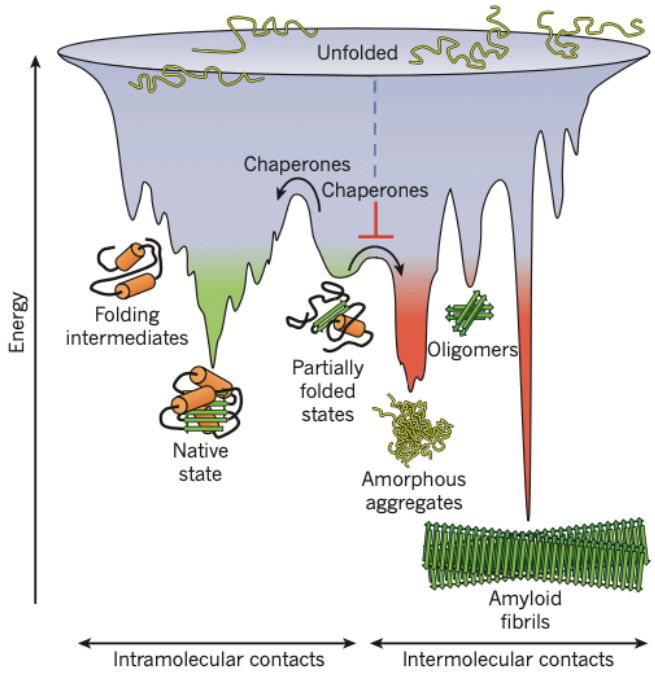


Fig. 1: Funnel-like energy landscape. Proteins attain, some with the aid of chaperones, their most stable conformation state. (From [4])

The amino acid sequence dictates the balance of thermodynamic (this still being related to Anfinsen), and interatomic forces that lead to the protein structure and kinetics, the folding routes, and pathways for a protein to go [11] from primary structure (the amino acid sequence) to secondary, tertiary, and for some quaternary structure [12].

Proteins may fold by first undergoing a rapid hydrophobic collapse, which restricts the conformational dynamics, leading to a globule in search of its native contacts or a misfolded state stabilized by non-native interactions. Folding intermediates are not rare for proteins larger than 100 amino acids. Since the free-energy landscape (depicted here as a funnel) often bears significant kinetic barriers for the folding protein [4]. At this point it is important to

mention the differences between a protein folding *in vitro* like Anfinsen's experiments for instance and the ones folding in the cell. The following table lists main differences between both (Table 1).

Differences	***In vivo***	***In vitro*** (Small, single domain protein)
Folding	Upon biosynthesis Environment: ribosomes, associated enzymes and chaperones Folding of chains: possibly co-translational	Unfolded ensemble of full-length/ one polypeptide Environment: folding conditions given
Time spend in the unfolded state	Unclear	Thermodynamic stability dictates dynamic equilibrium
Conditions to fold	High macromolecule concentrations, highly interactive surfaces all around	Highly diluted
How does it fold	Many fold with the help of chaperones	Folds on its own
Vulnerability intermolecular aggregation reactions with which to competing	Difficult to translate from in vitro studies	Correlated to aggregation prone species present in the test tube
Organization of folding reactions	Spatially	Difficult to mimic in in vivo studies

Table 1: Differences between *in vitro* and *in vivo* protein folding [13].

In this regard, it is important to mention that for instance an *E.coli* proteome has less than 15% of proteins that may fold on their own. This underlines the importance of *in vivo* studies and shows how crucial several *in vivo* aspects like the chaperones or the environment of the folding proteins are, so that the protein does not misfold or aggregate.

1.2 Protein Misfolding and Aggregation

Initial factors that may lead to protein aggregation include temperature, stress (mechanical as well as oxidative), pH and mutation [14]. On the protein level, a transient state of an unfolded

or native protein or an intrinsically disordered region of a protein, may lead to aberrant folding. When the concentration of these misfolded monomer is high enough, aberrant self-assembled oligomers form. There are non-toxic off pathway oligomers and toxic oligomers. Latter have spherical or doughnut-like morphology and aggregate further into insoluble, fibrous protein aggregates. Characteristically, those oligomers show in terms of secondary structure, β-loop-β folds, while mature fibrils are additionally rich in β-sheet structure (Fig. 2). These protein species can be further distinguished by immunological epitopes e.g. by the OC conformational antibody which recognize fibrillar elements excluding pre-fibrillar oligomers [14]. A table showing characteristic structural differences between oligomers and fibirls can be found below (Table 2).

Mature fibrils show how many amino acids can lead to a very similarly structured "end-product" (the mature fibril). This having the characteristic of non-covalent interactions between mainly the beta-sheets, leading to the formation of robust and usually unbranched fibrils by monomers [15].

	Oligomers	**Fibrils**
Hydrophobic surfaces	exposed	hidden
Size	small, diffuse easily	bigger, not so mobile
Open active sites	more	less
Stability, organization	Low, low	High, higher

Table 2: Structure of oligomers and fibrils in comparison – potential reasons for their differing level of toxicity [14].

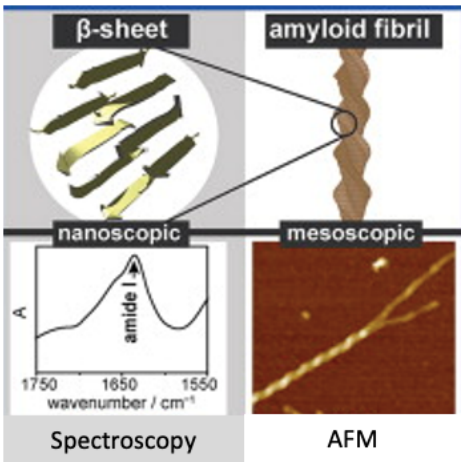


Fig. 2: Mature fibril. First row shows a mature amyloid fibril on the left and an amplified section of β-sheets on the right. On the bottom row corresponding experimental data are shown (Image modified from [15])

In the context of Alzheimer´s Disease, toxic gain of function aggregates may appear at the latter stages of life [16]. The buried protein patches of misfolding protein, become solvent exposed and lead to an elevated level of affinity to similar structures and, therefore, an increased risk of self-aggregation due to the formation of β-sheet structure. The β-sheet motif allows aggregation of elevated numbers of intermolecular aggregates. This agglomeration of protein aggregates leads to size heterogeneous protein aggregates classified more liberally into categories from oligomers (dimers-24mers) until fibrils (characterized by long, strait, unbranched structures, in the μm range in length and characteristically bind certain dye molecules such as thioflavin T) [17].

1.3 Mechanisms of Amyloid Aggregation

In fact, protein amyloid aggregation is a complex process that involves the self-assembly of proteins into insoluble aggregates, starting with primary nucleation. This initial nucleation phase is time-consuming. The macroscopic aggregation process is typically represented by a sigmoidal curve, which includes a lag phase, a growth/elongation phase characterized by the highest conversion rate into fibrillar mass, and a plateau phase, as illustrated in Figure 3. Therefore, it probably is not the main source of toxic species formation e.g. in AD. Secondary nucleation is the catalytic pathway oligomers undergo on the surfaces of already-formed fibrillar aggregates. In the context of AD, the rate of Aβ aggregation increases significantly through this secondary nucleation because this catalytic surface provides the

possibility of a constant generation of newly formed oligomer species, closing a circle between monomers and fibrils. Interfering with this catalytic pathway on the fibril surfaces is, therefore, of especial interest in AD [1].

On a microscopic scale, rate constants for fibril nucleation, fragmentation, and elongation can be determined [18]. The nucleation, or lag phase, is a self-assembly of monomers via aggregation-prone regions [19] into aggregates. This can then transition into a growth and elongation phase through fibril extension [20].

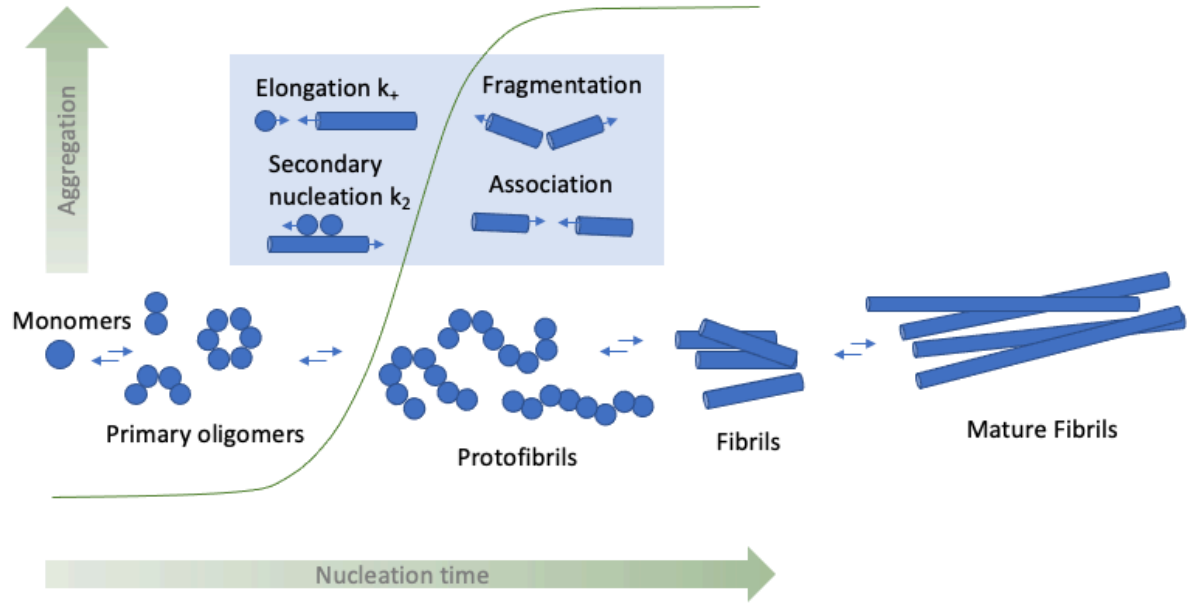


Fig. 3: Schematic representation of Aβ aggregation along nucleation time. After a (flat) lag-phase, with mainly Aβ oligomers, follows a growth phase. Elongation, fragmentation, secondary nucleation, and association occur during the growth phase. The following (again flat) plateau phase is mainly characterized by mature fibrils [21] [22].

Further, fibrils are formed by association of unfolded segments, namely the aggregation-prone regions [23]. Aggregation-prone regions are encoded in the primary sequence and usually constitute a short sequence segment of 5-15 amino acids [24], high hydrophobicity, low net charge, and a high tendency to form β structures [25] [26]. This goes hand in hand with amyloid fibrils from proteins associated with varying diseases forming a common cross-

β spine. The cross-β spine varies in steric zippers: residues of two neighboring β-sheet layers which are tightly interdigitated [27].

2. Protein Folding in Cells and Protein Quality Control Systems

2.1 Overview of Proteostasis

In the cell, protein is synthesized on ribosomes and the genetic information encoded in the DNA is transcribed. Folding may occurs in parallel. Other forming proteins fold in the cytoplasm or other compartments of the cell. Incompletely folded proteins, expose hydrophobic regions to the crowded environment of the cell, being prone to undergo inappropriate interactions [28].

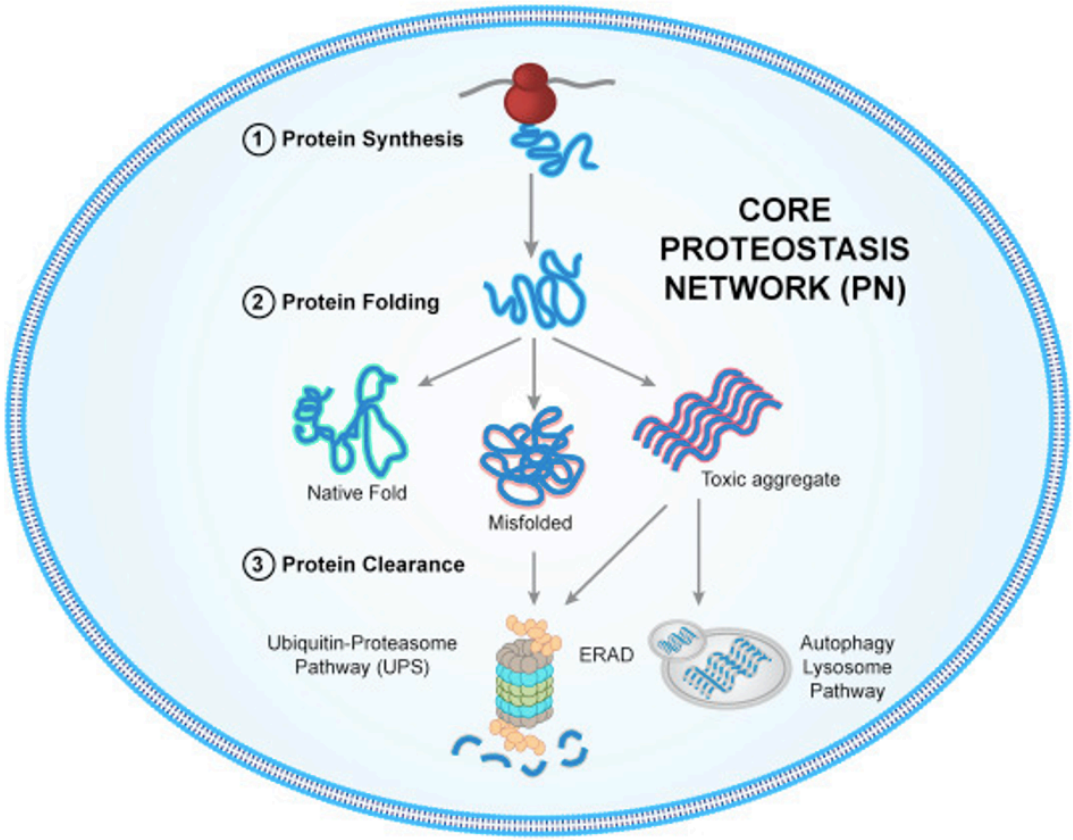


Fig. 4: The proteostasis network (PN) is directly involved in protein synthesis, folding, disaggregation or degradation. The PN is constituted of the machinery involved in translation, molecular chaperones and aiding factors, the ubiquitin-proteasomal system as well as the autophagy machinery [29]. (Image from [30])

Moreover, many proteins assemble into complexes and are modified by cleavage; many component-attachments are also decisive for protein localization [8]. In the crowded cell environment, molecular chaperones aid in the folding process. For some, their binding is crucial to stabilize proteins and allow/facilitate the folding process [8].
Still misfolded, aggregated, or damaged protein for instance, are degraded by the ubiquitin-proteosome system (UPS) and the autophagy-lysosome machinery [31] (Fig. 4).

2.2 Molecular Chaperones (including Extracellular Chaperones)

With the aid of molecular chaperones, the degradation machinery, and their activation factors, the cell regulates protein folding [32] in a time-efficient manner [16], keeping the protein in its native state and degrading it when due [32]. This quality control mechanism recognizes, for instance, upon an accumulation of unfolded proteins, the polypeptides [6] by their exposed hydrophobic residues [33] and a preliminary assessment of urgency and route of how to handle the mis/unfolded protein follows [6]. Depending on the amino acid sequence, changes in the folding of the amino acid sequence may lead to changes in its energy landscape: the shape acquired and the molecules it interacts with may change [34]. Still, the folding process and protein stability depend not solely on the catalytic activity of a molecular chaperones and the quality control system but also on the type of protein. There are stable and inherently unstable proteins. Since the latter is in the native state, when alternative energy is minimal. Proteins with alternative energy minima lead to confirmations that may have non-toxic functions for the cell. In case of protein misfolding however, in the crowded cell environments, previous events can lead to severe and wide-reaching perturbations [35]. To prevent that, molecular chaperones aid in folding by interacting, stabilizing, or helping a protein, but without making part of that protein once folded [4]. Molecular chaperone subgroups are involved in *de novo* folding, refolding posterior to environmental stress, and trafficking of other proteins, to mention some of their functions [36]. Chaperones of *de novo* protein folding and refolding, usually require a cycle of ATP or co-factor binding and release like e.g. HSP70. However, some may show functional cooperation with molecular chaperones independent of ATP-binding like small HSPs which have a ‘holdase’ effect [4] (Fig. 5).

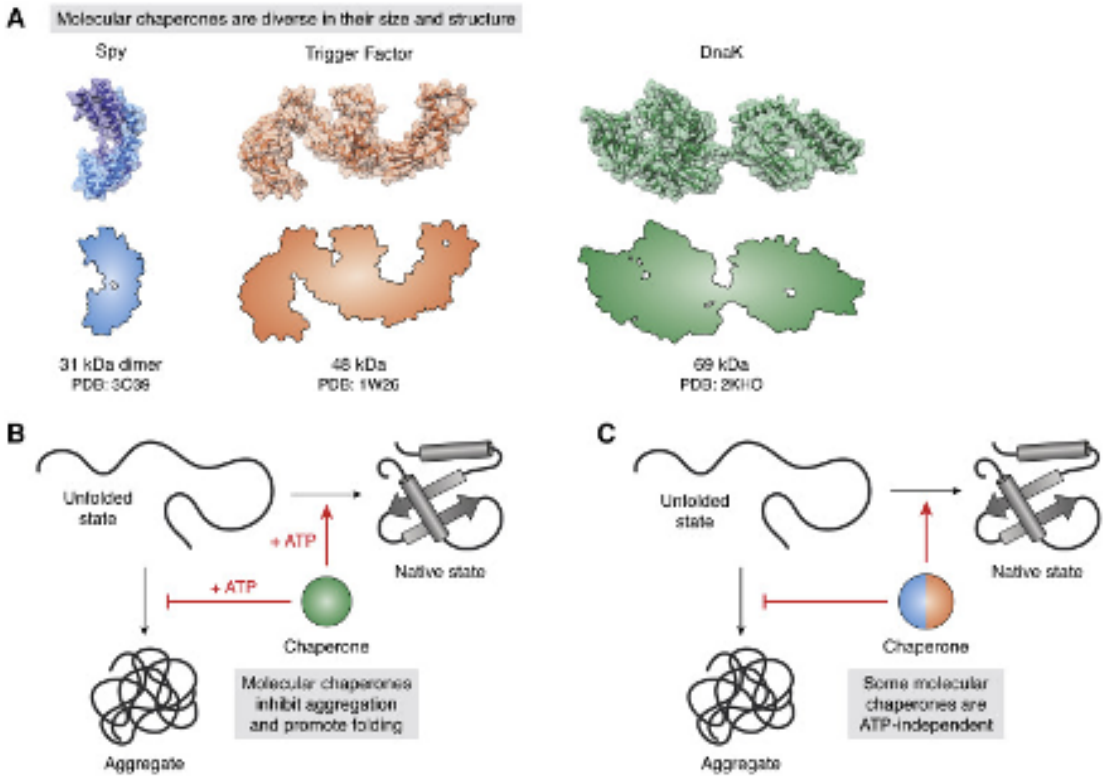


Fig. 5: (a) Different molecular chaperones with their size and structure and (b) Schematic ATP dependent and independent facilitating, of a folding process by a molecular chaperone (Image from [37] modified).

Molecular chaperones are usually classified by their molecular weight [4]. They can for instance be grouped in the following five chaperone families: HSP100, HSP90, HSP70, HSP60 (Group I and II) and sHSP. To give brief examples, HSP100 facilitates the unfolding of misfolded polypeptide or aggregated protein [38]. HSP90 and HSP70 are more classical in their function. They are highly abundant proteins and bind ATP. ATP hydrolysis then results in substrate binding. This process may occur several times before the client protein is fully folded. HSP60 chaperones play an important role in mitochondrial proteostasis among others and mammalian small HSP (sHSP) are all cytosolic, ATP independent, and facilitate the refolding of HSP70 client proteins for instance [29]. A more precise table of the different chaperones, their topology of binding, co-chaperones and known functions can be found below (Table 3).

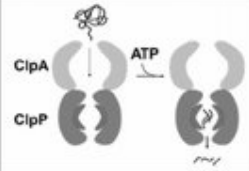
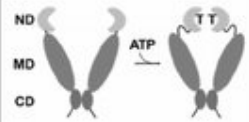
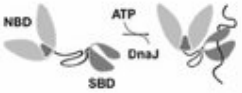
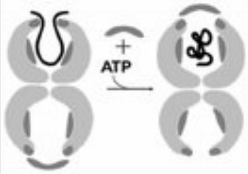
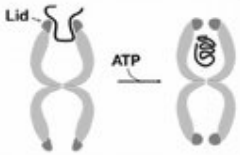
Chaperone Family	Topology of Binding	Co-chaperone	Known Function
Hsp100		ClpP, SspB, Hsp70, Hsp40	• Works with DnaK in ATP-dependent disaggregation and proteolysis • Prevents aggregation, degradation and turnover of unassembled mitochondrial proteins • Reactivates heat-damaged proteins • Establishes and maintains prion phenotype in yeast
Hsp90		Hop, Hip, Hsp70, Immunophilins, Grp78	• Refolds proteins in stressed cells. Probable secretory chaperone in prokaryotes • Major cytosolic chaperone in eukaryotes. • Cytoprotection and intracellular signaling • In ER, controls protein homeostasis, folding and assembly of secretory proteins
Hsp70		Hsp40, GrpE	• Ubiquitous Principal folding chaperone • Works with ClpB as disaggregase • Folding of newly synthesized proteins • Protein transport into ER and mitochondria
Hsp60 (Group I)		Hsp10	• Major chaperone for protein folding in prokaryotes • Stabilizes proteins during heat stress • Promotes folding of over-produced proteins • Major chaperone in mitochondria and chloroplast
Hsp60 (Group II)		Prefoldin/GimC	• Promotes folding of a cytosolic proteins in eukaryotes • Refolding of unfolded polypeptides in vitro
sHsps		-	• Stabilizes unfolded polypeptides • Prevents aggregation • Works with Hsp70 in protein refolding • Structural protein of eye lens

Table 3: Chaperone families, topology of binding, co-chaperones and their known function. (Image from [39])

Extracellular chaperones are crucial in maintaining protein homeostasis outside of cells, facilitating the proper folding and preventing aggregation in secreted proteins [40].

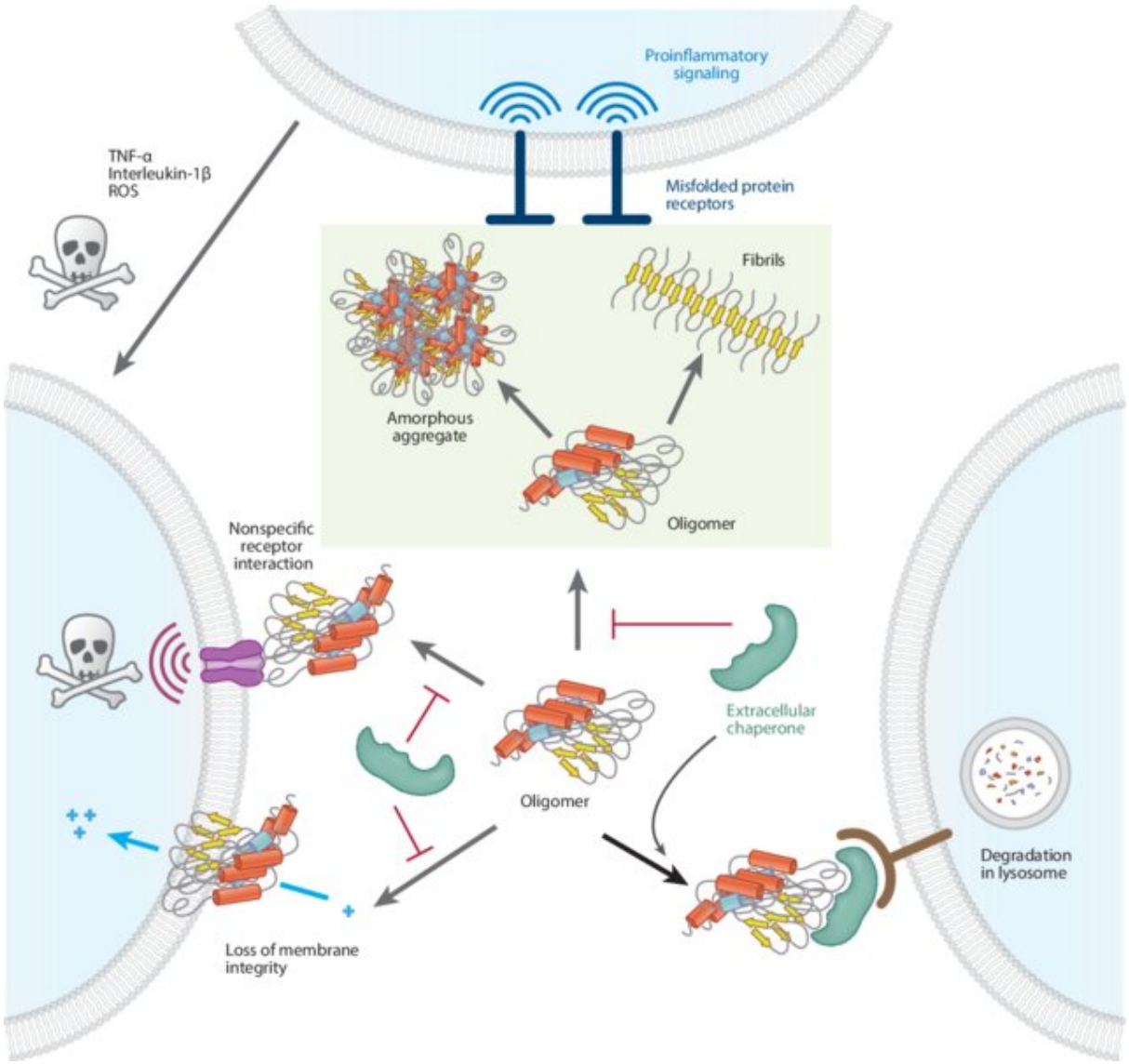


Fig. 6: Likely cytoprotective effects of extracellular chaperones in cellular processes involving misfolded extracellular proteins (Image from [41])

These chaperones facilitate the transport of newly synthesized proteins to their destinations while ensuring that they attain their functional conformations. Examples of well-known extracellular chaperones include clusterin, which is involved in lipid metabolism and neuronal protection, and alpha-2-macroglobulin, which is an example of a molecular chaperone playing a role in immune response and inflammation [2] (Fig. 6).

As indicated previously, recognition of non-native proteins is done by chaperones mainly via hydrophobic patches that are exposed on the surface [42]. Molecular chaperones increase the

efficiency of binding reactions upon protein folding by decreasing the probability of nonnative binding reactions occurring [28]. Is that not enough, amyloid disease may surge.

The proteins that aggregate in amyloid diseases vary from disease to disease, and the localization of the protein aggregates may be inside or outside the cell. However, amyloid disorders share the aggregation pathway of proteins aggregating in each disease, i.e., different proteins have similar aggregation pathways.

2.3 Chaperones in amyloid neurodegenerative diseases

Chaperones are critical players in maintaining protein quality control, and their significance is evident in various diseases associated with protein folding and aggregation, as extensively reviewed elsewhere. Several human diseases, known as protein folding disorders, arise from defects in protein folding and may involve misfolding or the formation of toxic protein aggregates, including Alzheimer's, Parkinson's, and Huntington's diseases. This section briefly overviews the evolving research on conventional molecular chaperones and their regulation of amyloid-beta and tau accumulation in the diseased brain. It serves as a primer for S100B, a newly identified molecular chaperone relevant to Alzheimer's disease, which is the focus of this thesis (see Chapter 3)

The heat shock protein chaperones (HSPs) in AD are under oxidative stress conditions, due to mitochondrial dysfunction and increasing levels of Aβ and tau. In this context, they have shown capable to directly prevent Aβ and neurofibrillary tangle (NFT) aggregation for instance. HSP70 has been shown able to reduce NFTs. HSP60 and HSP70 for instance exert neuroprotective functions. HSP70 in conjunction with HSP40 and HSP90 have shown to negatively influencing the formation of Aβ aggregates. HSP90 may further, trough microglia phagocytosis activation, lead to Aβ degradation. While Aβ in the AD context may influence chaperone expression [43].

An overview of diverse molecular chaperones that can interfere with $A\beta_{42}$ aggregation by a variety of microscopic mechanisms is presented in the figure below (Fig. 7)

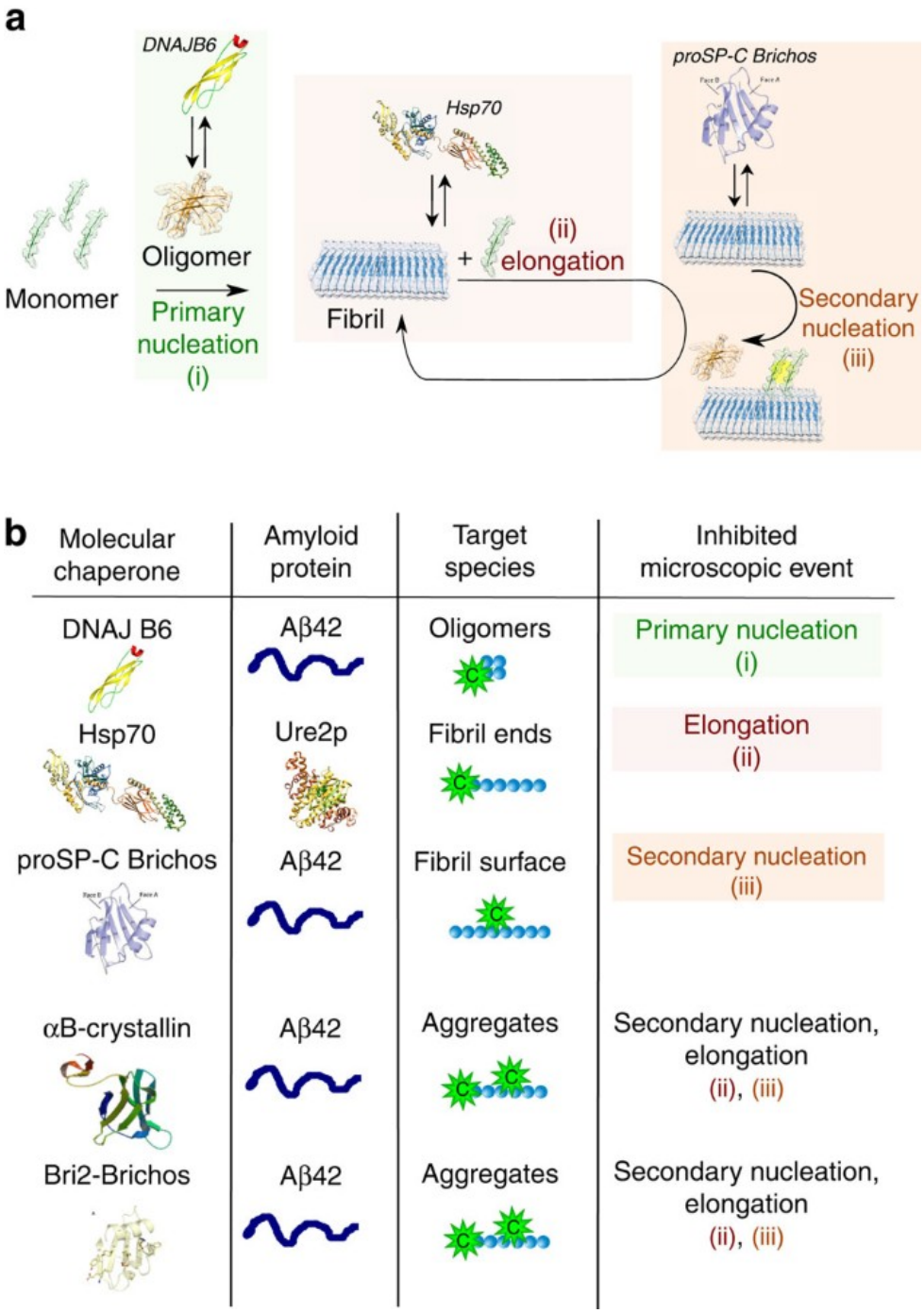


Fig. 7: Overview of diverse molecular chaperones that can supress $A\beta_{42}$ aggregation by a variety of microscopic mechanism. (Image from [44])

Understanding the molecular mechanism of $A\beta_{42}$ suppression by a molecular chaperone is challenging as it may interfere at any time step (e.g., primary nucleation, elongation, or secondary nucleation) in the folding process, and detailed attention has to be given to the specific chaperone-protein interaction, (in the case of Aβ) peptide interaction.

The molecular chaperone Bri, for instance (its 100 amino acid long protein domain Brichos), has been shown to interfere with the active catalytic site and interrupt the catalytic cycle of $A\beta_{42}$ secondary nucleation [1]. Prevention of $A\beta_{42}$ fibril maturation could also be shown for the molecular chaperone DNAJB6 of the heat shock protein 40 (hsp40) family [45].
Another protein introduced as molecular chaperone is S100B of the S100 protein family. S100B may act as a chaperone possibly able to bind and delay $A\beta_{42}$ aggregation [46]. And with the conditions given in the Alzheimer's Disease brain, it may even show potentiated capacity to do so. How, we will see further in chapter 3.

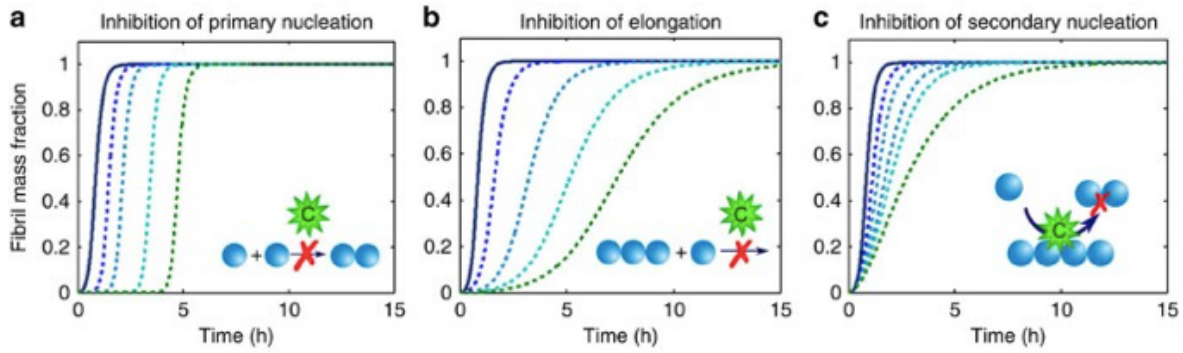


Fig. 8: Global kinetic profiles of microscopic aggregation events: Here represented is quantitative information on the interference of a molecular chaperone (green c) on microscopic events (primary nucleation, elongation, secondary nucleation, respectively) (Image from [44])

How to tackle the underlying mechanism? With kinetic studies, one may obtain global kinetic profiles that may shed light on the interaction mechanism. For that, dyes like the hydrophobic Thioflavin T may be used to track the increase in fibrillar mass [47].
One example of such a kinetic study is the kinetic profiles represented above, which give insight into the time step in microscopic events where the molecular chaperone intervenes (Fig. 8). In inhibition of primary nucleation, one can observe a slight delay (right shift) in protein/ peptide aggregation starting time, which means that the molecular chaperone, concentration-dependently, inhibited primary nucleation, i.e., the farther to the right the curve is, the higher was the chaperone concentration that was added [44] (Fig 8a).

Overall, much remains to be understood about the role of extracellular chaperones in Alzheimer's disease. The next chapter will provide an overview of the recently uncovered chaperone activity of S100B, an abundant protein in the brain that is upregulated under conditions that exacerbate Alzheimer's. S100B has been shown to interact with amyloid-beta, potentially modulating its aggregation and toxicity. This dual role of S100B as both a chaperone and a marker of neuronal stress makes it particularly relevant to understanding the mechanisms of Alzheimer's disease and will be the central focus of this dissertation.

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Chapter III

S100 Proteins in Alzheimer's Disease and their potential Regulation through Oxidation

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S100 proteins are emerging as critical players in the pathophysiology of Alzheimer's disease (AD), offering insights into the underlying mechanisms of neurodegeneration. These small, calcium-binding proteins are predominantly expressed in the brain and implicated in various cellular processes, including neuronal growth, differentiation, and inflammation. In the context of AD, S100 proteins, particularly S100B, have been shown to interact with amyloid-beta and tau, potentially influencing their aggregation and toxicity. Additionally, changes in the redox status of S100 proteins, particularly changes in their oxidation state, may modulate their functional roles, affecting neuroinflammation and synaptic function. This chapter overviews S100 proteins expressed in the brain and those implicated in AD, notably S100B, which has been recently discovered to have novel protective properties as a chaperone that can counteract amyloid aggregation. However, since in AD, protein aggregation occurs on a background of increased oxidative stress, and since S100 proteins are prone to regulation by oxidative modifications, this aspect will also be discussed.

1. The S100 Protein Family

1.1 Structural and Functional Properties.

S100 proteins are a family of small, calcium-binding proteins characterized by their EF-hand motifs, which allow them to bind calcium ions. The name S100 originates from the fact that the "S100 proteins" are soluble in 100% saturated ammonium sulfate solution at neutral pH [1]. These proteins are exclusively expressed in vertebrates, and the family comprises (at least) 24 members that may have extracellular, intracellular, and extracellular or predominantly extracellular effects. Their location of expression may change depending on the pathological conditions of the organism [2]. Structurally, they typically form dimers, although some can exist as tetramers, and they exhibit a high degree of conformational flexibility upon calcium binding (Fig. 1). This conformational change enables S100 proteins to interact with various target proteins, modulating processes like inflammation, cell growth, and cytoskeletal dynamics [2]. In addition to calcium, some S100 proteins can bind zinc and copper, further influencing their structure and function.

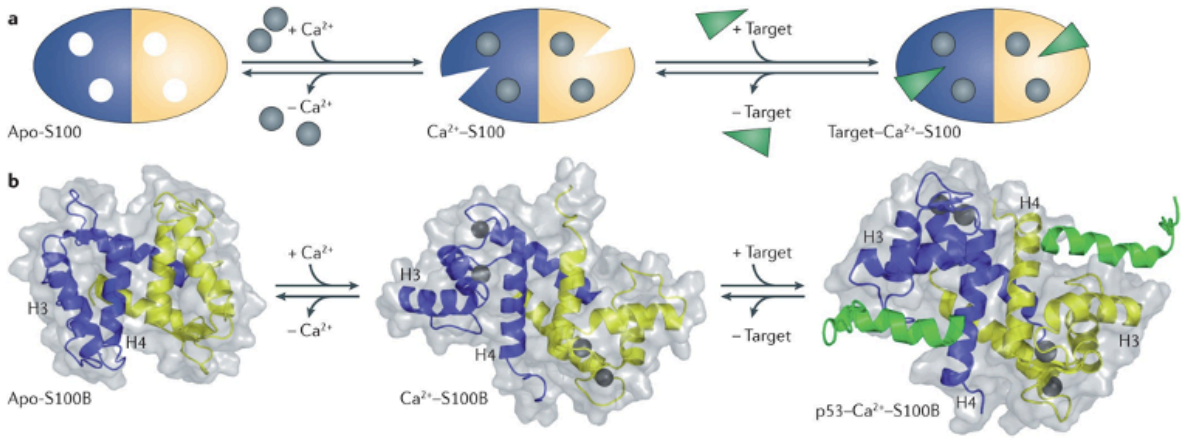

Fig 1.: S100B protein structure. From left to right, **a** and **b** show a scheme of, and apo-S100 protein that upon Ca^{2+} binding changes conformation and is able to bind several target proteins (green). (From [3])

Their structural versatility is key to their role in diverse cellular processes, and S100 proteins are implicated in multiple cellular functions e.g. Figure 2.

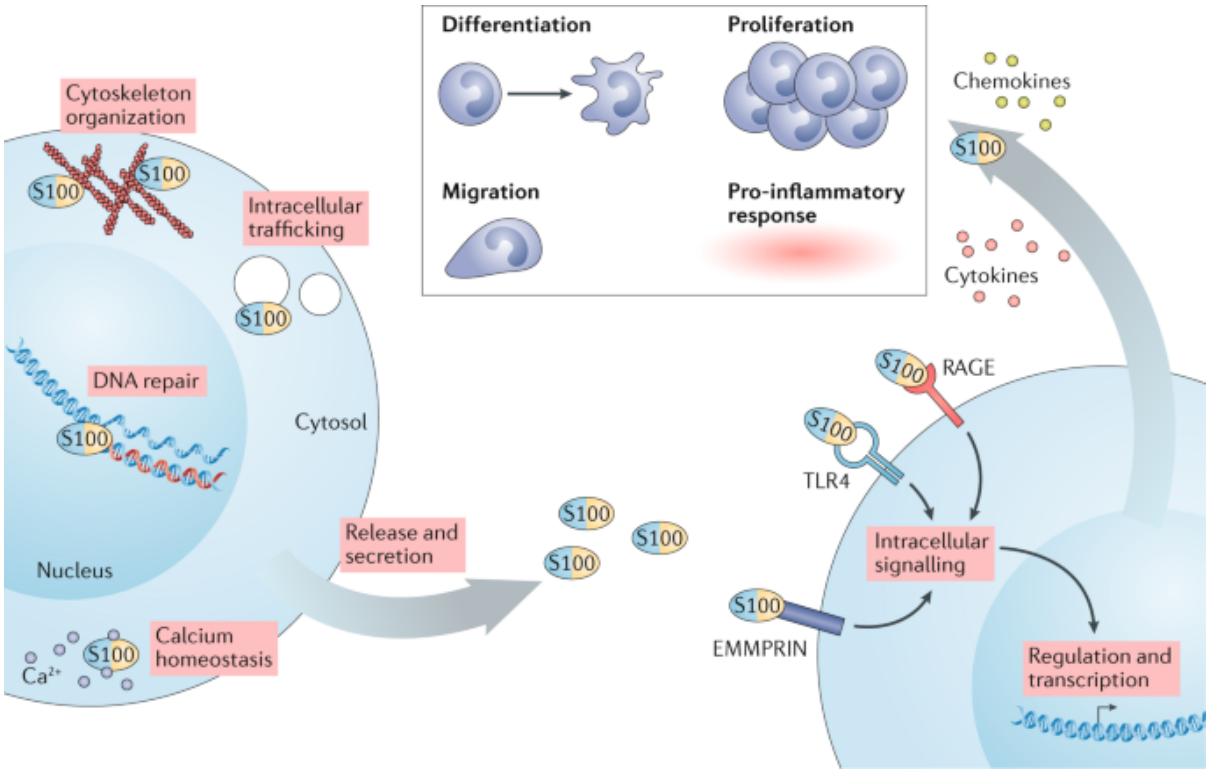

Fig. 2: Versatility of S100 functions. S100 proteins play a role in differentiation, proliferation, migration and are involved in pro-inflammatory response for instance. (From [4])

1.2 Impact of S100 Oxidation on Protein Function

As part of the post-translational modifications, it was hypothesized that oxidation may modify S100 proteins e.g. S100A8 [5] or S100A1 [6] regarding ion-binding properties and target protein interaction dynamics within the cell translocations and functions in the extracellular milieu [5]. Susceptibility may be enhanced for S100 proteins undergoing structural changes upon Ca^{2+} and Zn^{2+} binding [5].

Given the potential modifications that may occur upon oxidation (Table 1), the role of S100 proteins as solely pro-inflammatory agents remains controversial. Increasing evidence suggests that oxidative modifications of S100 proteins also regulate anti-inflammatory activity. Specifically, sulfur-containing cysteine and methionine residues are particularly reactive in proteins for redox modifications [7], [8], [9]. In this context, protein oxidation — particularly methionine oxidation — has been associated with the reduction or elimination of biological activity [10].

Oxidative modification	Definition	Reversibility of oxidative modification	Protein residue modified	Reference
S-nitrosylation	Addition of an NO group to the thiol side chain	Reversible	Cysteine	[11]
S-glutathionylation	Disulfide bonds formed between glutathione (a tripeptide) and cysteine residues	Reversible	Cysteine	[12]
Protein disulfides	Covalent linkages between thiol groups of two cysteines	Reversible	Cysteine	[13]
Methionine oxidation	Oxidized to a mixture of two diastereomers: methionine-S-sulfoxide and methionine-R-sulfoxide	Reversible	Methionine	[14]
Carbolylation	Addition of a carbonyl group to the side chain of an amino acids residue	Irreversible	including histidine, lysine, proline, threonine and cysteine	[15]

Table 1: Protein oxidative modifications.

2. S100 proteins in the brain and Alzheimer's disease

S100 proteins have been implicated in key functions in the brain and AD, notoriously S100B, which are discussed in detail later in this chapter. However, while it plays essential roles in normal brain function, in AD S100B is involved in both protective and neuroinflammatory functions. However, other S100 proteins, such as S100A1, S100A6, S100A7, S100A8, S100A9, and S100A12, have also been associated with AD (Fig. 3).

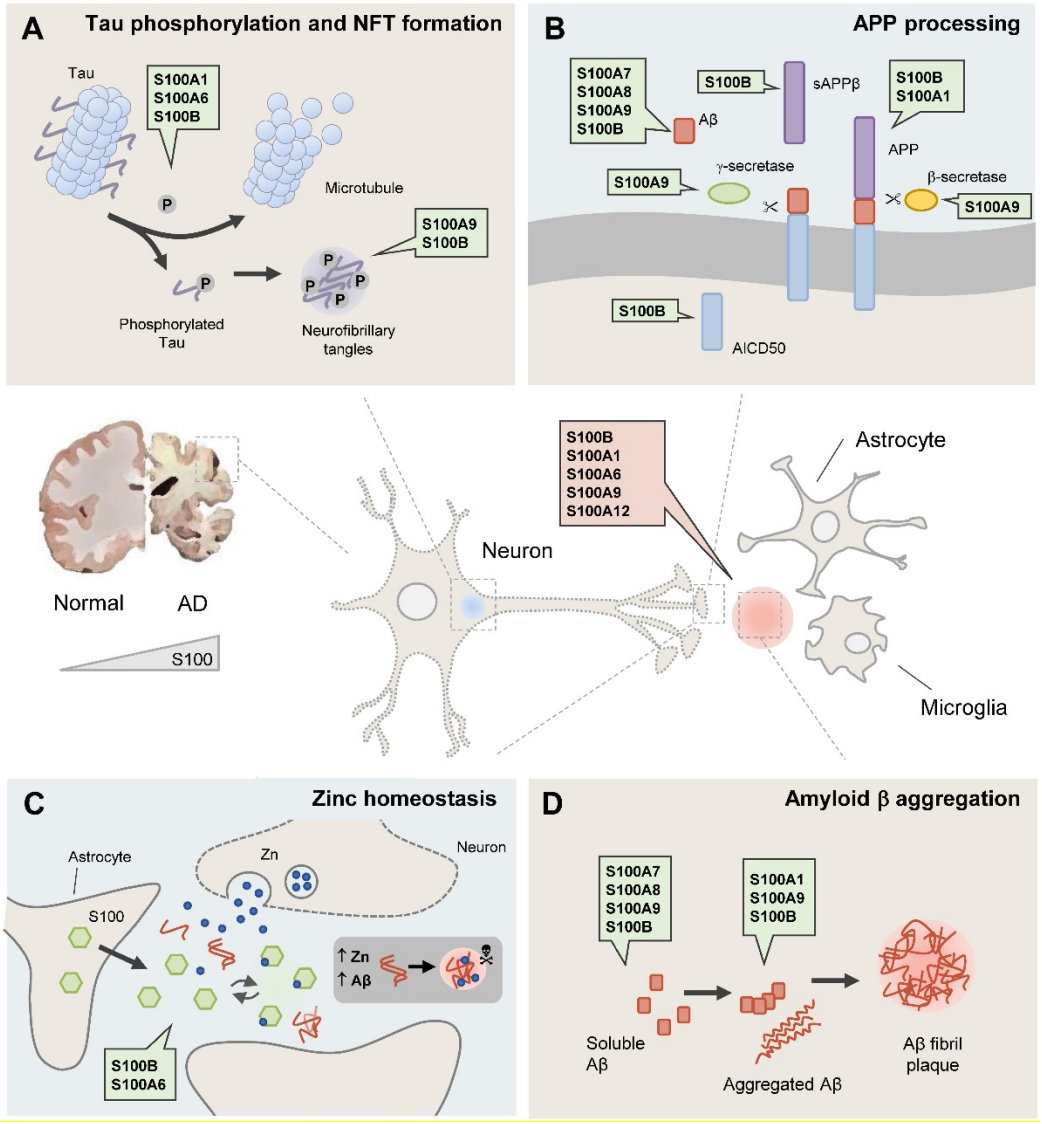

Fig. 3: S100 protein involvement in main processes associated with Alzheimer's disease. The central image presents a neuron, an astrocyte and a microglia and the extracellular space between them. Left to right, the four images are processes associated with Alzheimer's Disease that occur in the indicated regions of the central image, namely tau phosphorylation

and NFT formation, APP processing, Zinc homeostasis and amyloid beta aggregation in which the different S100 proteins play a role. All leading to brain cell death indicated by the shrunken AD brain half on the center, left. (From [16])

As depicted in Figure 3, this includes involvement in tau phosphorylation and NFT formation by S100A1, S100A6, S100B, S100A9, APP processing by S100A1, S100A7, S100A8, S100A9 and S100B, Zinc homeostasis by S100A6 and S100B and amyloid beta aggregation namely by S100A1, S100A7, S100A8, S100A9 and S100B.

To understand where S100 proteins are expressed and their functions, a few examples are presented. S100A1 is expressed in the cytoplasm of specific neuronal populations, which is found in association with the mitochondria to give an example [2]. S100A6 is only expressed by neurons, some limbic system nuclei, and some astrocytes. Upon normal aging, a slight upregulation of S100A6 could be verified [17]. S100A7 may interact with RAGE to activate NF-kB, leading, for instance, to the production of cytokines and the migration of neutrophils and macrophages [18]. S100A8 constitutes ca. 20% of the cytoplasm of neutrophils and is found in the nucleus of some cell types. S100A8 can be induced in macrophages and other cells by pro-inflammatory stimuli. S100A8 is, for instance, implicated in myeloid cell differentiation [2]. S100A9 functions include inhibition of differentiation (of myeloid cells) and different modify their phenotype. S100A12 is expressed in neutrophils and is inducible in macrophages, among others. For instance, S100A12 may also modulate cytoskeletal and membrane interactions [2]. Later in this chapter, we will elaborate on the role and function of these S100 proteins relevant to AD and what is known about them in their oxidized states.

2.1 S100 Proteins and Oxidative Stress

Oxidative stress is a key factor in the pathogenesis of Alzheimer's disease (AD), contributing to neuronal damage and protein aggregation (Fig. 4).

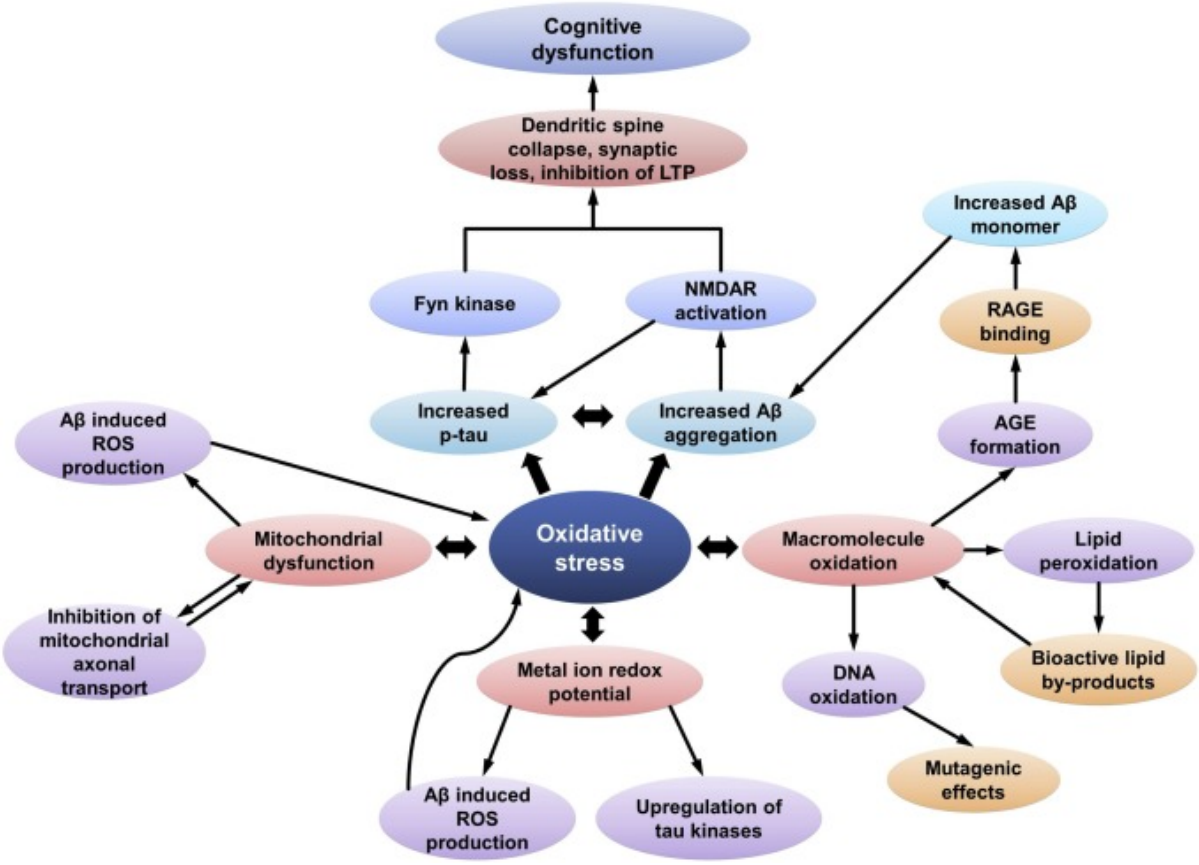

Fig. 4: Oxidative stress contributions to Alzheimer's Disease (From [19]).

Elevated levels of reactive oxygen species (ROS) in the AD brain disrupt cellular homeostasis, leading to oxidative modifications of proteins, lipids, and DNA [20]. Among the proteins affected are S100 proteins, particularly S100B, which play roles in neuroinflammation and calcium regulation and undergo functional changes under oxidative conditions. [20]. An intriguing question arises: could oxidized proteins act as additional antioxidants? This is one of the key questions addressed in this chapter. S100B has been shown to play a crucial role in AD, contributing to regulating tau and Aβ aggregation, Notably, S100B has also been shown to play a role in neuroinflammation, and the switch between its pro-inflammatory and anti-inflammatory effects may depend on its oxidation

state. Further, increased oxidant production due to a neuroinflammatory reaction to protein aggregation may increase S100B anti-aggregation activity. Thus, S100B, in its oxidized form (S100Box), may represent a critical link between protein aggregation and neuroinflammation in AD. To explore this possibility, the following sections will review the current knowledge on oxidative modifications of S100 proteins expressed in the nervous system.

2.2 Brain S100 Proteins and Their Oxidatized Forms

The study of oxidized S100 proteins remains in its early stages, though there is evidence linking their roles to AD, especially given the elevated oxidative stress in the disease. This chapter includes all S100 proteins tied to AD [16], [21], [22], [23], [24], even if oxidation has not been tested, as non-oxidized forms provide insight into potential oxidized functions. Results on oxidized S100 proteins are discussed in a historical context to show the progression of research over time. This section addresses the S100 proteins relevant to Alzheimer's disease — S100A1, S100A6, S100A7, S100A8, S100A9, S100A12, and S100B — as well as their oxidized forms (S100A1ox, S100A6ox, S100A7ox, S100A8ox, S100A9ox, S100A12, and S100Box). Additionally, we will consider S100 proteins that are oxidized but not yet linked to AD despite being expressed in the brain, such as S100A2, S100A4, and S100P. Given the scarcity of research on oxidized S100s, the following results are presented chronologically, highlighting gaps in the current understanding.

S100A1

S100A1 is highly expressed in the brain [25]. That is to this date, is not directly linked to AD. However, S100A1 may have an indirect involvement. Ryanodine receptors are altered in terms of expression level in AD [26], [27] since cellular processes, like the interaction with ryanodine receptors in the brain, are its primary target protein [28].

Another interaction partner of S100A1, that ties S100A1 to AD for its involvement in AD inflammation and oxidative stress induction [29] is the receptor for advanced glycation endproducts (RAGE), which is expressed abundantly in various cell types [30]. Moreover, S100A1 is involved in tau phosphorylation and APP expression [28] as well as Aβ production [27]. Regarding the interactions of these proteins and factors that may influence these interactions, the oxidation of the protein will be considered next.

S100A1ox has been suggested to be a link between the most important cell signaling pathways: calcium and redox [31], two signaling pathways altered in AD [32];[33]. This thesis was supported by experiments carried out in the last two decades. Zhukova *et al.* in 2004 could demonstrate cysteine-based redox modifications for S100A1 namely, the following ones. At physiological pH (7.4), metal-free S100A1 was close to nonreactive towards S-nitrosylation (in this case, GSNO, nitroso glutathione originating from NO and a glutathione molecule linked with a disulfide) and S-glutathionylation (see table 1). In the presence of calcium ions, GSNO was linked fast and efficiently. In fact, Ca^{2+} and Zn^{2+} binding enhanced the susceptibility of S100A1 to oxidation (S-nitrosylation) [34]. Interestingly, for apoS100A1 and nitrosylated apo S100A1, circular dichroism (CD) spectra were comparable. However, following the authors, an S-nitrosylated S100A1 was significantly less stable conformationally, as it turned out. This was tested by performing a denaturation curve treatment with urea. Results further suggest that solvent-accessible amide hydrogens increase in numbers in the case of S100A1 S-nitrosylation [34]. S100A1 S-nitrosylation has been demonstrated *in vivo* and influences the protein's overall conformation [35]. In a different study, S-glutathionylation of Cys85 residue of S100A1 enhances Ca^{2+} binding and, with it, the binding constant for C-loops and N-loops, around 10 folds and four orders of magnitude, respectively [31]. One has to consider that non-oxidized S100A1 likely only binds its Ca^{2+}-dependent target proteins upon Ca^{2+} saturation [31], [36]. Upon thiolation, the increased Ca^{2+} affinity and tuning of the structure of S100A1 may come from a rearrangement of the apo S100A1 hydrophobic core [36].
In 2016, Bajor *et al.* could also show that S100A1ox had an increased Ca^{2+} affinity and that this thiol/aromatic molecular switch involved Cys85 residue upon S-nitrosylation [6].
Since the physiological Ca^{2+} concentrations within cells ranges between 100-1000nM, it was hypothezised that, that could mean that intracellular S100A1 oxidation is a way to prime S100A1ox, for instance, for target regulation in physiological ranges by facilitating the open conformational state of the protein [37]. Regarding metal oxidation of S100A1, copper-oxidized S100A1 could not activate protein phosphatase 5 to the extent unoxidized S100A1 could [38].

Finally, it can be concluded that oxidized S100A1 has been shown *in vivo,* therefore possibly playing a role in AD. Ca^{2+} binding and oxidation may contribute to an open target binding

configuration of S100A1 and may increase the susceptibility of the protein to oxidation and Ca^{2+} binding, respectively. Both "contributions" are likely unstable by themselves [39], [37].

S100A6

S100A6 is another small S100 protein, which is highly correlated with the AD phenotype [40]. In comparison to the physiological state of the brain, astrocytic S100A6 is highly upregulated in the AD brain. It binds calcium and zinc ions. Zinc ion chelation is beneficial in inhibiting Aβ from aggregating. Zinc-related might also be, the finding that brain sections of AD mice model amyloid precursor protein/presenilin-1 (APP/PS1) co-incubated with human cells expressing S100A6 showed reduced plaque load. This was corroborated by the results showing that zinc toxicity in COS-7 cells could be rescued by exogenous S100A6 [41]. Another possibly crucial involvement point of S100A6 in AD [40] is that through protein phosphatase 5 catalytic subunit (PPP5C), S100A6 is capable of dephosphorylating tau [42]

The limited research on S100A6ox showed that in a lung and colorectal cancer cell line, S100A6 transited from S-glutathionylation to cysteinylation postexposure to ionizing irradiation, possibly for increased Ca^{2+} release and increased Ca^{2+} affinity. Following the authors, a well-regulated phenomenon suggests a link between Ca^{2+} and redox signaling, which may give a target interaction and localization orientation to the protein in the cell [43].

S100A7

Increased in the cerebral spinal fluid (CSF) of AD patients, S100A7 may promote the activity of alpha-secretase (non-amyloidogenic pathway) preventing Aβ monomer generation. This was corroborated by results obtained with a transgenic AD mouse model [44].

As for its oxidized counterpart, Cunden and Nolan's protocol showed that S100A7 can be oxidized. Results show that Cys47 and Cys96 exist as thiols in the reduced form and may form intramolecular disulfide bonds when oxidated [45].

S100A8

For S100A8, it has been claimed to make part of a distinctive group of damage-associated molecular pattern proteins (DAMPs), playing a role in inflammation *and* restoration of

homeostasis through inflammation resolution [5] by inhibiting and promoting anti-inflammatory properties. The latter, for instance, acts as an oxidant scavenger [46] and, with it, reduces oxidative stress, limits tissue damage, and promotes tissue repair. This would represent a compensatory mechanism of an anti-inflammatory process to restore homeostasis [9].

In the context of high levels of inflammation such as in AD, S100A8 was detected in patient blood sera compared to healthy age-matched control individuals [47]. Moreover, in a small cohort of human AD brain tissue, S100A8 *deposition* could not be observed [48].

In AD mice models, S100A8 is increased in hippocampi of AD mouse models during aging, prior to Aβ plaques deposition, marking different areas in the brain. Further evidence suggests a dependence between S100A8 and Aβ production [49]. In an APP/PS1 mice model, microglial S100A8 upregulation could be shown [50].

For the S100A8 oxidized counterpart, it was hypothesized that oxidatively modified Cys residues may be key in regulating ion-binding properties and target protein interaction dynamics of S100A8 [5]. Concerning inflammatory diseases, progression may show irreversible S100A8 modifications as relevant in terms of pathology because of the highly oxidized extracellular milieu due to ROS generation and transient nitrogen oxide (NO) [5]. For example, hypochlorite (HOCl) oxidation of human S100A8 (hS100A8) inactivated chemotactic activity for neutrophils, which was possibly due to cysteine oxidation (disulfide dimer formation) in a cell model [51], [9]. S100A8ox has further suggested to play a role in regulating blood flow [5]. And in cell culture, the oxidized mS100A8 was identified *in vivo* [52] as well as mice when they were in lipopolysaccharide (LPS) treated [53].

Secreted myeloid mS100A8 stimulates migration of myeloid cells towards inflammatory sites at picomolar levels by chemotaxis. Upon low hypochlorite level treatment, this mS100A8 with Cys41 70-80% oxidized formed disulfide-linked homodimers. (An additional 92 Da, however, suggests further oxidation that may be of Met residues.) Experiments *in vivo* showed that this oxidized mS100A8 dimer failed to recruit leukocytes by chemotaxis. A Cys41 by Ala41 substitution further showed that Cys substitution is not crucial but that the structural change may change the accessibility of the hinge domain and its function in chemotaxis [53]. HOCl oxidized mS100A8 oxidation products in a different experiment, including intramolecular sulfinamide-linked oligomers, which were also not chemotactic

[52]. At this point, it is important to note that S100A8 is more susceptible to HOCl oxidation than LDL [54].

Further, oxidation may play a role in hS100A8, which, when treated with NaOCl, inhibited neutrophile binding to fibrinogen by over 95%. (Similar could not be observed for S100A9 and S100A8/A9) [55], [54]. Results from 2005 suggest hS100A8ox presence in human reactive atherosclerotic carotid arteries. In 2006, it could be shown by Sroussi *et al.* that hS100A8 oxidation had a repellent effect on neutrophils [56]. These cells express high amounts of oxidative stress and are overexpressed in inflammatory disorders [5]. Interestingly, the effect was abolished when Cys42 was substituted for Ala42 [56]. The mutant seemed more efficient in ameliorating impaired wound healing than its oxidized counterpart [57], which suggests a kind of oxidative regulation of possibly an anti-inflammatory process. Further, S-nitrosylated S100A8, specifically, was shown to suppress the activation of mast cells, suggesting a role in NO transport. S-nitrosylated S100A8 is a stable NO adduct [46]. For HOCl-treated hS100A8, Cys-sulfenic and sulfonic acid adducts were found. This suggests a significant fold increase in modification susceptibility compared to murine S100A8 (mS100A8) [9].

To conclude, S100A8 may have pro and anti-inflammatory properties. In highly oxidized milieus, sulfinamide-linked S100A8 complexes are generated, as seen in human atheroma [54], [9]. These may be irreversible, possibly inhibiting anti-inflammatory activity depending on the oxidation of these proteins [9]. Therefore, oxidation may be a time-dependent and oxidant concentration-dependent regulatory switch dependent on the abovementioned factors and temporarily reversible.

S100A9

S100A9 has been characterized as an important amyloidogenic and pro-inflammatory protein in AD [58]. S100A9 is upregulated in AD brain [59],[60] cerebrospinal fluid (CSF) but not in blood plasma [50]. It is amyloidogenic (as observed *in vitro*) and colocalizes and interacts with Aβ (plaques) [61], but can also be found not colocalizing (with Aβ plaques) [58].

In an APP/PS1 transgenic mouse, S100A9 microglial upregulation was found around Aβ plaques and upon loss of function of S100A9; phagocytosis of fibrillar Aβ was found upregulated *in vitro* and *in vivo* [50]. Further, S100A9 deficient mice affected cytokines and

enzymatic activity and expression involved in Aβ production, showing, in the end, lower Aβ deposition [50]. In a further AD mouse model (Tg2576), knocking out S100A9 improved memory function and reduced amyloid plaque deposition [59] [60].

In cell culture, low molecular species of $A\beta_{42}$ led to a concentration-dependent decrease of S100A9 in human THP-1 monocytes, which led to an increase in intracellular Ca^{2+} levels and a decrease in antimicrobial activity [62]. S100A9 oxidized counterpart, was considered part of a distinctive group of anti-inflammatory DAMPs [63], as S100A8 as a pro and anti-inflammatory factor [5]. At least for murine macrophages in pathological circumstances, being able to act as a macrophage-deactivating factor [64].

Oxidative modifications may act as a regulatory switch, which may also apply to the oxidation of S100A9. A key may be oxidatively modified cysteine residues. Irreversible S100 modifications, like oxidation, may be relevant in inflammatory disease progression [5]. As S100A8ox, S100A9ox may be an anti-inflammatory DAMP in restoring homeostasis. For S100A9, it could be shown that Ca^{2+} and Zn^{2+} binding may enhance susceptibility to oxidation of the S100 proteins by inducing structural changes that facilitate former [5]. The previous could be shown for nitrosylation of S100A9 [46]. During inflammation, S100A9ox may be involved in controlling hemodynamics [5]. S100A9 and S100A8 may be considered a distinctive group of anti-inflammatory DAMPs [63].

S100A9, like S100A8, is expressed in cells like neutrophils that express high amounts of ROS and are overexpressed in inflammatory disorders [5]. When two oxidizable Met residues (Met63 and Met83) were substituted by Ala, it could be shown that this abolished the protein's chemo-repulsive effects on neutrophils [10] [56]. In 2009, it was shown S100A9 nitrosylation to be Ca^{2+} dependent [5]. S100A9 oxidized by another oxidant, HOCl, may give origin to Met sulfoxides [5]. HOCl-treated S100A9 shows 2kDa higher mass monomers than S100A9 [9]. Important to note is that there are two human isoforms of S100A9. Compared to the full-length form, the truncated isoform misses a Cys residue, possibly decreasing its susceptibility to oxidation [5].

S100A12

Pro-inflammatory S100A12 is also upregulated in AD [65]. However, published research on the role of S100A12 in AD is scarce. One paper reports that S100A12 has been linked with protein complex formation in AD. It has been associated with senile plaques, reactive glia, and neurons in sporadic AD [65]. As the two previous proteins, S100A12 is expressed in

cells like neutrophils that express high amounts of ROS and are overexpressed in inflammatory disorders [5]. However, S100A12 has no Met or Cys residues, excluding these oxidational targets [5].

Other examples: S100A2, S100A4 and S100P

Based on S100A1 and S100A4 interaction, results could show that the covalent, Cu^{2+} oxidized S100A4 failed to bind to protein phosphatase 5 (PP5) compared to the non-covalent S100A4 dimer. However, the non-covalent S100A4 bound S100A1 and prevented the activation of PP5. Further, hydrogen peroxide oxidated S100A4 inhibited PP5 activation by S100A1 in non-neural cell lines [66]. For instance, in a cell line exhibiting epithelial morphology, S100A2 and S100A4 crosslinked due to copper-induced cysteine oxidation, possibly potentiating the S100A4 effect on NF-kB, lead to TNF-α secretion [67]. In a lung, and colorectal cancer cell line S100A4 transited from S-glutathionylation to cysteinylation upon postexposure to ionizing irradiation for increased Ca^{2+} release and increased Ca^{2+} affinity, respectively. Following Orre *et al.*, a well-regulated phenomenon suggests a link between Ca^{2+} and redox signaling, which may give a target interaction and localization orientation to the protein in the cell [43].

Several copper-oxidized S100 proteins, among them S100A2 and S100P, were unable to activate protein phosphatase 5 to the extent that unoxidized S100A2 and S100P, respectively, could, possibly leading to a switch in signaling pathway towards an apoptotic ASK-1 mediated one [38].

3. S100B: A Link Between Proteostasis and Neuroinflammation in the Brain

3.1 Expression in the Brain in Health and Disease

S100B plays diverse roles in the central nervous system (CNS) and peripheral tissues [68]. In the CNS, S100B is expressed by astrocytes, maturing oligodendrocytes, neuronal progenitor cells, and certain neuronal populations. As mentioned previously, S100B has intracellular and extracellular functions. Intracellularly, S100B is localized by the cytoplasm in a soluble form and associated with intracellular membranes and the centrosome. There, it can act as a Ca^{2+}

sensor. Ca^{2+} binding causes the hinge region to move further apart, exposing various hydrophobic regions to the solvent. The resulting hydrophobic cleft allows S100B to bind to intracellular target proteins (as already seen in Fig. 1). (Binding independent form Ca^{2+}-ions has also been proposed for S100B, although by yet unknown mechanisms of target recognition) [68]. There (intracellularly) S100B can modulate cell proliferation migration and inhibit apoptosis and differentiation of cells [68]. For an overview of S100B suggested intracellular regulatory effects see Figure 5.

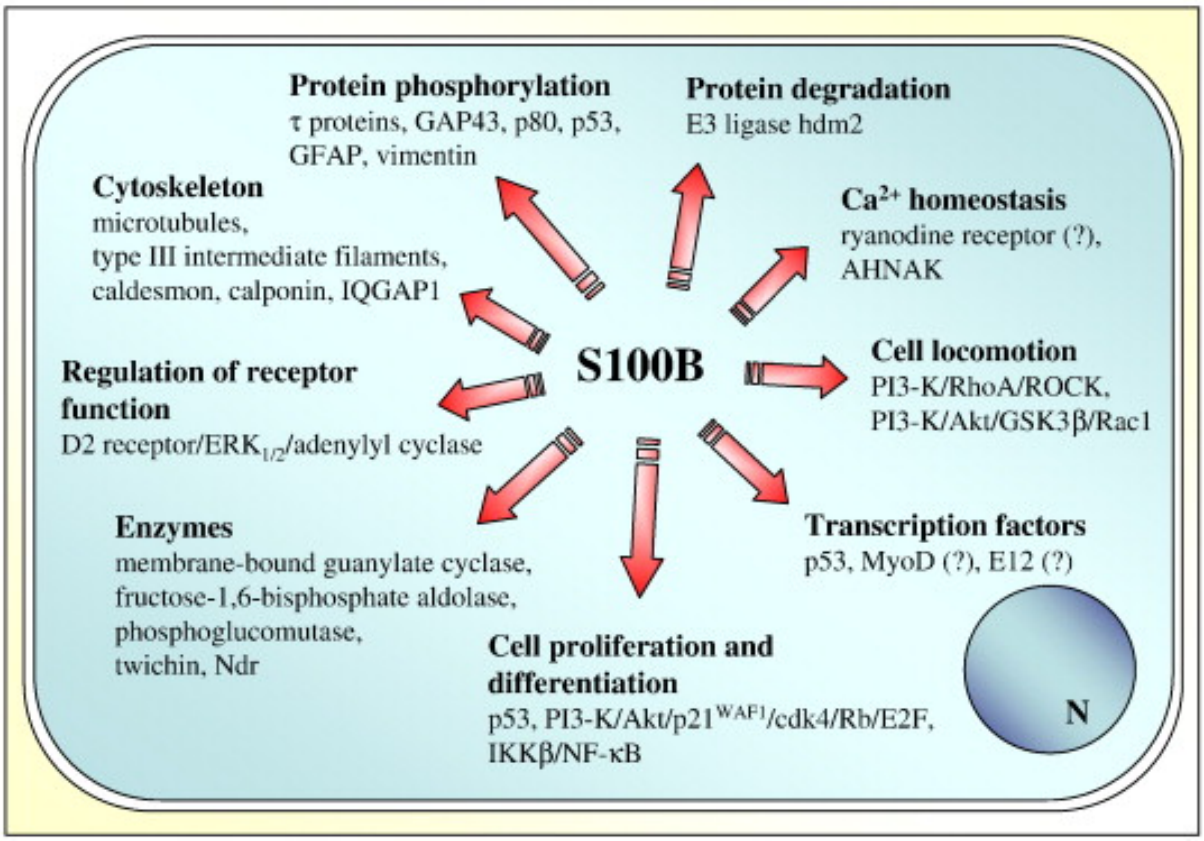

Fig. 5: Schematic representation of the Potential Regulatory Effects of S100B. (From [68])

For S100B to reach the exterior it is either secreted or released. For secretion internal Ca^{2+} concentrations have been suggested to be a mediator of S100B (Fig. 6). Since, when internal Ca^{2} concentrations are high and external decrease — an increase in cytosolic S100B may lead to its secretion [69]. The internal Ca^{2+} levels are mobilized by the endoplasmatic reticulum [69].

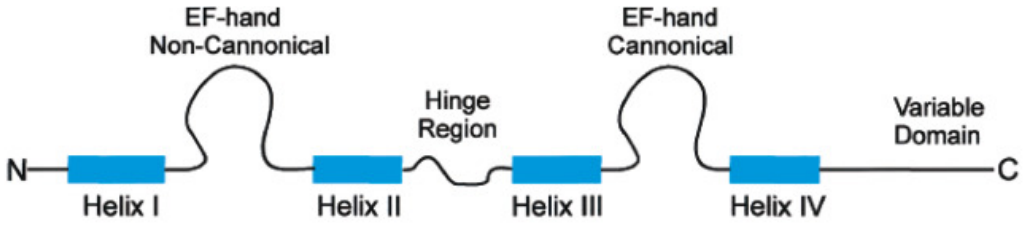

Fig. 6: S100 Protein Structure. The structure of each member of the S100 protein family consists of four α-helical segments, two calcium-binding EF-hand motifs (including one canonical site with high affinity and one non-canonical site with low affinity), a central hinge region of variable length, and variable domains at both the C- and N-termini. (From [70])

Under physiological conditions, astrocytes are the human brain cells, most abundantly expressing S100B and constitutively releasing it [71]. They are important in maintaining ion homeostasis, regulating oxidative stress, and repairing damaged brain tissue by concerting and remodeling blood vessels [72]. They can stimulate neurogenesis [73] or provide neurons with trophic support [74]. In fact, nM levels of S100B have been shown to stimulate astrocyte migration [75]. Under pathological conditions, astrocytic cells change morphology and proliferation, rapidly proliferating and migrating to the site of tissue damage, giving rise to the so-called reactive gliosis [71]. The released S100B has concentration-dependent effects: μM levels may lead to elevated ROS production, which may have toxic effects on the astrocytes [75] (Fig. 7).

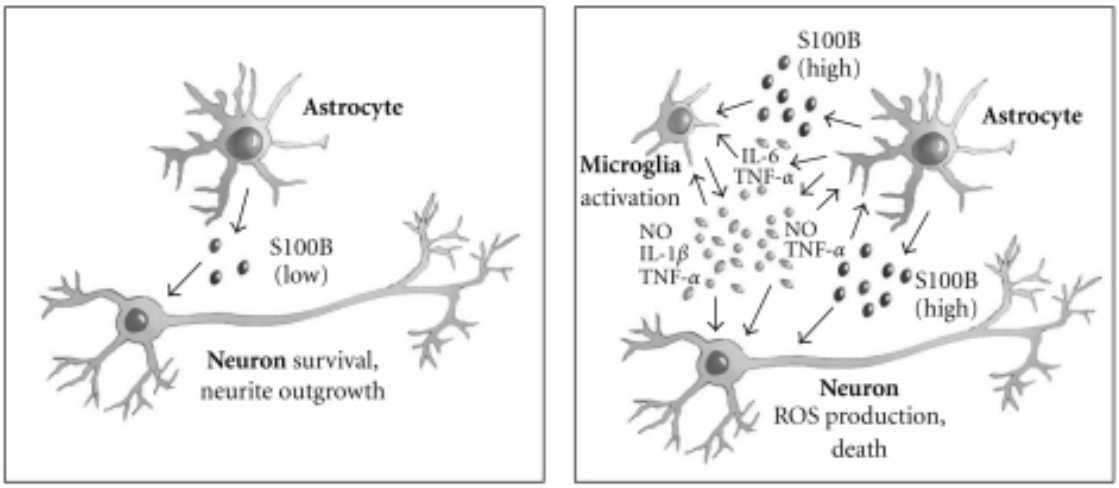

Fig. 7: Extracellular effects of S100B in brain. (a) at nanomolar levels, S100B acts neurotrophic, while at concentration levels in the μM range (b), neurotoxic effects are the consequence. (From [75])

Besides S100B having been suggested to be released upon Ca^{2+} levels, astrocytes can actively secrete S100B in response to the following specific stimuli, e.g., stress in terms of metabolism, natural antioxidants [68]. S100B leakage is another phenomenon of S100B reaching the extracellular space, this one reported upon cell damage [75].

In Alzheimer's Disease, Parkinson's Disease, multiple sclerosis, Schizophrenia, and epilepsy, to mention some, elevated levels of S100B can be found [76]. To counteract an increased S100B accumulation in the extracellular space astrocytes can, by endocytosis, take up S100B that has been secreted into the extracellular space to minimize unnecessary activation of inflammatory processes, which could be resumed in cell damage. Studies show that this may happen due to a partial involvement of RAGE. However, in AD, general trafficking is, to a vast extent, defective [77]. Extracellularly, S100B acts as a factor engaging with RAGE, depending on the microenvironment and concentration [68]. As a RAGE-activating ligand and upregulating factor, S100B potentiates the inflammatory response [75].
Secreted, extracellular S100B can activate microglia, the resident immune cells of the central nervous system (CNS), via poly(ADP-ribose) polymerase-1(PARP-1). PARP-1 regulates S100B, which in turn activates microglia [78]. RAGE-dependent regulation of microglia activation and migration was suggested to be one way for S100B to contribute to inflammatory events in the brain [79]. Upon inflammatory events, microglia morphological changes are accompanied by, for example, upregulation of TNF-alpha and release of NO [78].

3.2 S100B and the Alzheimer's Disease Patient's Brain

Concerning AD, elevated levels of S100B have been associated with AD progression [16]. In the human AD brain, S100B is highest in the hippocampus and temporal lobe [80]. S100B colocalizes with plaques close to reactive astrocytes [81]. AD patients' data from sera showed no difference in S100B levels [82]. S100B clearance from the brain to the blood possibly occurs through the cerebral spinal fluid (CSF) [83] in the case of an intact BBB. The S100B in the CSF has been reported to be higher in AD patients' brains than in control samples [84] while highest in the early/moderate stages of the disease [85]. For AD, S100B has been

suggested as a (complementary) CSF biomarker in humans [86]. S100B that reaches the blood has a half-life of 25 min [87]. While a slower clearance rate of up to 130 min was suggested for cranial S100B [88] a distinction between cranial and extracranial S100B half-lives has been suggested [89] (latter findings might need to be clarified [88]). Exclusion of sources of S100B that are exocranial is possible since they may not affect S100B serum levels significantly [90].

S100B crossing the BBB may be active or/and passive. Given that the S100B dimer [90] does not penetrate the BBB passively due to its size of 21 kDa [91]; [92]), S100B may be transported actively across the BBB [93]. Transport may occur passively in case of a no longer intact BBB [94]. Results suggest that the brain does produce more S100B than it requires (1), the ingressing S100B is modified, e.g., oxidized/conformationally changed and thus cleared into the blood or thus able to be transported across the BBB (2).

(1) Arundic Acid (AA), an agent that reduces astrocytic S100B expression in the brain, reached clinical trials for AD treatment [95], underlining the beneficial effect of decreasing the level of S100B in the brain. However, how S100B is controlled molecularly in the complex AD milieu is poorly understood. (2) Interestingly, S100B does undergo structural changes upon oxidation [6] [34], and overwhelming evidence shows that AD patients' brains endured elevated levels of oxidative stress [22] [23] [24], (evident in the abundance of e.g. oxidized proteins, advanced glycation end products or formation of toxic species [96]). In agreement, a higher antioxidant defense in the elderly correlated with better memory [97] or higher cognitive performance in general [98]. As shown by Ping *et al.* in 2018, oxidized S100B likely occurs in the human brain [99]. In a simplified redox model, authors tackled the relationship between myeloperoxidase (MPO), the potent oxidant produced by MPO, hypochlorite, and S100B [100]. As a side note, MPO is not present in the human brain of healthy individuals. Upon neuroinflammation, MPO enzyme function is contributed to by, for example, astrocytes, microglia, infiltrating macrophages, and neutrophils. In the AD brain, even neurons show myeloperoxidase-immunoreactivity [101]. Boyum *et al.'s* results suggest a possible redox buffering/balancing function for S100B [100]. Astrocytes in AD were suggested to become more rapidly activated by pro-inflammatory signals in the form of feedback loops. Then, exerting biphasic effects as reactive astrocytes [102].

Recent findings have, however, strengthened the hypothesis that early stages of Alzheimer's Disease make part of a disease-aggravating phase, while the late phase corresponds to a failed tissue resolution attempt [103] (Fig. 8). Inflammation, insoluble protein deposits, and death of specific brain cells are tightly correlated to AD [65]. As shown previously, in AD,

aggregation, and neuroinflammation may be linked by S100 alarmin functions and distribution in the brain [104].

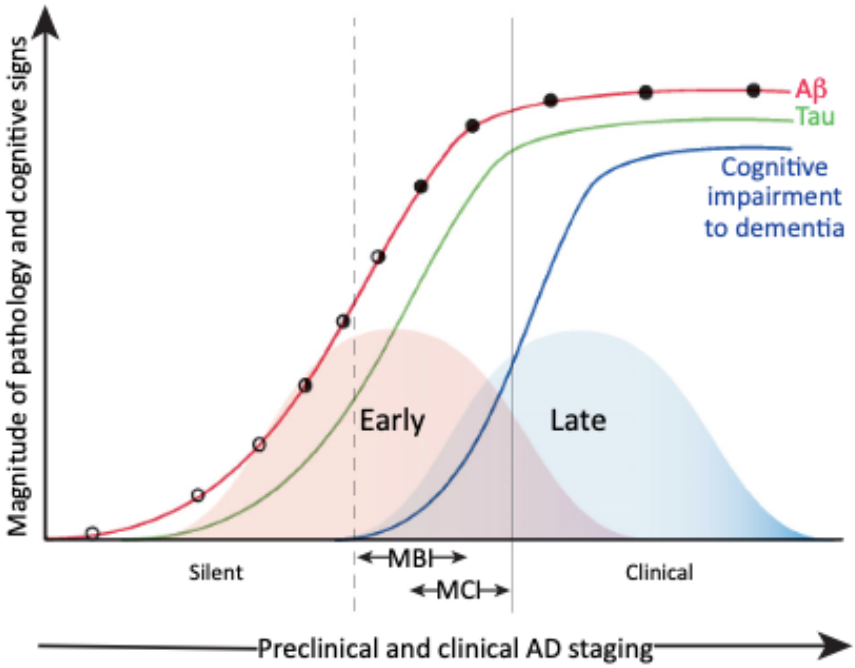

Fig. 8: Postulated graphic representations of two stages in the continuum of AD (Image from [103]) Abbreviations: MBI: mild behavioural impairment; MCI: mild cognitive impairment.

3.3 Chaperone Activity: Protective Functions Against Aggregation

In AD patients, S100B is chronically upregulated. S100B is expressed in the whole brain in wild-type mice brains, especially in the cortex and cerebellum (enriched in Purkinje cells). In AD mice models (APP23 mice), S100B is found in the periphery of AD plaques, where there is relatively fewer fibrillar Aβ than in the core [104]. S100B was localized close to/in Aβ plaques, and our lab could further show that S100B may bind $A\beta_{42}$ (Fig. 9).

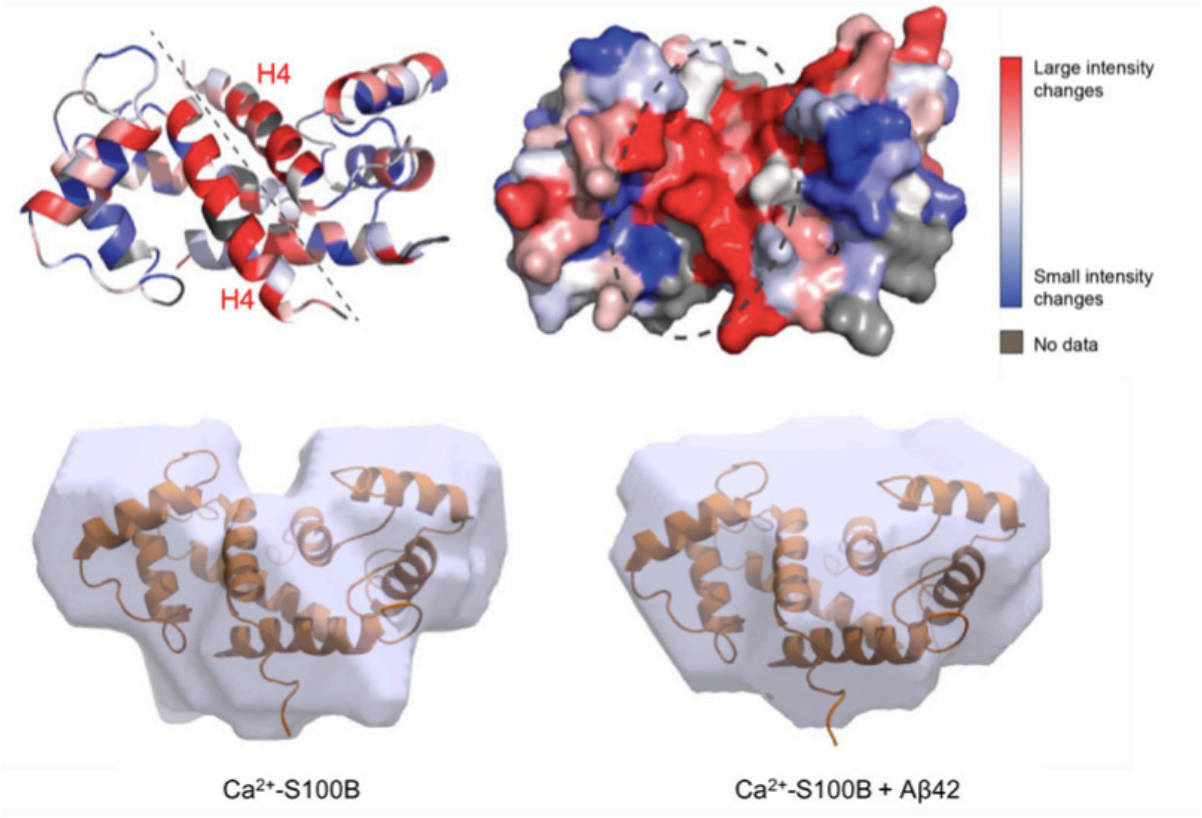

Fig. 9: Binding of $A\beta_{42}$ mapped on S100B. (From [105])

When bound to Ca^{2+}, S100B or the S100B::$A\beta_{42}$ complex is suggested to lead to conformational switching of $A\beta_{42}$ into α-helical conformers, locking aggregation, thus suppressing Aβ aggregation and toxicity [105].

For the chaperone-type holdase activity of S100B on $A\beta_{42}$, the Lys28(Aβ)/Glu86(S100B) pair was suggested to be significant to stabilize the interaction, given the coulombic forces. Met79(S100B) and Thr82(S100B) also were suggested to make part of the Aβ-binding triad of S100B [106]. S100B was shown to bind Aβ in the AD context and Zn^{2+}. Alzheimer's Disease patients' brains show elevated zinc and $A\beta_{42}$ levels. In this context, zinc levels correlate with cognitive decline. *In vitro* experiments show that zinc leads to polymorphic $A\beta_{42}$, less fibril formation, and consequently more toxicity. Down to sub-stoichiometric amounts of S100B may act as a dual chaperone, chelating zinc, allowing $A\beta_{42}$ to aggregate into fibrils more quickly and decreasing toxicity. Additionally, S100B is the holdase-type chaperone by suppressing Aβ aggregation [107].

Not only did our lab find evidence of the S100B alarmin binding Aβ and leading to a reduction of Aβ toxicity by dual chaperon activity, S100B also binds tau. S100B has been shown to suppress tau aggregation and seeding at sub-stoichiometric ratios. Tau:S100B

interaction was shown in microtubule-destabilizing conditions, which depended on Ca^{2+} [108].

In Alzheimer's Disease, extracellular S100B is involved in Aβ aggregation suppression. Often disregarded with this is the oxidizing milieu and the susceptibility of S100 proteins to oxidation. In the context of AD, oxidized S100B likely occurs in the human brain [99], possibly protecting it from oxidative damage while getting oxidized in the process ([109]; [5]; [110]).

Apart from S100B, other inflammatory cytokines play an important role in AD. IL-17 levels are elevated in AD. IL-17 (or IL-17A) is one of six members of the 17A-F cytokine family [111]. The function of IL-17A consists of mediating the release of pro-inflammatory molecules. Through the IL-17 receptor, IL-17 mediates activation of the transcription factor NF-kB and downstream kinases. In turn, these kinases lead to the generation of other pro-inflammatory signalling molecules and the attraction of immune cells to the site of inflammation [111]. In AD mouse models, beneficial and harmful effects have been reported concerning IL-17 ([112];[113];[114]). On the one hand, IL-17 overexpression has been shown to improve spatial learning without the aggravation of neuroinflammation [112]. However, depletion of IL-17 with an IL-17 antibody pre-treatment prevented $A\beta_{42}$ induced neurodegeneration and memory decline [113].

Neutrophils were identified in well-established AD mouse models by releasing IL-17 at Aβ deposition sites. Upon temporary depletion of these cells, cognitive improvements occurred [114]. In addition, Interferon-alpha (IFN-α) is a cytokine found to be elevated in the frontal cortex of AD brains [115]. Upon an insult to brain tissue, IFN-α is mainly expressed by astrocytes upon Toll-like receptor activation. In general, interferons are helical cytokines, and like IL-17, they play a role in mediating pro-inflammatory signalling [116].

3.4 Role and Functions of Oxidized S100B

S100B may have contradicting functions upon pathological AD disease progression (proinflammatory and anti-inflammatory). For this switch in functions, the susceptibility of S100B to oxidation [99] may play a role, shielding against excessive cell damage and promoting trophic activities of S100B [5].

Oxidative folding occurs in the endoplasmic reticulum (ER) **[117]** and in fact, the sulfur-containing amino acid cysteine is among the most reactive residues in proteins [34]. The pKa value of free cysteine residues is above the physiological pH 7.4. Only when free cysteine

residues gain a pKa that is lower than the physiological pH can their -SH group get thiolated, and those thiolated residues are susceptible to including oxidants, metals, and disulfides. (Disulfide bonds can be native (catalyzed in the ER or mitochondria) or non-native, i.e., induced by stress or non-physiological conditions [118]). Zhukova *et al*. could demonstrate cysteine-based redox modifications for S100B. At physiological pH (7.4), metal-free S100B was non-reactive towards GSSG and almost non-reactive with GSNO in comparison to S100A1 [34]. The oxidation of Cys residues 68 and 84 has however been shown to be able to enhances the neurotrophic effect of S100B. This is abolished when Cys are mutated to Ser residues [9] [119] [120]. Regarding metal binding for the oxidized S100B protein, zinc-binding could substitute calcium ions in terms of efficiency. With Cu^{2+}-ions, an intra- or intermolecular transfer of the NO group could be observed. S100B, $Cu^{2+,}$ and excess GSNO resulted in the non-formation of covalent dimers, and one to two times S-nitrosylated S100B monomers formed. For S100B, in this study, only Cys-84 could be nitrosylated *and* glutathionylated [34]. S-nitrosylation *and* S-glutathionylation may be a protection mechanism for irreversible S100B oxidation modifications [118]**.** For oxidation of S100B by chelating copper [109] protection of cells from copper toxicity was observed. Aβ for instance, binds copper, which may lead to resistant dimers, which cannot be further degraded into monomers and are neurotoxic for the cell [121]. S100B upon copper binding (it can bind up to 4 Cu^{2+} per dimer) results in a disulfide bridge also observed in S100A4 and S100A2. S100B-Cu^{2+} has (also in this study) been associated with changed biological activity [110]. Regarding protein phosphatase 5 activity, copper-oxidized S100B could not activate protein phosphatase 5 to the extent unoxidized S100B could [38]. Further, in a more recent study, Cu^{2+} binding to S100B showed induction of disulfide cross-liked tetramer formation, which increases anti-aggregation activity, uncovering relevant mechanisms in Alzheimer's disease, in which metallostasis and proteostasis are deregulated [110]. (However, the *in vitro* forming covalent disulfide S100B dimer has not yet been adequately shown *in vivo*) **[6]**.

In rats, S100B is a possible target of S-nitrosylation as an oxidative modification. In an astrocytic cell model, intracellular S-nitrosylated S100B could also be detected. S-nitrosylated S100B was stable, increased calcium affinity, and showed a more efficient Zn^{2+} S100B regulation **[6]**.

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Chapter IV

On the effect of S100B oxidation on its anti Amyloid-β Aggregation Activity

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Abstract

Protein aggregates accumulate in neurodegenerative diseases like Alzheimer's Disease (AD), triggering cellular defences and altering redox balance. The S100 family of pro-inflammatory cytokines, particularly S100B, is activated during AD. Recent findings reveal that S100B acts as an unconventional molecular chaperone, inhibiting Aβ aggregation and toxicity, suggesting a protective role at the early stages of Aβ proteotoxicity in an environment prone to oxidative stress. In this study, we investigated the impact of oxidative conditions on S100B's protective function. Mild oxidation of S100B ($S100B_{ox}$) led to methionine oxidation, as detected by mass spectrometry, without cysteine-mediated crosslinking. Structural analysis confirmed that oxidation did not alter S100B's folding, structure, stability, or quaternary assembly. Interestingly, oxidized S100B was more effective in preventing Aβ aggregation, potentially due to enhanced interactions linked to oxidation of Met residues within the S100β binding cleft.

$S100B_{ox}$ significantly delayed Aβ aggregation *in vitro*, potentially even modulating Aβ aggregation at sub-stoichiometric levels. Additionally, it was demonstrated that this oxidation does not interfere with the Aβ aggregation mechanism involving secondary nucleation.

1. Introduction

In a physiological state, cellular redox balance is maintained by a highly reducing cytosol and nucleus, contrasted by an oxidizing extracellular environment [1]. This balance is primarily regulated and controlled by the upregulation of intracellular antioxidant systems. However, during inflammation, this equilibrium is disrupted as increased levels of reactive oxygen species (ROS) promote cytokine production [2]. Cytokines, in turn, may function as damage associated molecular pattern molecules (DAMPs). DAMPs are biochemical entities produced and secreted in response to tissue damage or cellular stress, triggering an immune response [3]. S100 proteins can also be classified as DAMPs signaling molecules.

Excessive ROS acts as a pathological stimulus for brain cells and may lead to tissue damage, including the disruption of structural proteins [2]. ROS can originate from multiple endogenous sources that may induce mitochondrial dysfunction and peroxidation, among

them of proteins and lipids, which cause cell damage [4]. During this process, proteinaceous DAMPs are targets for oxidative modifications, the consequences of which are still unclear. Cytosolic proteins with DAMP activity are folded in a reducing environment within the cell. Upon secretion, i.e., crossing the cell border, susceptible residues may become oxidized. This oxidation can lead to inactivation of the protein, resulting in the loss of its DAMP activity. While DAMP properties are essential for pathogen killing, oxidation may alter the inflammatory properties of the protein in question. For example, monomeric S100A8 acts as a DAMP that stimulates recruitment of myeloid cells to the location of inflammation, while dimeric S100A8 is inactive, and it might be speculated that oxidative modifications might pay a role in the process. Also, oxidation of High Mobility Group Box 1 (HMGB1) by ROS alters its functionality, depending on the exposed cysteine residues, with treatment by H_2O_2 completely inactivating it. Carta *et al.* suggest that oxidation serves as a mechanism to control bioactivity, functioning as a switch to inactivate DAMPs. In the context of inflammation, the short-lived bioactivity of DAMPs, when high levels of oxidizing agents are present in the extracellular environment, may help restrict the inflammatory response both spatially and temporally [2].

In AD, ROS-producing enzymes were found to be increasingly expressed and ROS generated in the brain includes hypochlorous acid, leading to its suggestion as a potential biomarker for the disease [5]. Indeed, brains affected by AD exhibit significant oxidative damage, with reported reductions in antioxidant levels [4]. Consequently, the effects of oxidative stress extend beyond mere damage, playing a role e.g. in the formation of stress granules that persist as inclusions, disrupting neuronal function and adversely impacting proteins essential for neuronal health.

To explore how oxidative modifications of proteins may affect DAMP function, it is essential to understand the types of post-translational modifications that can occur. Proteins can be oxidized through reactions between amino acid residues and reactive oxygen or nitrogen species. These modifications can be classified as either irreversible or reversible, depending on their nature, and they are linked to functional changes in the target proteins.

Irreversible modifications include the formation of carbonyls, while reversible modifications encompass methionine oxidation, disulfides, and various cysteine oxidation products, such as

S-glutathione and S-nitrosothiols [6]. Notably, all these modifications have been associated with proteins altered in Alzheimer's Disease (AD) [7].

S-nitrosylation, induced by nitric oxide, is analogous to simpler processes like protein (de)phosphorylation and (de)nitrosylation, which occur on cysteine residues rather than tyrosine or serine. Increased S-nitrosylation of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been observed in postmortem AD tissue [7]. S-glutathionylation modifies proteins through mixed disulfide formation (Protein-S-S-G), potentially altering their function. As the major antioxidant in cells, glutathione can exert both detrimental and protective effects on target proteins. In AD, altered levels of S-glutathionylated proteins, including Enolase 1 (ENO1) in postmortem tissue, GAPDH in blood, and Transthyretin (TTR) in cerebrospinal fluid, have been reported. Elevated levels of oxidized GAPDH in the blood have also been correlated with AD progression [7]. Protein disulfides caused by ROS for instance (i.e. not the native disulfide bonds formed usually in correct protein folding), play a beneficial role in the cellular stress defense system [6]. Disulfide bonds formation in AD patients GAPDH has been shown elevated [7]. Another reversible oxidative modification is methionine oxidation, where methionine residues can be oxidized to methionine sulfoxide (MetO), resulting in two diastereomeric forms. Methionine oxidation has been implicated in intracellular signaling, making it particularly relevant in pathologies like AD. In fact, methionine oxidation has been detected in postmortem brains of AD patients [8].

Carbonylation, an irreversible oxidation, occurs on over six amino acid residues and is less restricted to specific residues, making it easier to detect. While carbonylation is often associated with detrimental effects, it has also been reported to play a role in signal transduction [6]. Its association with oxidative stress renders it an intriguing target for AD research. For instance, Aksenov *et al*. found elevated carbonylation levels in the hippocampal area and temporal gyri of a small cohort of former AD patients, while no carbonylation was detected in the cerebellum [9]. This suggests that carbonylation primarily affects intracellular proteins [10]. Additionally, carbonylation has been shown to be elevated in AD plasma and cerebrospinal fluid, with superoxide dismutase 1 (SOD1) being one of the proteins affected in postmortem tissue [7].

This chapter investigates the biological effects of oxidized S100B compared to non-oxidized S100B on Aβ aggregation. As discussed in the introductory chapters, S100B is upregulated in

Alzheimer's Disease (AD) and during cellular damage, exhibiting both deleterious and protective functions, including its role as a holdase-type chaperone that inhibits Aβ aggregation. In the brain, S100B, secreted by astrocytes, is exposed to an oxidizing environment, and it is known that oxidation can alter S100 protein's metal ion-binding properties, protein interactions, and extracellular functions. Therefore, here I will explore the possibility that oxidative modifications of S100B in the human brain may impact its chaperone activity.

2. Material and Methods

Recombinant Protein Generation

Human S100B was expressed in *E. coli* cells, and dimeric S100B was purified following a previously established protocol [11]. To obtain apo S100B, S100B holo was incubated with a 300-fold excess of dithiothreitol (DTT) and 0.5 mM EDTA for 2 h at 37 °C. Afterward, it was injected and eluted in a Superdex S75 column (GE Healthcare, Chicago, IL, USA). To prevent trace metal binding, solutions were prepared with water passed through a Chelex resin (Bio-Rad, Hercules, CA, USA). Oxidation of S100B was carried out in a manner similar to a previously described protocol [12]. Hypochlorite (49.08 µL, 1.913 mmol) was added to S100B (4.3 mg, 201 nmol) diluted in 500 mM Tris-HCl pH7.4, incubated for 10 min at room temperature, and centrifuged for 5 min at 12,400 rpm, before being eluted in an S75 column (GE Healthcare). The $S100B_{ox}$ protein was concentrated and stored at −20 °C. Human recombinant $A\beta_{42}$ was expressed and purified as previously described [13]. The $A\beta_{42}$ expression plasmid was kindly gifted by J.Presto (Karolinska Institutet, Solna, Sweden). $A\beta_{42}$ expressing E. coli cells were harvested after 4 h by centrifugation and resuspended in 20 mM Tris-HCl pH8.0. For purification, $A\beta_{42}$ cells were lysed by sonication. A centrifugation step was followed at 14,000 rpm for 20 min to isolate inclusion bodies. Next, the pellet was dissolved, sonicated for 3 min, 65 amplitude in a cycle of 0.5, and centrifuged again at 14,000 rpm for 20 min. Urea-solubilized inclusion bodies were then purified by anion-exchange chromatography and centrifugal filtration using a DEAE-cellulose column (GE Healthcare). Fractions containing solubilized $A\beta_{42}$ were lyophilized and stored at −20 °C in low-binding tubes (Axygen Scientific, Corning, NY, USA). To obtain the low molecular species of $A\beta_{42}$, 2.48 mg of lyophilized $A\beta_{42}$ was dissolved in 1.7 mL 100% DMSO. For the lyophilized $A\beta_{42}$ to dissolve, it was vortexed for 30 min, at a speed of 8, and water bath sonicated (pulses) at a

frequency of 37 kHz for 3 min. The supernatant was kept following centrifugation at 8000 rpm. The concentration was determined by Nanodrop, and the $A\beta_{42}$ stock was then stored at −20 °C. To obtain monomeric $A\beta_{42}$ for ThT aggregation assays, about 2 mg was dissolved in 7 M guanidine hydrochloride and eluted in a Superdex S75 (GE Healthcare) with 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, NZYtech, Lisbon, Portugal) pH 7.4.

Mass Spectrometry

Intact protein analysis: The liquid chromatography–mass spectrometry (LC-MS) runs were realized using a Dionex Ultimate 3000 UHPLC+ system equipped with a Multiple-Wavelength detector, an imChem Surf BIO C4 300 Å 3 µm 150 × 2.1 mm column connected to Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific™ Q Exactive™ Plus, Waltham, MA, USA). The mobile phase consisted of water with 0.1% FA (mobile phase A) and acetonitrile with 0.1% FA (mobile phase B). The following gradients were applied at a flow rate of 200 µL/min: precondition with 10% B for 5 min, linear gradient from 10% B to 80% B in 20 min. The electrospray source was operated with a Spray Voltage (+): 3.5 kV, Capillary Temperature 320 °C, Sheath Gas 47.50 (a.u.), Aux Gas 11.25. Deconvolution of peaks was performed using MagTran1.03 [14].

Digestion procedure with trypsin: 90 µL of NH_4HCO_3 (16 mg/mL), 18 µL TCEP solution (16 mg/mL in 30 mg/mL of NH_4HCO_3), and 72 µL of protein solution (4–6 µM) were combined and incubated at 60 ºC for 60 min. After cooling to room temperature, 21 µL of activated trypsin (0.1 µg/µL in NH4HCO3 (16 mg/mL) solution) was added and incubated at 37 °C for 16 h. The digestion was quenched with 15 µL of formic acid, vortexed briefly, and then centrifuged. Then, 20 µL of the solution was injected for MS analysis. Digestion procedure with formic acid: 100 µL of protein solution (100 µM) was added to 2 µL of formic acid and 3 µL of acetonitrile. The solution was incubated at 108 °C for 6 h, allowed to cool to room temperature, and then analyzed by LCMS. Analysis of digested peptides: The Liquid chromatography–mass spectrometry (LC-MS) runs were realized using a Dionex Ultimate 3000 UHPLC+ system equipped with a Multiple-Wavelength detector, an imChem Surf C18 TriF 100 A 3 µm 100 × 2.1 mm column connected to Thermo Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo ScientificTM Q ExactiveTM Plus). Tryptic peptides were separated with a 0.2 mL/min and a mixture of water with 0.1% formic acid (buffer A), and acetonitrile with 0.1% formic acid (buffer B), using a gradient mixture of A:B solvents from 97:3 until 5:95 during 90 min. The Exactive mass spectrometer was operated in

positive ion mode with alternating MS scans of the precursor ions and AIF (all ion fragmentation) scans in which the peptides were fragmented by HCD. Both scan types were performed with 100,000 resolution (at m/z 200), with each scan taking 1 s, and the maximal fill time was set to 1 s. The m/z range for MS scans was 300–1600, and the m/z range for AIF scans was 150–1600. The target value for the MS scans was 106 ions, and the target value for the AIF scans was 3 × 106 ions. HCD collision energy was 50 eV. A database search of possible modifications of digested peptides was performed using Skyline (64-bit) 20.2.0.343. The reaction of S100B and S100Box with maleimide: To a solution of S100B (106 uM approx.) (5 µL, 0.00053 µmol) or S100Box (151 uM approx.) (5 µL, 0.00076 µmol) in ammonium acetate at 20 mM pH 7 (106 µL) dansyl maleimide [15] was added at 10 mM in ACN (1.060 µL, 0.0106 µmol) and allowed to react for 4 h.

Aggregation Kinetics

$A\beta_{42}$ aggregation kinetics were performed by recording Thioflavin-T (ThT) fluorescence intensity as a function of time in a plate reader (FLUOstar Optima, BMG Labtech, Ortenberg, Germany) with a 440 nm excitation filter and a 480 nm emission filter. The fluorescence was measured using the bottom optics in half-area 96-well polyethylene glycol-coated black polystyrene plates with a clear bottom (Corning, 3881, Corning, NY, USA). The microplates were sealed with foil to avoid evaporation. Monomeric $A\beta_{42}$ was diluted to a final concentration of 5 µM in 50 mM of HEPES pH 7.4 and the indicated concentrations of S100B and $S100B_{ox}$. Then, 10 µM of ThT (Sigma, St. Louis, MO, USA) was added to each condition. All assays were performed at 37 °C, under quiescent conditions, with fluorescence measurements taken every 400 s and with three technical replicates (n = 3).

ANS Fluorescence

Fluorescence measurements were performed on a Jasco FP8200 spectrofluorometer. The temperature was kept at 25 °C by a Peltier-controlled cell support. For ANS analysis, S100B and $S100B_{ox}$ were incubated with 2 molar equivalents of ANS for 30 min. ANS emission spectra were recorded using 10 nm excitation and emission slits upon 370 nm excitation.

Analytical Size-Exclusion Chromatography

Analytical size-exclusion chromatography was performed at room temperature on a Superdex 75 Tricorn high-performance column (GE Healthcare, bed volume = 24 mL) eluted at 1 mL/min, with 50 mM TrisHCl pH 7.4 using imidazole as internal standard.

Circular Dichroism

Circular dichroism (CD) measurements were performed on a Jasco J-1500 spectropolarimeter equipped with a Peltier-controlled thermostated cell support at 25 °C. Samples were prepared by diluting S100B to a final concentration of 5 μM (dimer equivalents) in 50 mM Tris-HCl pH 7.4. Far UV-CD spectra were recorded between 200 nm and 260 nm using a 1 mm pathlength quartz cuvette (Hellma Analytics, Müllheim, Germany) and a minimum of 8 scans of average accumulation.

Fourier-Transformed Infrared Spectroscopy

Fourier-Transformed Infrared Spectroscopy (FTIR) measurements were performed on a Bruker Tensor II FTIR Spectrometer (Billerica, MA, USA) equipped with a nitrogen-cooled MCT detector and a thermostatized Harrick BioATR cell at 25 °C. Before spectra acquisition, 20 μL of untreated and oxidized apo-S100B (≈250 μM) in 50 mM Tris-HCl pH 7.4 were pipetted into the ATR cell and equilibrated for 5 min. FTIR spectra between 900 and 4000 cm^{-1} were acquired with 120 technical accumulations, 12 mm of aperture, 20 kHz scanner velocity, 4 cm^{-1} spectral resolution, and buffer background correction.

Limited Proteolysis

S100B and S100Box were incubated with 0.4μM Trypsin at 37ºC in 50mM Tris-HCl pH=7.4. Aliquots were taken at different time points (up to 45min). The reaction was stopped upon addition of SDS-PAGE loading buffer by 2% SDS and 5% β-mercaptoethanol. The products of the proteolysis reaction were analyzed by 8% SDS/PAGE, stained with Bluesafe. As an internal control for the quality of loaded protein in the gel slots, 2.5 μM bovine serum albumin (Sigma) final concentration, was also added to loading buffer solution.

3. Results

Identification and structural mapping of S100B oxidized residues

To mimic S100B *in vivo* oxidation, we chemically oxidized S100B using a mild hypochlorite oxidation protocol, herein designated $S100B_{ox}$. From mass spectrometry (MS) analysis of HClO-treated S100B, we observed an oxidation of up to three methionine residues (Fig. 1a)

per $S100B_{ox}$ monomer. Methionine oxidation to methionine sulfoxide and the formation of intra- or intermolecular disulfides are considered reversible oxidative modifications. These modifications can help protect proteins from irreversible oxidation and permanent damage. [6].

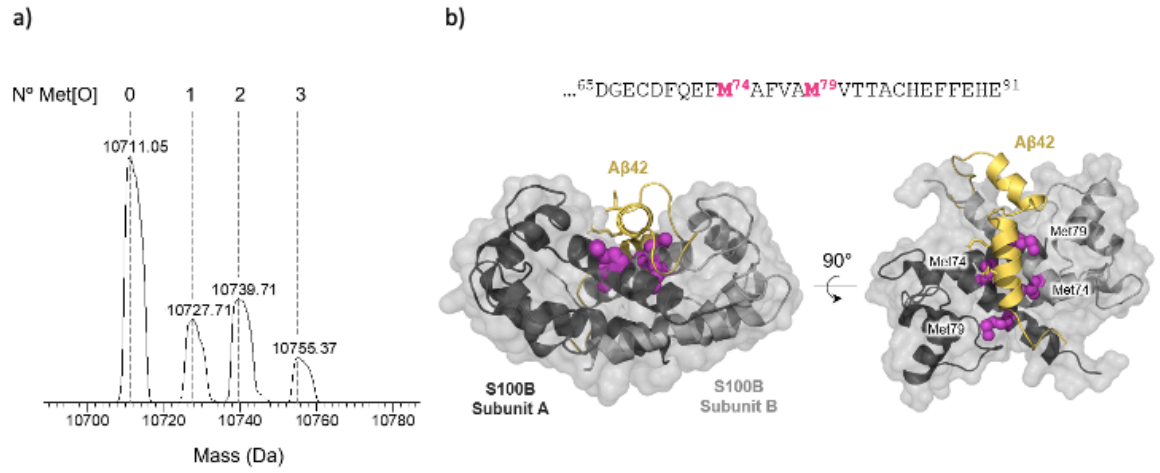

Fig. 1: Oxidized S100B. Left (**a**) MS spectrum of $S100B_{ox}$ depicting the peaks corresponding to the different degrees of methionine oxidation identified. Right (**b**) Structural representation of a model of S100B (grey) bound to $A\beta_{42}$ (yellow) generated in [16] highlighting S100B-Met residues (magenta) within the chaperone–client binding cleft, identified in [17].

Supporting such modifications, we also noted that the FTIR spectra of oxidized and non-oxidized S100B were distinct in the 1000–1100 cm^{-1} region in which vibrational modes associated with methionine sulfoxide have been identified [18]. MS analysis did not provide evidence for Cys oxidation, as confirmed by the reaction between $S100B_{ox}$ and dansyl-maleimide [19], which introduced a 0.373 kDa mass shift per free sulfhydryl group. Notably, two out of five methionine residues are located at the interfacial cleft of the S100B protein (model with PyMOL), including Met-79 and Met-74 (Fig. 1b). The interfacial cleft of Ca^{2+}-S100B is of particular interest in the context of AD since it is the binding site of $A\beta_{42}$ [17].

Investigation of structural changes upon S100B oxidation

We subsequently conducted a comprehensive biophysical characterization to compare the structural properties of oxidized S100B with its unmodified form.

To assess changes in secondary structure, we employed attenuated total reflectance Fourier-transform infrared spectroscopy (ATR FTIR) and far-UV Circular Dichroism (Far-UV CD). FTIR analysis (Fig. 2a) revealed that the amide I band centered at 1655 cm^{-1} is preserved in oxidized S100B, showing no significant changes compared to unmodified S100B. Similarly, far-UV CD analysis indicated that $S100B_{ox}$ maintains the characteristic α-helical topology of S100 proteins, evidenced by negative CD bands at 208 and 222 nm, with spectra from $S100B_{ox}$ and S100B being superimposable (Fig. 2b).

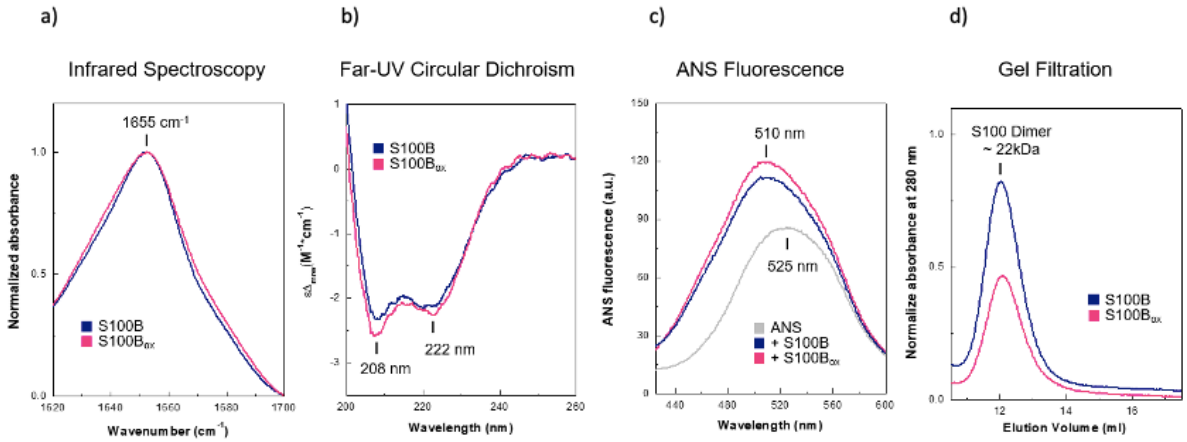

Fig. 2: Structural similarities of $S100B_{ox}$ and S100B. $S100B_{ox}$ structural characterization using (**a**) FTIR [20], (**b**) CD, (**c**) ANS fluorescence, and (**d**) size-exclusion chromatography.

To investigate whether oxidation would lead to a decrease in conformational stability of S100B, we conducted thermal denaturation analyses comparing the oxidized and unmodified forms of the protein. Additionally, we examined the effects of calcium binding on S100B during this process (Fig 3). The results indicate that oxidation does not significantly impact the thermal stability of S100B. In fact, S100B is a highly thermostable protein, maintaining its folded structure even at 90°C. A comparison of the thermal denaturation profiles of the oxidized and unmodified proteins reveals essentially no changes. Interestingly, when the same experiment is conducted with calcium-bound S100B, the far-UV CD spectra show some mild differences, particularly in the ratio between the negative bands at 222 nm and 208 nm. This suggests potential rearrangements in the α-helical structure, possibly due to slight changes in the tilting angles between the α-helices.

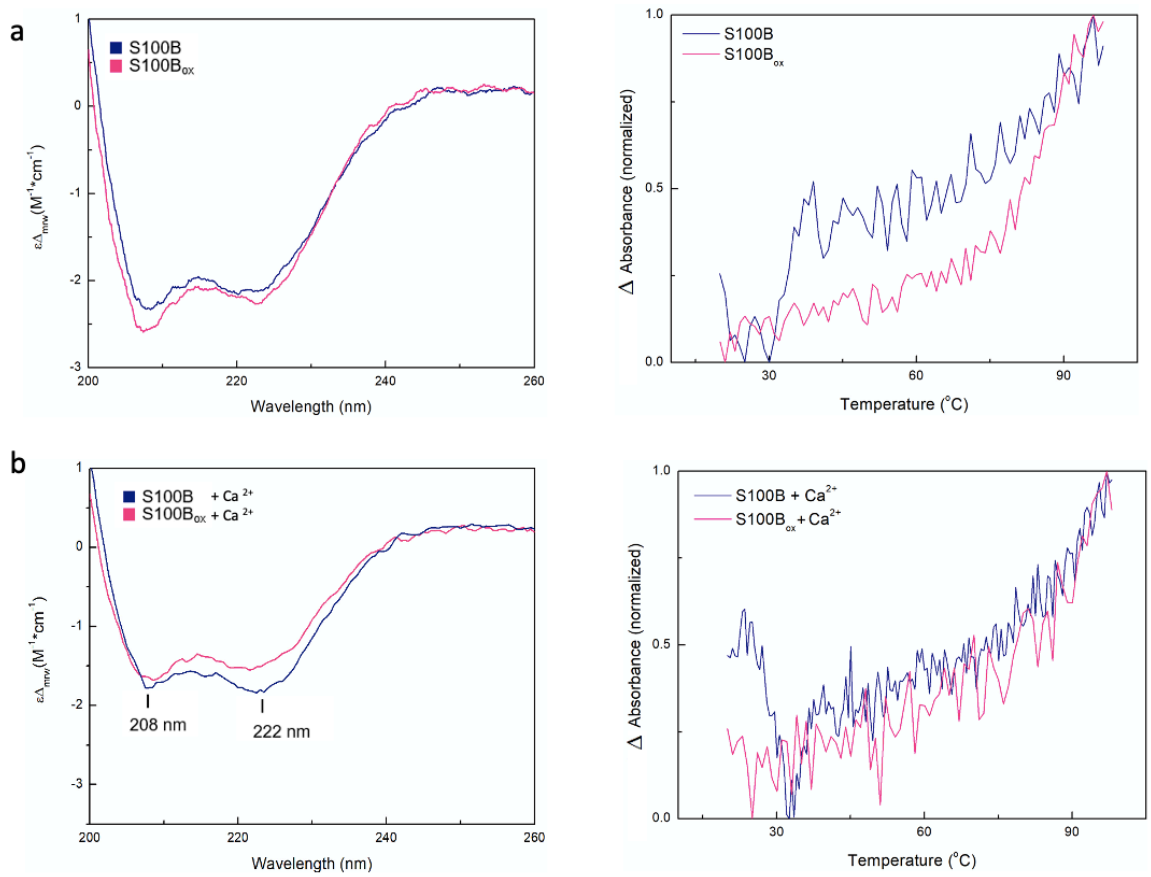

Fig. 3: CD spectra similarities of apo and Ca^{2+} bound $S100B_{ox}$ and S100B. a) shows apoS100B and b) Ca^{2+} bound S100B Far-UV CD spectra and thermal denaturation curve upon tracing ellipticity at 222 nm as a function of temperature.

To further examine these subtle conformational differences, we employed ANS fluorescence to determine whether oxidation would lead to an increase in calcium-binding-induced conformational changes. This phenomenon is known to enhance the exposure of hydrophobic patches on the protein surface, as ANS specifically binds to these regions [21]. We observed that S100B oxidized (S100Box) binds ANS, resulting in a slight blue shift from 510 nm (ANS) to 525 nm (S100Box) upon interaction with hydrophobic moieties after excitation at 370 nm [22] (Fig. 2c). This behavior is similar to that of non-treated S100B. Additionally, the increase in fluorescence emission suggests that there are no significant conformational changes in the tertiary structure.

We next assessed whether oxidation would lead to changes in the protein's quaternary structure. Specifically, copper binding to S100B induces a polymerization process, resulting in the formation of high-order multimers [23]. To investigate if oxidation would similarly affect this process, we employed size exclusion chromatography to monitor changes in the hydrodynamic radius of the protein depending on its oxidation state (Fig. 2d). The typical size exclusion chromatography (SEC) chromatogram for apo S100B displays a peak at 12.5 mL. Both the non-treated and oxidized forms of S100B exhibit peaks at this canonical elution volume, indicating that there is no alteration in the dimeric state of oxidized S100B.

To evaluate whether S100B oxidation affects the stability of protein dynamics, we conducted limited proteolysis experiments comparing the modified and unmodified proteins. The rationale behind this setup is that modifications impacting protein dynamics would render it more susceptible to proteolytic degradation. The results from the trypsin degradation assays indicate that apo S100B, even in its oxidized form, maintains a stable conformation over various time points, showing no significant differences from non-treated apo S100B (Fig. 4).

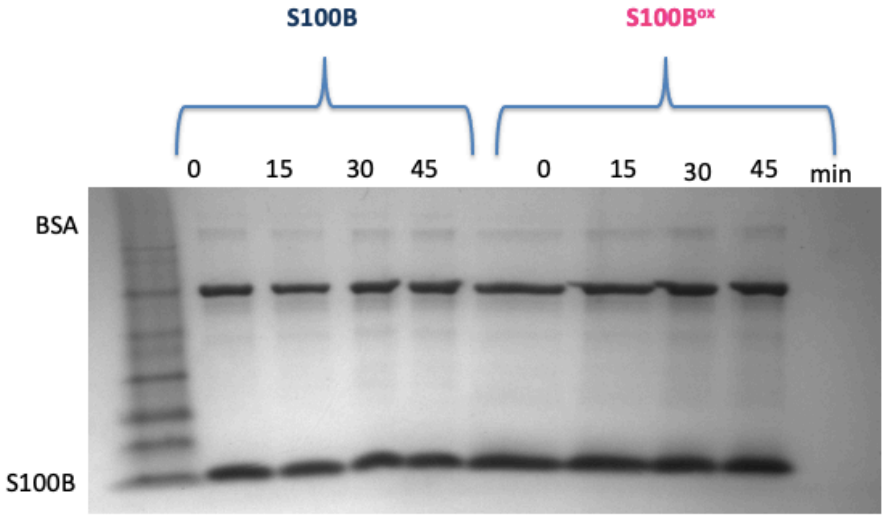

Fig. 4: Comparative Limited proteolysis of $S100B_{ox}$ and S100B. Limited proteolysis was performed at 37ºC employing trypsin digestion (0.4µM) of 20µM S100B/ S100Box, and aliquots were sampled at 0, 15min, 30min, and 45min. The reaction was stopped by adding the SDS/PAGE loading Buffer at these time points. The internal standard of loaded protein/well was acquired by 2.5µM bovine serum albumin (BSA) addition

Determination of effects of oxidized S100B on amyloid β aggregation

To determine whether oxidation impacts S100B's anti-aggregation chaperone activity, we performed kinetic experiments. To provide context for these results, it is necessary to briefly discuss the mechanistic insights that can be extracted from such experiments.

The aggregation of $A\beta_{42}$ is driven by a positive feedback loop stemming from interactions between its monomeric and fibrillar forms, known as the secondary nucleation mechanism. To elucidate the dominant mechanisms underlying aggregate proliferation and to characterize how a specific compound, in this case, S100B, influences amyloid beta aggregation, we studied the kinetics of $A\beta_{42}$ aggregation under quiescent conditions. We employed thioflavin T as a detection probe for amyloid formation. These experiments produced the characteristic sigmoidal transitions associated with the multiple stages of $A\beta_{42}$ self-assembly from which the reaction of times can be extracted, and the scaling exponent which is the slope from the negative linear correlation between the varying half-times and increasing monomer concentration when the secondary nucleation is the dominating microscopic process (Fig. 5b)

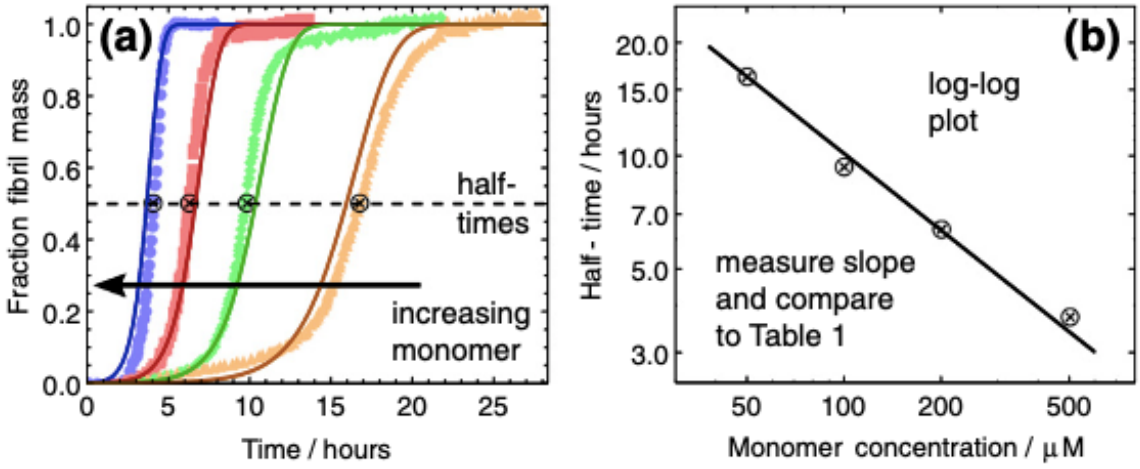

Fig. 5: Half-time scaling and global fitting of exemplary kinetic data. a) fitted data of increasing initial monomer concentrations, b) a log-log plot showing the scaling exponent (slope). (From [24])

By varying the concentrations of monomers, we can gain insights into how specific conditions affect the reaction halftime and the slope of the transition. These parameters provide valuable information about the aggregation process, which can be extracted through global fitting of the data according to domain mechanistic models, namely: primary

nucleation with no secondary pathways (Fig. 6b), fragmentation in addition to primary nucleation (Fig. 6c); and secondary nucleation, in addition to primary nucleation, (Fig. 6d) [25].

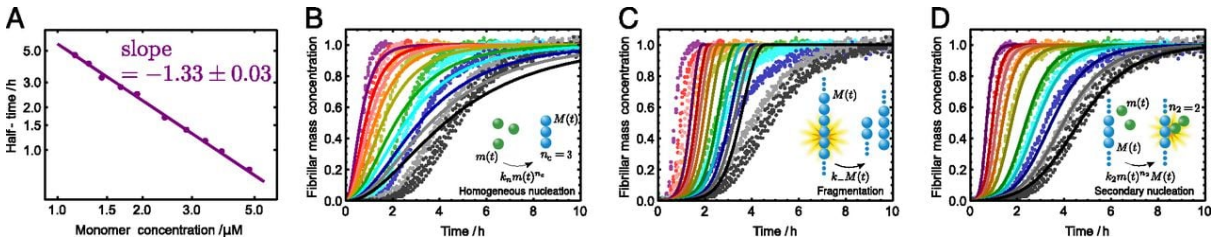

Fig. 6: Experimental kinetics of $A\beta_{42}$ aggregation (A) Power-law scaling of the time to half-completion with the initial monomer concentration. The slope gives the scaling exponent discussed in the text. (B–D) Global fits to the normalized experimental data, using the analytical solutions for systems where (B) the dominant nucleation mechanism is primary nucleation, and there are no secondary pathways; (C) a (dominant) fragmentation process is active in addition to primary nucleation; and (D) secondary nucleation, in addition to primary nucleation, creates new aggregates (From [25])

To verify if the microscopic aggregation mechanism is dominantly dependent on secondary nucleation or fragmentation, in comparison to complex primary nucleation that might only be poorly understood, one may add pre-formed seeds at the beginning of the kinetic reaction and compare both. As shown in Figure 7, a shift should be visible of the aggregation curve towards a more rapid creation of aggregates [24].

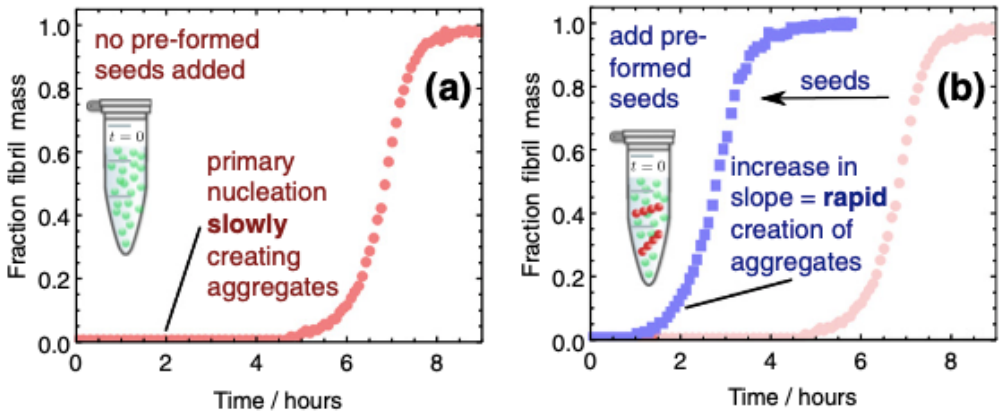

Fig. 7: Illustrates a sigmoidal curve exemplifying aggregation reaction in vitro from a solution of a) only monomers and b) a solution with pre-formed seeds added at the beginning of the reaction (Image from [24]).

These experimental approaches were employed to characterize how S100B oxidation might influence its chaperone activity, specifically by uncovering differences compared to the non-oxidized form. Considering the structural similarities of $S100B_{ox}$ and S100B and that oxidation is highly probable occurring in the $A\beta_{42}$ binding cleft, we speculated that oxidation of S100B might also be causing altered $A\beta_{42}$ fibrillation. To test this possibility, we analyzed the kinetics of $A\beta_{42}$ upon incubation with $S100B_{ox}$ using ThT emission as a fluorescent dye for protein fibrillation [17] [26] [27].

We observed that $S100B_{ox}$ further delayed $A\beta_{42}$ aggregation compared to its non-treated counterpart, half-times increasing from 4.5 h (+S100B) to 8.5 h (+$S100B_{ox}$) versus 0.9 h for $A\beta_{42}$ alone (Fig. 8). Next, we compared the effect of S100Box versus that of S100B on $A\beta_{42}$ fibril elongation using Aβ seeded aggregation assays. Briefly, $A\beta_{42}$ aggregation into fibrils proceeds through a series of microscopic states that involve the self-assembly of $A\beta_{42}$ monomers (primary nucleation, at rate k_1), with the formation of early fibrillar oligomers [28] that evolve to mature fibrils, upon the addition of Aβ monomers to fibril ends (elongation phase, at rate k_+) [25] [29] Interaction of the Aβ monomers with the fibrils prompt the formation of more oligomers (secondary nucleation, at rate k_2) [30].

Experimentally, the influence of a chaperone (or any other aggregation modulator) on fibril elongation can be accessed by employing conditions that accelerate primary nucleation, which can be achieved by adding pre-formed Aβ fibrils that will seed aggregation [28] [31]. In this case, aggregation assays in the presence of a high concentration of pre-formed $A\beta_{42}$ fibrils were then employed to assess how $S100B_{ox}$ affected fibril elongation. We noted that $S100B_{ox}$ is about three times more efficient than S100B in blocking fibril elongation (Fig 8b).

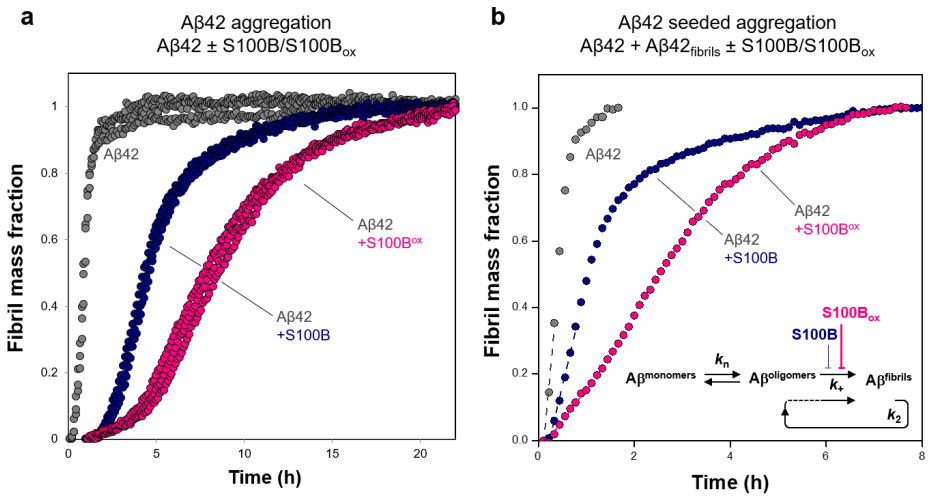

Fig. 8: Effects of oxidized and non-oxidized S100B on $A\beta_{42}$ aggregation. (**a**) Kinetic traces of ThT-monitored aggregation of monomeric $A\beta_{42}$ (5 μM) in the absence and presence of a 10-fold excess ratio of S100B (blue) and $S100B_{ox}$ (magenta) (in all cases n = 3). (**b**) Kinetic traces of ThT-monitored aggregation of monomeric $A\beta_{42}$ (5 μM) in the absence and presence of a 4-fold excess ratio of S100B (blue) and $S100B_{ox}$ (magenta) and seeded with 0.04 μM of pre-formed $A\beta_{42}$ fibrils (average traces, n = 3) [20].

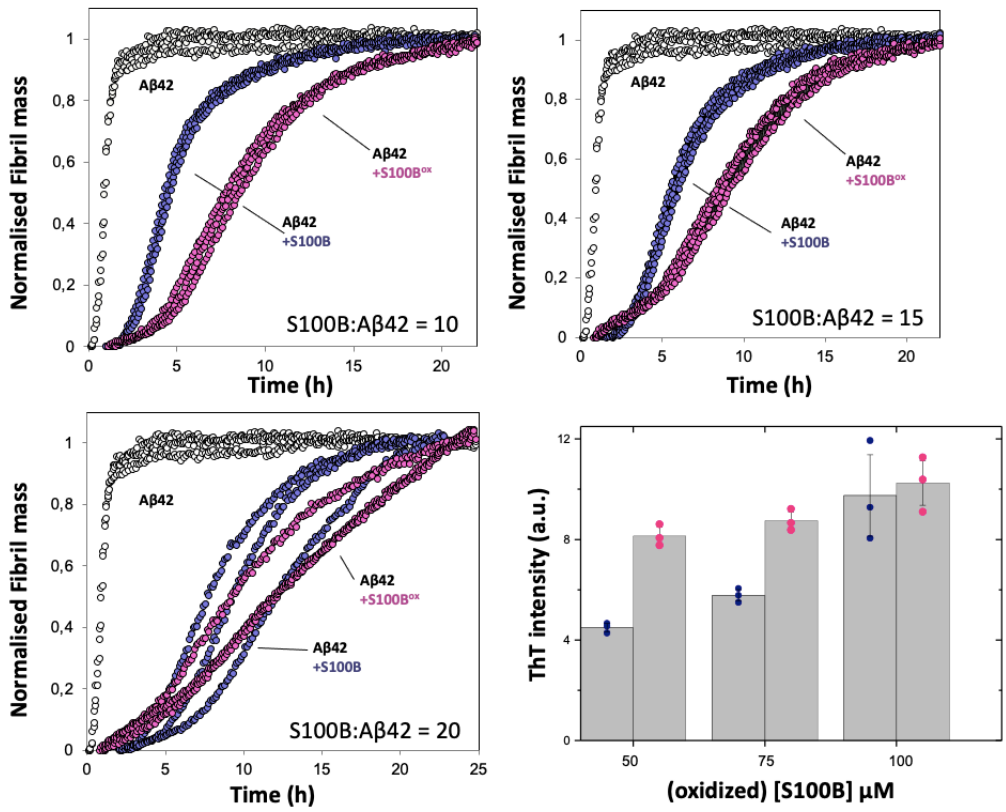

Fig. 9: Effects of oxidized and non-oxidized S100B on $A\beta_{42}$ aggregation at various concentrations. (**a**) Kinetic traces of ThT-monitored aggregation of monomeric $A\beta_{42}$ (5 µM) in the absence and presence of a 10-fold excess ratio of S100B (blue) and $S100B_{ox}$ (magenta) (in all cases $n = 3$) in comparison to **(b)** 15-fold and **(c)** 20-fold (average traces, $n = 3$). (**d**) Shows the corresponding, average half-times in hours by the concentration of (oxidized) S100B used, explicit values can be found in the table below. S100B (dark blue) and $S100B_{ox}$ in (magenta) ($n = 3$).

Half-times values

S100Box			S100B		
50 µM	**75 µM**	**100 µM**	**50 µM**	**75 µM**	**100 µM**
8.6 h	9.2 h	9.1 h	4.7 h	6.1 h	8.0 h
7.8 h	8.7 h	10.4 h	4.5 h	5.8 h	9.3 h
8.1 h	8.4 h	11.3 h	4.3 h	5.5 h	11.9 h

Table 1: Half-times. Values of half-times at concentrations 50µM, 75µM and 100µM of S100Box and S100B.

We further performed the same kinetic analyses in the ratios of 1:15 and 1:20. Upon an excess of calcium and under quiescent conditions at physiological pH 7.4 with 5µM $A\beta_{42}$. We found that $S100B_{ox}$ further delayed $A\beta_{42}$ aggregation compared to its non-treated counterpart and that half-times increased correspondingly (Fig. 9).

For S100B, it has been shown that the protein affects $A\beta_{42}$ fibrillation by affecting the primary and secondary nucleation processes of $A\beta_{42}$. For $S100B_{ox}$, what could be observed was a delay in $A\beta_{42}$ aggregation upon $S100B_{ox}$ addition. Reaction half times increase even at sub-stoichiometric ratios (Fig. 10).

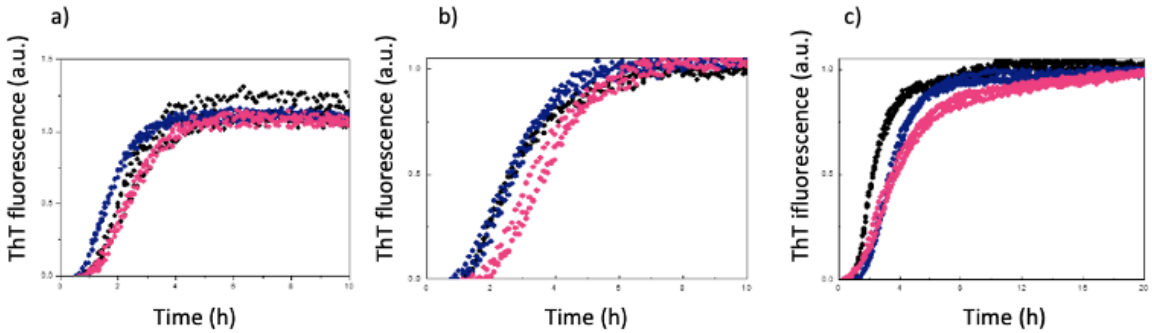

Fig. 10: $S100B_{ox}$ modulate $A\beta_{42}$ aggregation, even at sub-stoichiometric amounts. Shown is a sigmoidal aggregation reaction of primarily monomeric $A\beta_{42}$ into mature fibrils monitored by the probe: ThT (thioflavin-T) (black curve) in HEPES pH 7.4 at 37º under quiescent conditions. $A\beta_{42}$ with (**a**) half as much S100B or $S100B_{ox}$ than $A\beta_{42}$. (**b**) Equal as much S100B or $S100B_{ox}$ than $A\beta_{42}$. (**c**) Four times more S100B or $S100B_{ox}$ than $A\beta_{42}$. The plots

represent normalized intensity curves, each representative of the ratio tested, given that five concentrations were tested for each ratio. S100B is always shown in dark blue and $S100B_{ox}$ in magenta.

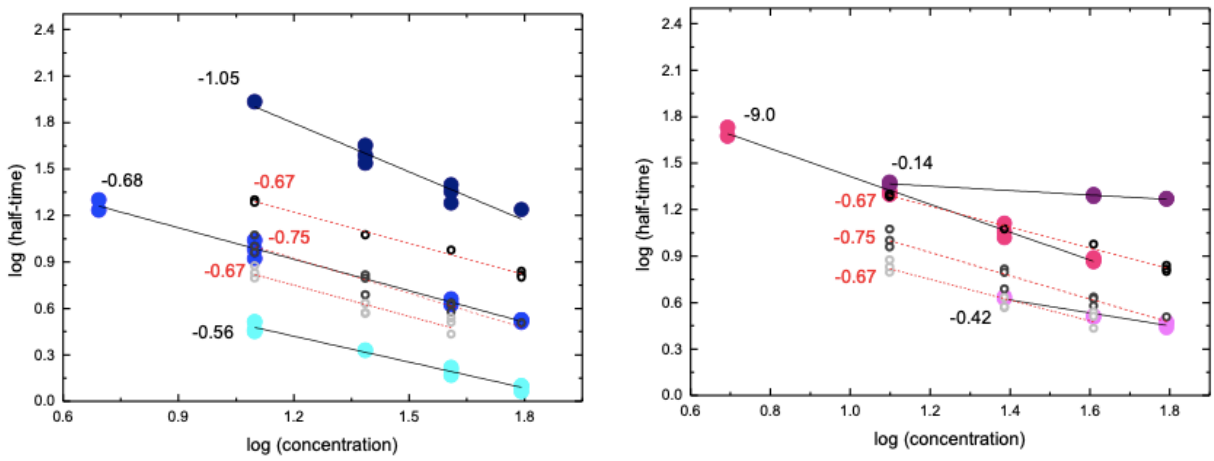

Fig. 11: Oxidized S100B modulates $A\beta_{42}$ aggregation. Log-log plot of the half-time of the $A\beta_{42}$ aggregation reaction as a function of the initial $A\beta_{42}$ monomer concentration in the presence of non-oxidized (blue) and oxidized (magenta) S100B while $A\beta_{42}$ shows a scaling exponent of -0.67, -0.75, -0.67 (red dashed line, grey circles). $A\beta_{42}$ with S100B or $S100B_{ox}$ also shows a scaling exponent that indicates domination of secondary nucleation events. The reaction occurred in HEPES at 50mM, pH 7.4, and excess of $CaCl_2$ (1.1 mM) and was monitored by ThT. Light colour corresponds to $S100B_{(ox)}$: $A\beta_{42}$ 0.5:1, a darker tone corresponds to $S100B_{(ox)}$: Aβ42 1:1, whereas dark blue, purple and dark grey corresponds to $S100B_{(ox)}$: $A\beta_{42}$ 4:1.

Previous was not observed when untreated S100B was added to $A\beta_{42}$. To determine if $S100B_{ox}$ changes this $A\beta_{42}$ aggregation mechanism under physiological conditions, the scaling exponent (γ) was determined (Fig. 11), suggesting that oxidation does not affect the dominant aggregation mechanism. Upon increasing $S100B_{ox}$, an increase of the half-times can be observed in the ratios 1:1 $A\beta_{42}$: $S100B_{ox}$, but the linear plot is maintained, suggesting that $S100B_{ox}$ delays $A\beta_{42}$ aggregation without altering the dominant nucleation events. Instead, secondary nucleation seems to be the dominant aggregation mechanism at ratios 0.5:1 $A\beta_{42}$: $S100B_{ox}$ and 1:4 $A\beta_{42}$: $S100B_{ox}$. To further investigate the role of $S100B_{ox}$ on $A\beta_{42}$ seeding potency, we performed a seeding experiment (in the presence of calcium). To

investigate if $S100B_{ox}$ acts as an aggregation inhibitor in the case of seeded $A\beta_{42}$, we monitored ThT aggregation kinetics of $A\beta_{42}$ seeds added to calcium-bound S100B $_{ox}$: $A\beta_{42}$ at ratios of 1:1 (low seeding) and 1:4 (high seeding) to see the effect of elongation in the aggregation mechanism. In addition, the slightly biphasic sigmoidal $S100B_{ox}$ curve could indicate presence of two MetO diastereomeric forms of S100B [8] [32]. One possibly being more efficient in inhibiting Aβ aggregation, showing the biphasic behavior in Aβ aggregation (Fig. 12-13). In fact different diastereomeric forms often show a difference in bioactivity [33].

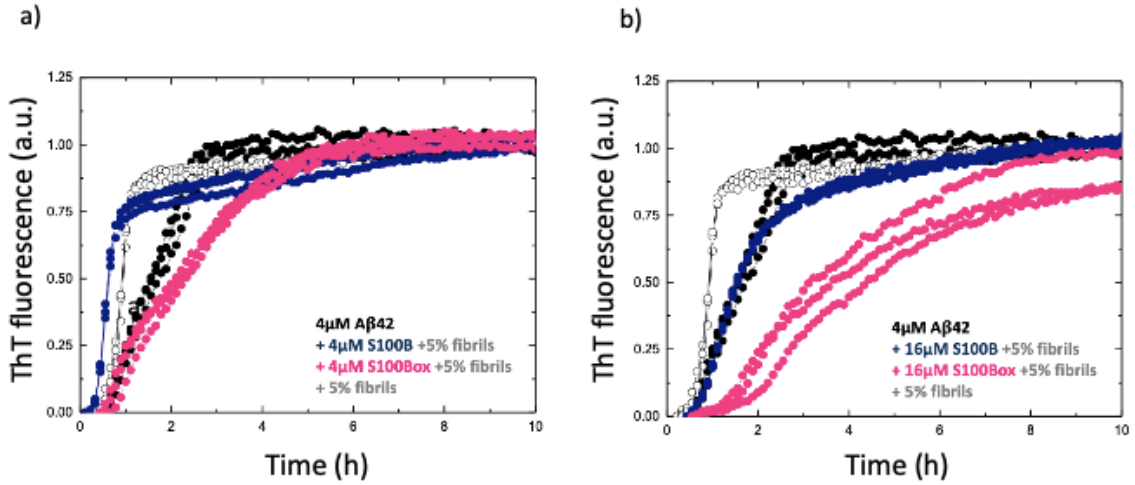

Fig. 12: $S100B/_{ox}$ with low $A\beta_{42}$ seeding. Shown is a sigmoidal aggregation reaction of primarily monomeric $A\beta_{42}$ into mature fibrils monitored by the probe: ThT (thioflavin-T) (black curve) in HEPES pH 7.4 at 37º under quiescent conditions. $A\beta_{42}$ with (**a)** Equal amounts of S100B or $S100B_{ox}$ than $A\beta_{42}$ plus seeded with 5% fibrils or only $A\beta_{42}$ with 5% fibrils. (**b**) Four times more $S100B/_{ox}$ than $A\beta_{42}$ seeded with 5% fibrils. The plots represent normalized intensity curves, each representative of the ratio tested. (S100B (dark blue) S100B-OX (magenta)).

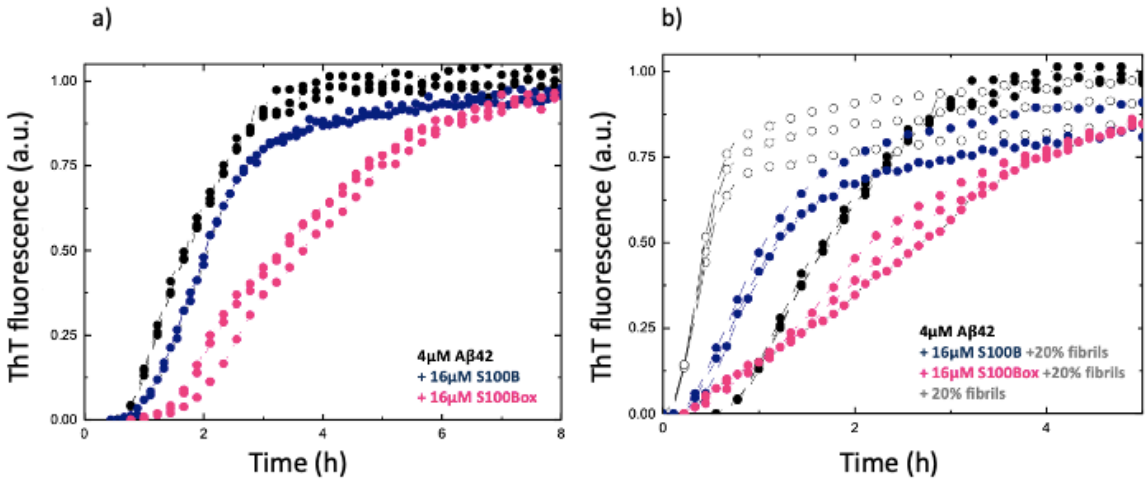

Fig. 13: S100B or $S100B_{ox}$ with high $A\beta_{42}$ seeding. Shown is a sigmoidal aggregation reaction of primarily monomeric Aβ42 into mature fibrils monitored by the probe: ThT (Thioflavin-T) (black curve) in HEPES pH 7.4 at 37º under quiescent conditions. Aβ42 with **(a)**: four times the amounts of S100B or $S100B_{ox}$ than $A\beta_{42}$ (unseeded). **(b)** Four times more $S100B_{ox}$ than $A\beta_{42}$ seeded with 20% fibrils. The plots represent normalized intensity curves, each representative of the ratio tested. S100B is always shown in dark blue and $S100B_{ox}$ in magenta.

4. Conclusion

Molecular chaperones play key roles in proteostasis regulation, preventing misfolding and the accumulation of toxic aggregates which are a hallmark in several age-related neurodegenerative diseases. Molecular chaperones are potentially neuroprotective, given their ability to modulate initial aberrant protein interactions that prevent toxic conformers that trigger pathogenic cascades. This is commonly achieved by establishing protective interactions between a chaperone and its client that prevent or recover a misfolded conformed. One such holdase-type activity has been recently uncovered for S100B which is able to mitigate the pathological self-assembly of Aβ42 and to decrease toxicity. This novel activity is particularly interesting, given the fact that S100B has both an intra- and extra-cellular function, being found to be associated with amyloid plaques in AD animal models upon their expression and secretion by activated astrocytes. This suggests its potential

relevance in the biological setting and potential as a drug target, given the limited number of known secreted chaperones. Importantly, the fact that S100B, like DNAJB6 and the Brichos domain, are able to inhibit Aβ42 secondary nucleation, which is the main route to generate toxic oligomers, makes this type of proteins attractive inspirations for translational AD therapies. Our results suggest a unique way of modifying the S100B function using oxidation that may occur in response to specific pathologies: through oxidation, the pro-inflammatory activity of S100B may be turned into an anti-inflammatory activity during the '$A\beta_{42}$ detoxification' by S100B, and, through oxidation, its own expression levels may be controlled.

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Chapter V
Oxidized S100B increases cell health in an astrocytic AD model

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Abstract

This chapter presents the relationship between the oxidized/non-oxidized forms of S100B and their inflammatory response. How functions of glial cells are changed upon pathological stimuli, leading to oxidative stress and altered cytokine expression, will be discussed. How such a cytokine, in its oxidative state, can be studied in an AD model will further be addressed. To that end, we will test the effect of oxidized S100B in an astrocytic AD model using a dynamic, real-time, and label-free way. Adding low molecular $A\beta_{42}$ species to astrocytic cells and cotreating them with oxidized S100B ($S100B_{ox}$) shows promising effects on cell health. The expression of cytokines in this AD model was also tested (for IL-17, INF-α and S100B). We are further corroborating a beneficial role for $S100B_{ox}$ and opening the possibility of a negative feedback loop of S100B expression.

1. Introduction

Astrocytes are the most numerous cells in the central nervous system (CNS). They are essential in including neuronal maintenance and support, synaptic activity, and amyloid β (Aβ) clearance. Based on morphology and function, astrocyte populations vary and distinguish themselves in white and grey matter. Since protein homeostasis is perturbed in AD, dysregulation of astrocytes and new subtypes can be observed, which may be related to neuronal death [1]. Astrocytes may undergo changes in morphology and function, termed reactive astrogliosis, which can be triggered by inflammation. Astrocytes, even though they may show an elevated level of heterogeneity in an inflammatory context, are involved in promoting or counteracting the inflammatory response [2].

In the presence of Aβ, disruption of glia-transmission and changes in Ca^{2+} signaling could be found [3]. Signaling pathways like the NF-kB signaling pathway, which may lead to the transcription of cytokines in astrocytes, among other signaling pathways, are prominently activated [3]. Oxidative stress is also a co-appearance when Aβ is present, and astrocytes also seem to be involved in that i.e. producing, upon Aβ, reactive oxygen and nitrogen species affecting intracellular calcium levels, NF-kB signaling and glutamate uptake for instance [3]. In more detail, astrocytes express many receptors that Aβ can interact with e.g. RAGE, acetylcholine receptors, complement and chemokine receptors, T-cell receptors to name some. Binding and mainly effects are not thoroughly studied yet, however binding seems to

depend on the aggregation form of the binding Aβ peptide(s). RAGE, a receptor that was studied in this context, induces an increase in reactive oxygen species production via activation of the NADPH oxidase complex. Independently, through the ERK1/2 pathway and cytosolic phospholipase A2 phosphorylation astrocytes can cause mitochondrial dysfunction by decreasing its membrane potential, in this case, NADPH oxidase overactivity and increased reactive oxygen species production follow [3].

1.1 Neuroinflammation

Data from AD research shows an immune response is triggered by proteins being misfolded or aggregated, binding to pattern recognition receptors on glial cells releasing inflammatory mediators. This immune response contributes to disease progression and aggravation. Neuroinflammation is an active system that may contribute much or even more to AD pathogenesis than plaques and tangles [4]. Mild cognitive impairment is related to the involvement of inflammation already at early stages. Microglia and astrocytes are, upon pathological stimulia in the brain, one of the primary cells, playing a role in neuroinflammation [4].

1.1.1 Microglial and Astrocytic Brain Cells

Microglia

Microglia are known to be the phagocytes of the CNS. However, they also play a role in the plasticity of neuronal circuits, such as tissue surveillance and the remodeling of protection of synapses, partly mediated by trophic factors [4]. They make up, brain region dependent, up to 12% of the total number of brain cells [5].

Once pathological triggers like aggregated proteins are activated and detected by danger-associated molecular patterns (DAMPs), microglia dislocate to the injury site and initiate an immune response [4]. Microglia retract extended filopodia and form condensed structures [6]. The dislocation then occurs with the help of chemokines. Microglia, now involved in aiding clearance and promoting the restoration of protein homeostasis, have their other non-inflammatory functions compromised [4]. Sustained exposure to these triggers leads to chronic inflammation, proinflammatory cytokine exposure, and the absence of the usual beneficial functions of microglia, leading to the degeneration of neurons [4]. Extracellular

S100B has been shown to affect microglial activities, concentration dependently. At high concentrations S100B has been shown to activate microglia, stimulating inducible nitric oxide synthase (iNOS), interleukin (IL)-1beta release, for instance and possible lead to the upregulation of proinflammatory genes via NF-κB, later, in a RAGE dependent manner [7].

Astrocytes

The crosstalk between microglia and astrocytes is intimate [8]. Astrocytes are best known for their interactions with neurons, neurotransmitter recycling ion homeostasis, remodeling of synapses, and oxidative stress modulation. With they enveloping (as many as) 100,000 synapses, (grey matter) astrocytes can have neuro-supportive effects. However, a pathological stimuli results in a drastic change and may severely affect the integrity of CNS neuronal tissue. Upon such a pathological stimulus, astrocytes change rapidly in morphology and function, for instance. Being in this reactive state, they may show both beneficial and detrimental effects [9]. The reactive, hypertrophic astrocytes in AD were found to accumulate around senile plaques [4], which reminds one of astrocytic scar formation, a way to create a barrier between healthy and injured tissue [9]. However, no data supports astrocytic scar formation in the classical sense [4]. Monocyte chemoattractant protein-1 (MCP1) is chemotactic for adult astrocytic cells and very abundant in Aβ plaques, besides expressing receptors like receptors for advanced glycation endproducts (RAGE) that may bind Aβ. Aβ clearance may happen upon engulfment and degradation of Aβ by astrocytes. In turn, Aβ may be detrimental to astrocytic calcium homeostasis, and reactive astrocytes are shown to lead to neurodegeneration in astrocyte-neuron cocultures [9].

Notably, a specific correlation has been found between cognitive changes and the development of neuroinflammation [3]. Further, *in vitro*, exposing cultured astrocytes to Aβ had different effects depending on the Aβ aggregation state, be it oligomeric or fibrillar [9]. In line, it has been suggested that a correlation exists between cognitive decline and the level of astrogliosis [10]. Besides the changes astrocytes undergo, they are also known to release cytokines and nitric oxide, among other factors [4].

In more detail, Cytokines may stimulate inducible nitric oxide synthase in glial cells, producing high concentrations of reactive nitrogen species (RNS) that may be toxic to the neuronal cells, causing post-translationally modified proteins (/peptides), for instance, Aβ. Oxidized Aβ has been shown to increase Aβ aggregation propensity and found localized to

the core of Aβ plaques. Oxidized Aβ suppresses LTP more efficiently than its non-oxidized counterpart [4].

1.1.2 Cytokines

Cytokines are secreted proteins crucial in several steps of the immune response, including regulation. Further ones are involved in pro-inflammatory or anti-inflammatory processes [11]. They are immune proteins, soluble communication factors cells use to communicate. However, as seen for S100B (Chapter 3), their role is not restricted to inflammation. They may regulate the proliferation and differentiation of cells in the CNS. The consequences of cytokines on brain functions upon AD are not well studied yet, but mice studies suggest potent effects. Cytokine mRNAs or proteins may be up or downregulated/expressed in AD brains, and AD mouse models show partly beneficial effects on amyloidosis, among them IL-6 [9].

Cytokine Il-17

IL-17 is a proinflammatory cytokine [11]. In AD patients' brains and AD mouse models, IL-17 levels are three-fold these of age-matched controls. Il-17's physiological role includes, besides its involvement in immunity and extracellular pathological defense, a role in learning, possibly because of its capacity to up-regulate BDNF [12]. In AD, however, multiple evidence suggests it plays a pro-inflammatory role. Astrocytes express the IL-17 receptor IL-17RA/IL-17RC and trigger the NF-kB signaling response [13]. IL-17 in AD has been suggested to promote impairments in olfactory senses, cognition, and involvement in the mediation of Aβ and possibly the inhibition of Aβ clearance [12].

Cytokine IFN-a2

Helical cytokine superfamily Interferons (IFN) are best known for their involvement in pro-inflammatory activities. Different types – I, II, III – exist based on their receptors. Type I is subdivided into 14 types, some α and some β. These bind to receptors IFN-α/β [14] and operate via the JAK-STAT signaling pathway [15]. In the brain, astrocytes are one of the main producers of IFN type I. IFN type I may downregulate the expression of BDNF, among other neurotrophic factors. In AD and AD models, IFN-α levels were increased, including mRNA levels [14].

Cytokine S100B

S100B makes part of the pro-inflammatory cytokines and triggers glial cell proliferation dependent on RAGE [16]. In a transgenic AD mouse model (APPV717F), S100B could be shown to increase with age. This increase stops just before Aβ deposition. However, S100B overexpressing mice showed a leveled-up inflammatory response to Aβ when intracerebroventricularly-infused compared to control mice [9]. As seen in the previous chapters, this research still leaves many open questions.

2. Astrocyte model culture and its analysis in real time

The AD cell model used in this thesis consists of DI TNC1 cells treated with $A\beta_{42}$. Adherent DI TNC1 astrocytes, origin from primary cultures of type 1 astrocytes from one day old sprague-dawley rat brains (https://www.culturecollections.org.uk/nop/product/di-tnc1), to immortalize the cell line, it was transfected by placing a SV40 large T antigen under the human promoter of GFAP. DI TNC1 cells retain characteristics of the phenotype of type 1 astrocytes [17]. They express S100B and secret cytokines in a similar manner to primary astrocytic cultures [18]. They fulfill the requirements of showing astrocytic properties as mentioned above. They allow reproducible results, even after a few culture cycles and are cost-effective in comparison to primary astrocytic cell cultures. Besides being easy to handle which reduces error, they reduce animal use.

To investigate an insult on the above mentioned cell model an xCELLigence, a commercial instrument for Real Time Cell Analysis was used. In comparison to other methods it allows the combination of several aspects (advantages): continuous monitoring of the cells which allows early detection of cellular responses for instance. It is non-invasive and lable-free which allows cells to maintain cell physiology. In addition, it is automated which makes it less prone to experimental artifacts. It allows following cell health in real-time and makes high-throughput screening possible [19]. In such a model, astrocytes can be plated in a monolayer on cell culture microplates with special microelectrodes at the bottom of the well. Adherent astrocytic cells attach on the well bottom, allowing the measurements of changes in electrical impedance with the xCELLigence RTCA Systems (Agilent/ACEA Biosciences). The result analysis can then be done with the RTCA DPlus Software. Together, they allow

cell health data among others to be obtained in a dynamic, real-time, lable-free way. Upon an insult, measurements of cell death (in this case, detachment) from the well bottom, for instance, may be collected [20]. The readout is a unitless “cell index” [19].

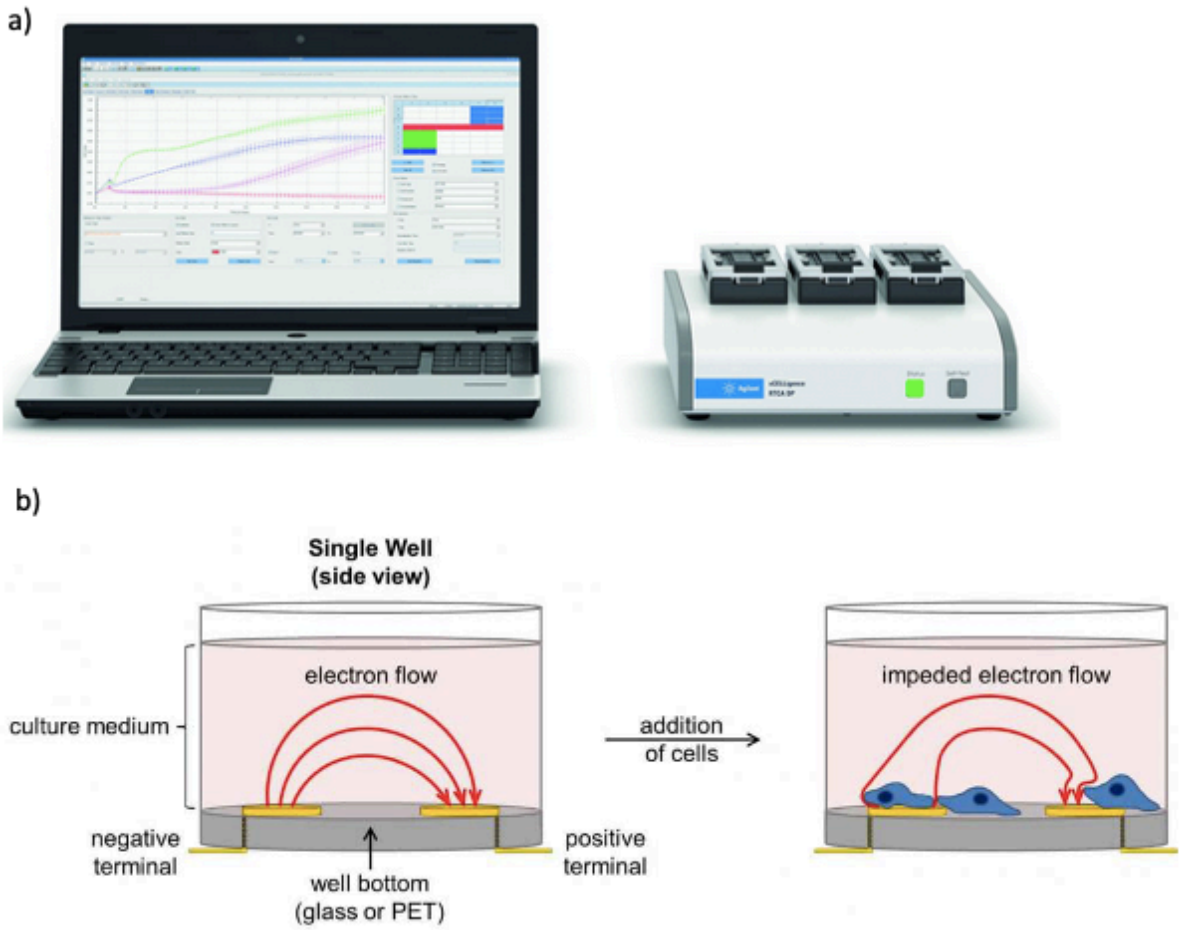

Fig. 1: xCELLigence. a) Real time cell analysis being done by the commercially available instrument, xCELLigence. b) Gold electrodes at the bottom of the wells measure an electrical impedance generated by cell attachment. The cell index is then provided, a unitless readout [19]. (Image from https://www.ols-bio.com/xcelligence and https://www.accela.eu/agilent-technologies/xcelligence-s16)

As discussed in chapters 3 and 4, ROS and RNS are highly abundant in the AD brain, and the high abundance of S100B in the AD brain makes it an easy "target" for an oxidative post-translational modification. Here, we wanted to *understand the impact of an oxidatively modified S100B on the above astrocytic AD cell model.*

3. Material and Methods

Cell Culture

DI TNC1 (ATCC, Manassas, VA, USA) rat astrocytes were cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 2% glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin. Cultures were maintained at 37°C with 5% CO_2 until they reached the 80% confluence necessary to perform the experiments.

Quantitative Real-Time PCR (qRT-PCR)

DI TNC1 cells were seeded on poly-L-Lysine (PLL) (0.1 mg/mL) coated Petri dishes 10 cm in diameter and stored in the incubator until a monolayer (80% confluency) was reached. Afterward, the medium was replaced with a new medium for control or one already containing the treatment. The treatment of the $A\beta_{42}$ condition was 5 µM $A\beta_{42}$. For the S100B or S100Box plus $A\beta_{42}$ condition, a co-administration of 10 µM S100B or oxidized S100B occurred, respectively. Then, 72 h later, total RNA isolation was performed using the RNeasy Mini kit (Qiagen, Manchester, UK), as described by the manufacturer. Isolated RNA was eluted in 30 µL RNAse-free water and kept at −80 °C. Quantitative real-time PCR was performed using the Rotor-Gene SYBR Green RT-PCR kit (Qiagen). First-strand synthesis and real-time qRT-PCR amplification (Roche LightCycler 480 II, Basel, Switzerland) were performed in a one-step, single-tube format. Validated primer pairs from Qiagen (Quantitect primer assay) were used. For the internal standard, obtained data were analyzed using the hydroxymethylbilane synthase (*hmbs*) gene. All reactions were run at least in technical triplicates. Virtual mRNA levels were calculated from mean ct values according to virtual mRNA level = 10 ((ct(target) − ct(standard))/slope of the standard curve.

Impedance-Based Cell Health Assay

DI TNC1 cells were seeded at 5,000 cells per well on a PLL-coated E-16 plate (Agilent /ACEA Biosciences, San Diego, CA, USA). After attachment and 24 h of growth, the cells were treated with 5 µM $A\beta_{42}$ or 5 µM $A\beta_{42}$ co-administered with 10 µM S100B/10 µM S100Box. Controls were left untreated. Impedance was measured every 5 min in the following 70 h, employing the xCELLigence RTCA Systems (Agilent/ACEA Biosciences) with RTCA DPlus Software. A decrease in microelectronic impedance measured as (delta)

cell index indicated a decrease in proliferation and detachment of the cells from the bottom of the wells as a sign of cytotoxicity.

Statistical Analysis

For statistical analysis of the data sets, GraphPad Prism 8.0.2 was employed. All data are shown with standard error of the mean (SEM). Data were analyzed by one-way ANOVA and appropriate *post hoc* tests (e.g., Tukey's test). Statistical significance corresponded to a significance level of $\alpha \leq 0.05$. Significances are stated with p values < 0.05 *; <0.01 **; <0.001 ***.

4. Results

Effects of oxidized and non-oxidized S100B on cell health in AD

We investigated whether S100B oxidation can modify $A\beta_{42}$-induced toxicity using an astrocytic cell line in the following experiments. To that end, the health of DI TNC1 cells was assessed by measuring the impedance of astrocytes grown for 24 h (Figure 2a, b). In real-time, cell proliferation/cell viability was monitored after adding a low molecular species of $A\beta_{42}$ (5 μM) [21] [22], with or without S100B or oxidized S100B in a ratio of 1:2 for another 46 h. The results show that $A\beta_{42}$ treatment induces evident cell toxicity. S100B co-administration did not result in a significantly lower $A\beta_{42}$-induced toxicity. In contrast, co-treatment with $S100B_{ox}$ led to significantly lower $A\beta_{42}$-induced toxicity (Fig. 2b) after 46 h of treatment.

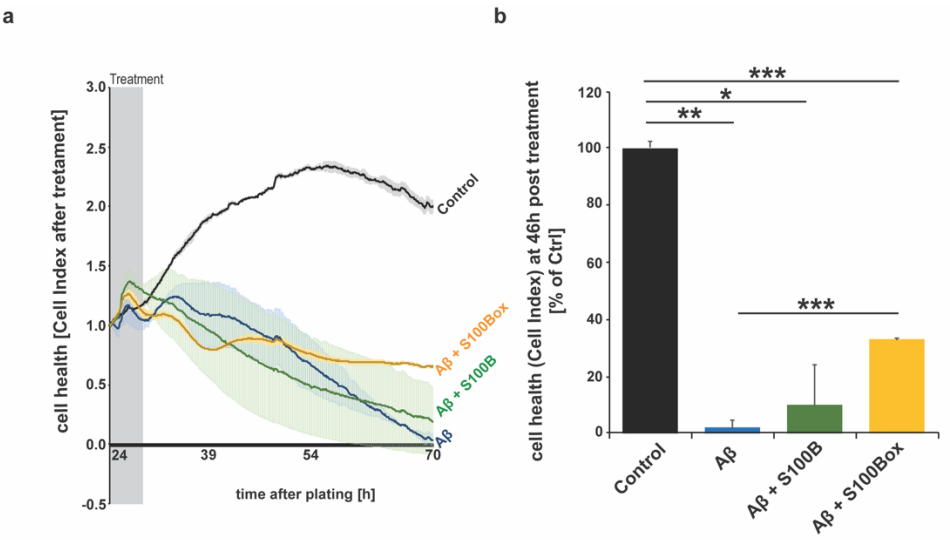

Fig. 2: Cell health assessments using E-Plates and an xCELLigence RTCA instrument. (**a**) Real-time impedance traces were obtained using real-time monitoring of astrocyte cell adhesion and proliferation treated with $A\beta_{42}$, $A\beta_{42}$ + S100B, and $A\beta_{42}$+$S100B_{ox}$ (n = 2–3 wells per condition). While in the first phase, no difference between the treatment conditions was observed, cells exposed to $A\beta_{42}$+$S100B_{ox}$ showed recovery starting at around 15 h post-treatment. Untreated control cells proliferate until 100% confluency, and cell health decreases after reaching this point (54 h). After $A\beta_{42}$ treatment, a continuous decrease in cell health was observed due to the detachment of dead cells. (**b**) After 46 h, significant differences between treatment conditions were observed (one-way ANOVA, F = 23.3995; p = 0.0023). $A\beta_{42}$ treatment significantly reduced cell health compared to untreated controls (p = 0.0013). Additional S100B treatment did not significantly reduce the toxicity of $A\beta_{42}$ ($A\beta_{42}$ vs. $A\beta_{42}$+S100B: p = 0.64). In contrast, $S100B_{ox}$ partially rescued $A\beta_{42}$ toxicity (Ctrl vs. $A\beta_{42}$+$S100B_{ox}$: p = 0.0001; $A\beta_{42}$ vs. $A\beta_{42}$+S100Box: p = 0.0005).

The effect of S100B and its oxidized counterpart on cytokine gene expression

As mentioned before, the release of inflammatory cytokines has been reported in AD model systems as well as in patients [23] [4]. IL-17 levels are elevated in AD [24]. Through the IL-17 receptor, IL-17 mediates activation of the transcription factor in NF-kB and downstream kinases. These kinases, in turn, lead to the generation of further pro-inflammatory signaling molecules and the attraction of immune cells to the site of inflammation [25]. Depleting IL-17 with an IL-17 antibody pre-treatment prevented $A\beta_{42}$-induced neurodegeneration and memory decline in mice [26]. In addition, Interferon-α (IFN-α) is a cytokine found to be elevated in the frontal cortex of AD brains [27]. Astrocytes mainly express IFN-α upon Toll-

like receptor activation. Interferons are helical cytokines, and, like IL-17, they mediate pro-inflammatory signaling [28].

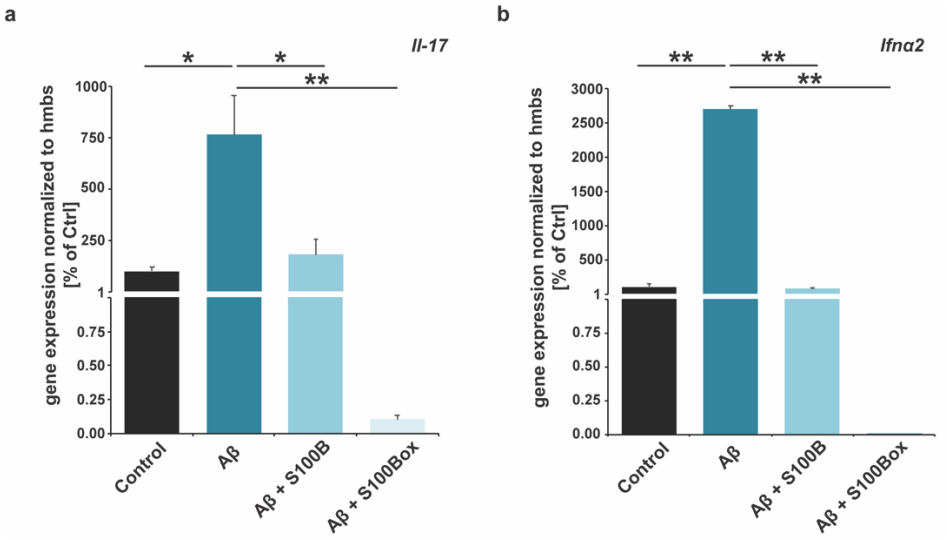

Fig. 3: Inflammatory cytokine expression after exposure to $A\beta_{42}$ with and without S100B or oxidized S100B. DI TNC1 astrocytes were treated with 5 µM $A\beta_{42}$ and 10 µM S100B or $S100B_{ox}$ plus $A\beta_{42}$. RNA expression levels were normalized to *hmbs* and presented in the percentage of control (n = 3). (**a**) Significant changes were detected in *Il-17* expression (one-way ANOVA: F = 9.4102; p = 0.0075). $A\beta_{42}$ treatment significantly increases *Il-17* expression (Tukey's test, p = 0.0136). The addition of S100B normalizes *Il-17* expression levels (Tukey's test, Aβ vs. Aβ + S100, p = 0.0252; Ctrl vs. Aβ + S100, n.s.). $S100B_{ox}$ also down-regulates $A\beta_{42}$-induced *Il-17* expression (Tukey's test, Aβ vs. Aβ + $S100_{ox}$, p = 0.0065; Ctrl vs. Aβ + $S100_{ox}$, n.s.). (**b**) Significant changes were detected in *Ifn-α2* expression (one-way ANOVA: F = 989.4808, p = 0.0000034). $A\beta_{42}$ treatment significantly increases *Ifn-α2* expression (Tukey's test, p = 0.00101). The addition of S100B normalizes *Ifn-α2* expression levels (Tukey's test, Aβ vs. Aβ + S100, p = 0.0010053; Ctrl vs. Aβ + S100, n.s.). $S100B_{ox}$ also down-regulates $A\beta_{42}$-induced *Ifn-α2* expression (Tukey's test, Aβ vs. Aβ + $S100_{ox}$, p = 0.0010053; Ctrl vs. Aβ + $S100_{ox}$, n.s.).

Next, we investigated the anti-inflammatory mechanism following exposure to $S100B_{ox}$ (Fig. 3). Upon inducing $A\beta_{42}$ cytotoxicity, we analyzed mRNA levels of IL-17 and IFN-α2. IL-17

mRNA levels are significantly increased in the experimental group in which the astrocytes were treated with $A\beta_{42}$ alone. In comparison, upon S100B or $S100B_{ox}$, a significant decrease in mRNA was documented. For IFN-α2, the same could be observed.

IFN-α2 is secreted by dying cells. Its receptor is ubiquitously expressed in humans, making IFN-α2 capable of acting on an extensive range of body cells. Inflammatory cytokines can decrease the proliferation rate of dividing cells and work in an immunomodulatory way [29]. Interestingly, in the described experiment, IFN-α2 mRNA levels were significantly increased upon cell treatment with $A\beta_{42}$ alone. However, upon S100B or $S100B_{ox}$, significantly lower mRNA levels could be registered.

Therefore, we determined $A\beta_{42}$ induced cytokine S100b mRNA level release from astrocytes upon S100B and $S100B_{ox}$. When comparing the S100B expression of cells treated with $S100B_{ox}$ plus $A\beta_{42}$ to astrocytic cells treated with $A\beta_{42}$, only a significant difference in S100B mRNA levels was found. However, $S100B_{OX}$, but not S100B treated astrocytes, had a significantly reduced $A\beta_{42}$-induced expression of S100B (Fig. 4).

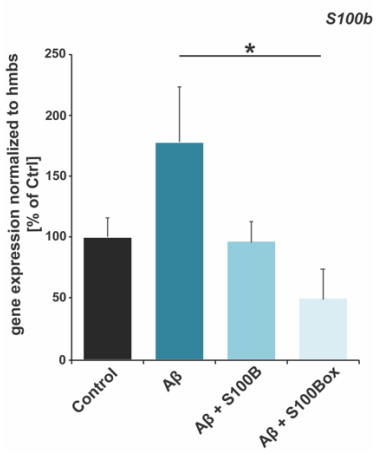

Fig. 4: *S100b* gene expression after exposure to $A\beta_{42}$ with and without S100B or oxidized S100B. DI TNC1 astrocytic cells were treated with $A\beta_{42}$ and $A\beta_{42}$ plus 4 µM S100B and oxidized S100B peptides (ratio 2:1). RNA expression levels were normalized to *hmbs* and are shown in percent of control (n = 3–6). $A\beta_{42}$ treatment increases *S100b* expression. Only $S100B_{ox}$ leads to a significant rescue (one-way ANOVA: F = 3.5366, p = 0.05, with Tukey's test: Aβ vs. Aβ + $S100_{ox}$, p = 0.0395671; Ctrl vs. Aβ + $S100_{ox}$, n.s.).

5. Conclusion

In our study cell health was assessed using DI TNC1 astrocytic cells. Cell coated E-plates and an XCELLigence instrument, showed control cells proliferating until confluency at 54 h after plating (Fig. 2). They reached over double the cell index, at that time point. S100Box + $A\beta_{42}$ treated cells seem to relatively maintain cell number, (how/why this may be the case will be discussed further in Chapter 6). $A\beta_{42}$ + S100B cotreated cells seem to oscillate in cell number. However, the mean value shows a decrease in astrocytic cell number and therefore in cell health. To note is that the xCELLigence E-plate allows the cells to attach at the bottom of the well, which they do. Upon $A\beta_{42}$ and an increase of S100B however, a pathological and "alarming" stimulus, astrocytes may lead to the retraction of astrocyte filipodia as discussed previously. They may acquire a condensed 'ameboid' shape, ready to dislocate, possibly decreasing their attachment to the well bottom at differing rates and hereby increase their susceptibility to $A\beta_{42}$ toxicity of the remaining filopodia. The $A\beta_{42}$ treated wells show, as expected, a decrease in cell health. All tendencies become more pronounced at 46 h of treatment.

Considering the importance of cytokine expression in the context of AD, we tested for S100B, IL-17, and INF-α expression, all pro-inflammatory signaling mediators. The anti-inflammatory mechanism found following an Aβ insult and the additional exposure to $S100B_{ox}$ by analyzing mRNA levels of S100B, IL-17, and IFN-α2 showed a significant decrease in mRNA levels compared to control (non-oxidized S100B). The reduced *S100b* expression upon $S100B_{ox}$ is particularly interesting, as it might indicate astrocytic S100B autoregulation, also further discussed in the next chapter.

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Chapter VI

Discussion

Abstract

Molecular chaperones play a key role in neurodegenerative diseases and may be neuroprotective. S100B has been shown to have a holdase-type activity, binding $A\beta_{42}$ and reducing its toxicity. In the present work, it could be shown for the first time that mild hypochlorite (HOCl) oxidation potentiates this chaperone activity (*in vitro)* and possibly adds anti-inflammatory activity to the protein, reducing cytokine expression among them *S100b*. The two crucial methionine residues identified for this chaperone activity are conserved across species, underlining their significance. Low doses of S100B have been shown to lead to astrocytic cell proliferation, possibly further elucidating the underlying mechanism of the results, showing an increase in cell health of $A\beta_{42}$ and $S100B_{ox}$ in comparison to S100B cotreated cells. Notably, oxidative stress is high in the AD brain. Anti-inflammatory drugs have only shown effects at early stages (before cognitive compromise) [1]. Increases of hypochlorite oxidized S100B might be a later event (after cognitive compromise), possibly serving (in combination with further specific AD biomarkers) as an AD progression biomarker and able to contribute to further characterize the "turning point" suggested in [1].

1. Introduction

As part of the cellular quality control system, molecular chaperones may play a potentially neuroprotective role in a pathology like AD by acting as suppressors of amyloid formation [2]. S100B has been shown to act as such a chaperone and probably bind $A\beta_{42}$ [3] and suppress primary as well as secondary nucleation of $A\beta_{42}$ [2]. Our results show that methionine oxidation of S100B ($S100B_{ox}$), very probably occurring in the $A\beta_{42}$ binding cleft, had no significant structural effect on S100B. However, $S100B_{ox}$ delayed $A\beta_{42}$ aggregation *in vitro* more efficiently than its non-oxidized counterpart, which could be shown with ThT kinetic assays — possibly even delaying aggregation in substochiometric ranges. In agreement, a novelty of this work is that cotreatment of $S100B_{ox}$ and $A\beta_{42}$ showed more cell health than the same conditions with non-oxidized S100B and $A\beta_{42}$ in an astrocytic AD cell model. This was tested in a dynamic, real-time, and label-free system.

Further, a significant downregulation of *S100b* transcription was evident upon an $S100B_{ox}$ cotreatment, opening the possibility of a negative autoregulation of S100B upon oxidation. Further, pro-inflammatory cytokines IL-17 and IFN-α were also found to be downregulated in the $S100B_{ox}$ and $A\beta_{42}$ treated astrocytes — another potentially crucial novelty of this study.

2. Methionine oxidation of S100B

In our study, as far as we know, the second study of oxidized S100B, we show that the *mild* oxidation protocol led to a significant percentage of oxidized S100B. The mild oxidation protocol could have been the reason for the ratio between oxidized and unoxidized S100B to be 0.87 and not 1. Methionine oxidation (MetO) is a reversible oxidative modification and Met74 and (even more) Met79 are conserved residues across species (https://www.ncbi.nlm.nih.gov/Structure/cdd/cd05027). Therefore, this is possibly a conserved way of preventing further irreversible oxidative modifications in addition to scavenging oxidants from its environment. This 'behavior' in a cellular environment would signify an attempt to restore homeostasis. To show that S100B is susceptible to MetO upon only a *mild* oxidation protocol is promising for the previously mentioned and following reasons. The structural representation of a model of S100B bound to $A\beta_{42}$ presented in Chapter 4, Fig. 4 highlighting S100B-Met residues (magenta) within the chaperone–client binding cleft, identified in [3] and with that crystalized the importance of Met74 and Met79. These two Met residues might be why $S100B_{ox}$, based on our ThT kinetic assay results, shows an even more efficient chaperone activity than its non-oxidized counterpart. The $S100B_{ox}$ is up to three times oxidized at Met residues, possibly giving origin to two MetO diastereomeric forms of S100B [4] [5]. One may be more efficient in inhibiting $A\beta_{42}$ aggregation, showing the biphasic behavior in $A\beta_{42}$ aggregation (Chapter 4, Fig. 8a). Different diastereomeric forms often show a difference in bioactivity [6].

3. Hypochlorite potentiates chaperone activity and is present in the AD brain

Our results suggest mild hypochlorite oxidation potentiates S100B's chaperone activity. In line, HOCl has been shown previously to potentiate the holdase-like chaperone activity of hsp33 [7] and lead to a marked increase of human chaperone α2-macroglobulin (α2M) [8], which, when oxidized, may decrease $A\beta_{42}$ toxicity. $A\beta_{42}$ hypochlorite oxidation has been shown to promote aggregation and reduce toxicity [9].

Hypochlorous acid is a potent ROS generated by myeloperoxidase enzymes that may dissociate partially into hypochlorite [10]. Enzymatically active myeloperoxidase and its oxidation products have been shown in human brains. Elevated levels of myeloperoxidase could be verified in brains affected by AD. Immunoreactivity is partly localized to active microglia and mostly to amyloid plaques and neurons (including pyramidal neurons of the

hippocampus). Several neurons and primary neuronal cultures express this protein as well. Myeloperoxidase was also detected in neuronal cell lines like SK-N-SH and SK-5SY [11], suggesting the potential of HOCl oxidation of S100B in those cells as well.

4. $S100B_{ox}$ possible anti-inflammatory activity?

In $S100B_{ox}$ and $A\beta_{42}$ treated astrocytic cells, our results show a downregulation of pro-inflammatory cytokines, among them *S100b* mRNA expression itself. This suggests an autoregulation of S100B, dependent on its oxidation. In cancer cells, it could be shown that the S100B - p53 interaction leads to an inhibition of p53 and an increased expression of S100B, resulting in a positive feedback loop for the S100B [12]. Blocking S100B with the drug pentamidine blocks S100B by inhibiting its interaction with p53 and causes a reduction of RAGE protein expression in an AD mouse model [13]. Oxidation of S100B can affect the p53 binding [14]. Suggesting a way $S100B_{ox}$ from our study could modulate S100B expression.

Our results further show a significantly higher level of cell health in $S100B_{ox}$ and $A\beta_{42}$ cotreated cells compared to non-oxidized S100B and $A\beta_{42}$ cotreated astrocytic cells. Both may suggest that $S100B_{ox}$ downregulates S100B production. When taking this as premise and that DITNC1 astrocytic cells also proliferate upon low levels of S100B [15] (Fig 1.), then this could raise the question of $S100B_{ox}$'s potent chaperone activity (in comparison to its non-oxidized counterpart) or possibly its anti-inflammatory activity has more weight in leading to the increase in cell health in $S100B_{ox}$ and $A\beta_{42}$ treated astrocytic cells in comparison to controls.

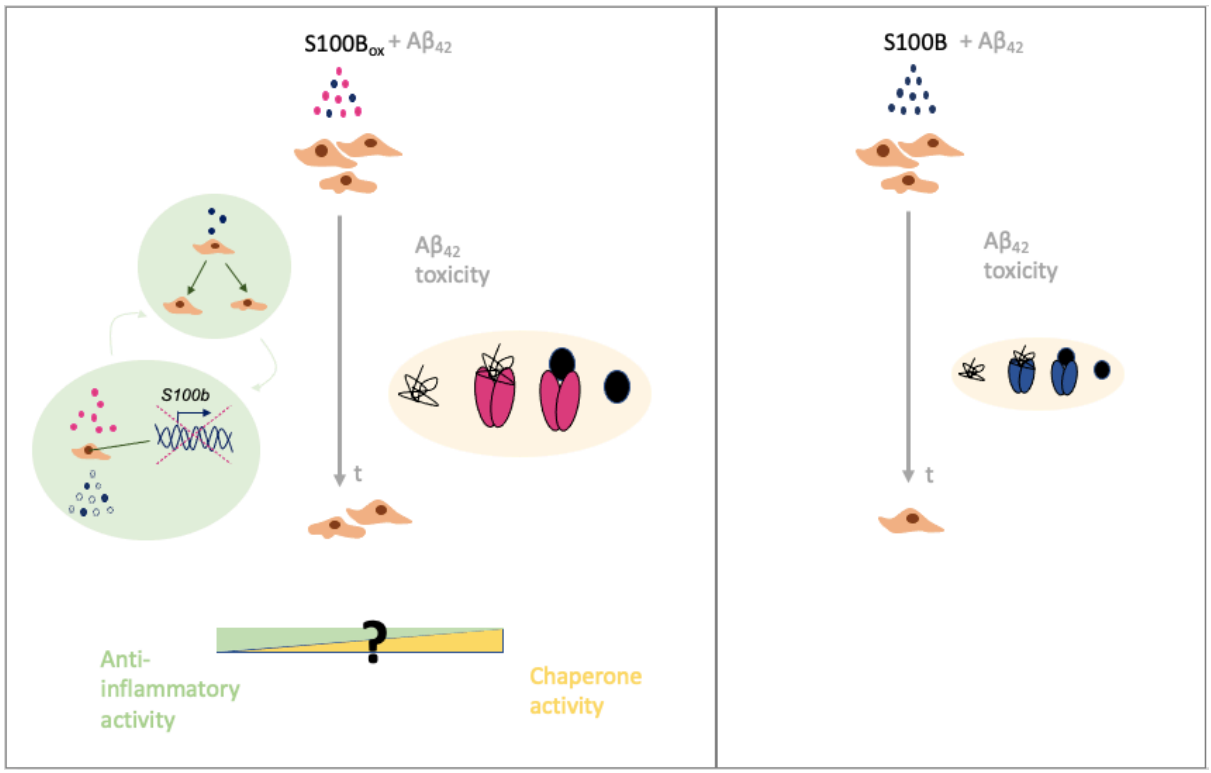

Fig. 1: A possible schematic representation of S100B$_{ox}$ pro- and anti-inflammatory activity in a DITNC1 astrocytic cell culture AD model in comparison to non-oxidized S100B and Aβ$_{42}$ treatment. First section: S100B$_{ox}$ is together with Aβ$_{42}$ added to the DI TNC1 cells. Low doses of S100B have been shown to stimulate astrocyte proliferation, as mentioned above. Therefore, if this is true for DI TNC1, then the small amount of S100B in the S100B$_{ox}$ sample, may lead to cell proliferation. S100Box may further downregulate S100B expression as was shown in this work, and with it potentially keep S100B levels low, even in new astrocytic cells. The above would represent S100B$_{ox}$'s anti-inflammatory activity and can lead to the measured increase in cell health, when comparing to S100B. Its chaperone activity or a conjunction of chaperone and anti-inflammatory activity could equally lead to an increase of cell health, since the oxidation of the methionine residues *in vitro* was more efficient in suppression Aβ$_{42}$ aggregation, would that be the same in DI TNC1 cells, then it could also explain the difference in cell health between S100B$_{ox}$ (increase) and S100B.

If oxidation is a way to control bioactivity as seen in Chapter 4, 'switching off' proinflammatory activity also in the case of this study, then astrocytes in S100B$_{ox}$ and Aβ$_{42}$ cotreated cells may suffer from less 'chronic' activation, from the start, possibly leading to less cell death.

Further, the literature suggests that low molecular $A\beta_{42}$ species in cell culture are more potent inflammatory agents than fibrillar $A\beta_{42}$ [16]. Therefore, $S100B_{ox}$ cotreated cells may have less pathological stimuli ($A\beta_{42}$ oligomers or increased concentrations of pro-inflammatory cytokines, for instance) than control wells, further underlining S100B's enhanced chaperone activity upon oxidation.

5. Possible toxicity of S100B?

In M.E. Clementi, 2023, S100B cell expression in a human astroglioma cell line upon Aβ exposure for 48h was tested. Cell death was around 30%, and S100B expression was 40% higher than control. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels showed a significant increase in these cells after exposure to Aβ of the 48h. Interestingly, however, is that upon (down to 40%) silencing of S100B, no Aβ toxicity and no significant increase in ROS or RNS levels could be measured. They suggested that Aβ stimulates S100B expression in these cells and that cell death results from heightened S100B expression and, consequently, elevated ROS/RNS [17]. However, one must keep in mind that low doses of extracellular S100B have been shown to stimulate astrocytic cell proliferation in a RAGE-dependent manner [12]. Upon downregulation of S100B to 40% and the RAGE stimulus of Aβ, it would have been interesting to see in this study that the extracellular S100B protein levels are not different in control and Aβ treated samples. Thus, proliferation not being the factor to compensate for the number of dying cells in Aβ treated wells.
Further, it has been shown that S100B levels in astrocyte cell lines reduce migration and acquisition of a differentiated phenotype (i.e., stellation) [18]. Thus, this unnatural astrocytic state analyzed [17] shows a lack of the capacity to produce as much oxidative stress, possibly not due to 'silenced' S100B but since S100B functions are concentration-dependent and low S100B levels show different effects. Further heterogeneity of astrocytes is a factor to consider [19] [20]. Finally, results suggest that S100B was not oxidized at methionine residues of the Aβ binding region.

6. Conclusion and Outlook

We found that S100B has chaperone activity, but that this activity is potentiated through mild hypochlorite oxidation is novel. It is also novel that this applies even to substoichiometric

ranges. Furthermore, we could show in this work that oxidation may be a way to downregulate S100B, possibly through a negative autoregulation. In more detail, $S100B_{ox}$ leads to the downregulation of *S100b* transcription — the downregulation of *S100b* and that of the tested pro-inflammatory cytokines IL-17 and INF-α. Future work could investigate how this downregulation of transcription and possible negative feedback loop works, whether through missing RAGE interaction, p53 interaction, or a mechanism that is so far unknown. This is to come closer to possibly another way of pharmacologically controlling S100B levels in the AD brain and possibly with less toxic secondary effects. Another hypothesis would be to consider $S100B_{ox}$ as a potential biomarker.

6.1 S100Box as a potential biomarker for AD

Oxidative stress, an imbalance between oxidants and antioxidants towards an increase of oxidants in the extracellular milieu, [21], may, in the context of Alzheimer lead to the oxidation of proteins like S100B [22]. S100B can be found in the cerebrospinal fluid (CSF) and blood (Chapter 3). Concern was raised regarding S100B being suggested as a blood biomarker, as S100B originates from further extracranial sources [23] [24]. This is, however, neglectable (if additional multiple trauma can be excluded) [25]. S100B being expressed by different extracerebral tissues *upon* AD [26] is an interesting thought. However, the only study doing research in this direction we found was not in line with what was inferred by the authors [26] and only tested for lymphocyte-localized *S100* protein expression [27]. Together, the fact that in the blood, no significant differences in S100B levels have been found between AD and non-AD patients [28] suggests that that might not be the case for S100B. S100B clearance from the brain to the blood possibly occurs through the CSF [29] in case of an intact blood-brain barrier (BBB). However, with time, S100B may contribute to opening the blood-brain barrier via cytokine upregulation [30]. One possible explanation for why the S100B in the CSF of AD patients is elevated in the earlier stages of Alzheimer's disease in comparison to later stages of AD [31], but blood levels stay the same [28].

Taking the above into account, it rests the question of whether the oxidative state of the S100B proteins varies in AD and if this modification could indicate whether $S100B_{ox}$ could be a biomarker for AD progression in the context of further AD markers.

S100B is a pro-inflammatory cytokine, and on a broad scale, anti-inflammatory therapy was reported to be efficient in the absence of cognitive compromise [1], possibly also attenuating pro-inflammatory S100B expression and secretion at early stages (before cognitive

compromise). Given that oxidative stress is high in postmortem tissues (late stages), as shown before, S100B oxidized at methionine residue levels may increase upon AD progression.
Following the method in [32], methionine oxidation may be detected in AD patients' serum using a label-free mass spectrometry approach, where the mass spectra intensity ratio of the oxidized and non-oxidized protein in serum tryptic proteins gives reproducible results about the redox changes. Necessary for this approach would only be a single drop of a patient's serum.

Therefore, research guidelines to validate $S100B_{ox}$ as a blood biomarker may include the following. Based on the work presented in this thesis, a first step could be to repeat the results from Chapter 5 in primary human astrocytes. It would be of importance to investigate how mRNA down-regulation and chaperone activity is regulated in primary human astrocytes and further analyze the pathway in AD animal models.
In a following step it would be necessary to confirm that S100B in its oxidized form can be reliably detected and quantified in blood samples using the method mentioned above (based on mass spectrometry). Further, patients with mild cognitive impairment and more advanced stages of AD could be followed over time and $S100B_{ox}$ blood levels measured. Given its validity as a biomarker so far, to assess its potential for clinical utility, one would have to test reproducibility and refine cutoff values for clinical use. The details of which are beyond the scope of this outlook.

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