Chapter 9

Can agrin CSF concentration be used as an early biomarker for Alzheimer's disease?

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Abstract

The need for effective treatments halting Alzheimer's disease (AD) urges the discovery of the earliest possible biomarkers. Agrin is increased in early stages of AD tissue and is involved in amyloid-β (Aβ) fibrillation and synaptogenesis. Here, we investigated the potential of agrin as an early AD cerebrospinal fluid (CSF) biomarker. We analyzed agrin CSF concentration in non-demented controls (n=20), mild-AD (n=20) and severe-AD (n = 20) patients. The levels of agrin CSF were not significantly divergent between the different patient groups; and it did not correlate with the concentration of $A\beta_{42,}$ total tau, phosphorylated tau or MMSE scores. However, agrin strongly correlated with age in the demented cases. The results indicate that agrin cannot be used as an early AD CSF biomarker using the current immunoassay. However, our population was relatively young; thus, the correlation between agrin and age suggests that stronger differences in agrin concentrations might be found in older groups with more heterogeneous AD pathology.

Key words: Agrin, CSF, Biomarker, Diagnosis, Aging.

Introduction

Alzheimer's disease (AD) is the most common form of dementia with unknown etiology. It is pathologically characterized by the accumulation of amyloid β (Aβ) in senile plaques and phosphorylated tau (p-Tau) in neurofibrillary tangles (NFT). In addition, a remarkable loss of neurons and synapses is also observed in AD[1,2]. It is widely accepted that interventions to slow down or halt the disease should be administered at the earliest possible stage, before neuronal damage has occurred. Hence, there is a strong need for the discovery of novel biomarkers that can better detect this critical phase.

The current core CSF biomarkers for AD diagnosis reflect the classical hallmarks of AD pathology: a decrease in CSF $A\beta_{42}$ levels reflects senile plaque pathology and increased total tau (t-Tau) and p-Tau levels reflect axonal degeneration and NFT formation[3,4]. Their sensitivity and specificity for AD is high and they can reasonably predict the transition of mild cognitive impairment (MCI) to AD[5]. Nevertheless, AD CSF biomarker patterns are often present in cognitively normal subjects and can be influenced by the age of the patient, resulting in loss of sensitivity at higher age[6,7]. Thus, the quest for the earliest and most sensitive marker is ongoing. In this sense, interest in biomarkers reflecting synaptic dysfunction is growing since synaptic damage occurs in very early pathological stages preceding neuronal loss and it strongly correlates with cognitive symptoms[8,9]. In a previous hypothesis-free proteomics study, several potential CSF markers for early AD diagnosis were identified, including one of the major heparin sulfate proteoglycans (HSPG) named agrin (Fig. 1A).

Agrin is expressed in neurons and glia cells[10] and its suppression in mature rat hippocampal cultures severely reduced the number of synapses formed, indicating a role of agrin in synaptogenesis[11,12]. In addition, agrin is an important component of the basal lamina where it can regulate the expression of other proteins and influence the permeability of the blood-brain barrier[13,14]. Several studies have already implicated agrin in AD aetiology[10,15], as it is the predominant HSPG associated with fibrillar and non- fibrillar senile plaques, cerebral amyloid angiopathy and neurofibrillary tangles in post-mortem AD tissue[16–18]. Agrin is an amyloid-associated protein able to enhance Aβ fibril formation and to halt Aβ degradation[19], suggesting that agrin may not only be a structural component of senile plaques, but it may also contribute to Aβ fibrillation. Importantly, increased soluble agrin levels were found in post-mortem hippocampus and prefrontal cortex of AD patients compared to controls[14], with significant hippocampal changes observed in very early stages of AD (Braak III-IV for NFT)[20,21].

Given the early changes found in human AD tissue and our recent proteomics data, we hypothesized that agrin is a potential early synaptic biomarker for AD. Thus, we analyzed

the levels of agrin in CSF from AD patients and control cases using a specific ELISA immunoassay.

Materials and Methods

Human CSF samples

CSF material (n = 60) was obtained from Alzheimer Center Memory Cohort, NUBIN/ (NeuroUnit Biomarkers for Inflammation and Neurodegeneration) VUmc biobank (Amsterdam, The Netherlands). For the initial pilot proteomic study we selected age matched AD patients (n =5; MMSE: 20.8 ± 5.6) and non-demented subjects with subjective memory complaints (SMC; n = 4; MMSE: 29.5 ± 1), patients with mild cognitive impairment (MCI) who after two years follow-up converted to AD (MCI-AD; n = 5; MMSE: 25 ± 2.5) or remained stable (MCI-S; n = 4; MMSE: 28.4 ± 2). For the analysis of agrin in a larger cohort using an specific immunoassay, non-demented subjects with subjective memory complaints (SMC; n = 20; MMSE: 27 ± 1.78), mild AD patients (n = 20; MMSE: 21.1± 1.0) and severe AD patients were selected (n = 20; MMSE: 15.3± 1.9). Diagnoses were defined in a multidisciplinary meeting as described[22]. The diagnostic accuracy of specialized centers as the Alzheimer Center Amsterdam has been previously reported[23,24]. Patients were matched for age and sex. Collection, storage and analysis of CSF biomarkers (Aβ1-42, t-Tau and p-Tau) were done as previously described[25]. CSF samples were stored in agreement with JPND-BIOMARKAPD guidelines[26]. Age, sex, biomarker levels as well as Mini Mental State Examination (MMSE) scores of all cases are listed in Table 1. The ethical review board of the VUmc approved the study and all subjects gave written informed consent.

Mass spectrometry analysis of CSF

CSF samples were analyzed by label-free GeLC-MS/MS-based proteomics and normalized spectral counting as previously described[27]. The data obtained were processed and analyzed as described before[28]. The global protein profiling results of the CSF proteomics screen will be reported elsewhere (Chiasserini et al., manuscript submitted). BRI2 was one of the identified proteins.

Agrin CSF analyses

The concentration of agrin was determined using sandwich human agrin (AGRN) ELISA kit (Wuxi donglin Sci&Tech, Jiangsu, China) following manufacturers instructions. Performance of the assay was evaluated using CSF pools as internal controls. The coefficient of variation

Table I 1. Demographic data of CSF sample

Data are reported medians and interquartile range unless indicated. SMC = Subjective memory complaints, AD-M = Mild AD, AD-S = Severe AD. * = at least $p < 0.05$ from SMC, † = at least $p < 0.05$ from MCI-S or AD-M.

	Proteomics analysis				ELISA		
	SMC	**MCI-S**	**MCI-AD**	**AD**	**SMC**	**AD-M**	**AD-S**
Age (mean ± SD)	60.3 ± 4.5	62.1 ± 3.2	66.2 ± 6.4	63.9 ± 6.6	60.2 ± 2.61	60.9 ± 3.00	60.4 ± 3.21
No (M/F)	4(2/2)	4(1/3)	5(2/3)	5(2/3)	20(7/13)	20(8/12)	20(10/10)
MMSE baseline (mean ± SD)	29.5 + 1.0.	27.4 ± 2.2	27.0 ± 1.4	21.4 ± 6.3	27 ± 1.78	21.1± 1.0*	15.3± 1.9*,†
MMSE follow-up (mean ± SD)	/	28.4 ± 2.0	25.0 ± 2.5	20.8 ± 5.6	n.a	n.a	n.a
$A\beta_{42}$ (pg/mL)	838 ± 133	875 ± 201	499 ± 78	384 ± 146*,†	888± 211.5	491.5± 185*	390.5± 149.5*,†
t-tau (pg/mL)	200 ± 76	421 ± 347	1071 ± 248*	526 ± 120	228± 161	729± 599*	679.5± 856.25*
p-tau (pg/mL)	47 ± 16	73 ± 48	138 ± 37 *	102 ± 40	48± 18.5	98.5± 48.5*	95.5± 81.25*

(CV) was calculated for each sample in duplicate as the standard-deviation divided by the mean. The mean CV of all the samples was calculated to establish the intra-assay CV which was 8.6%. Samples with an intra-assay CV higher than 15% were excluded from further analysis (n = 11). Inter-assay CVs were calculated using two internal quality control CSF samples in two plates from one batch, which was 11.4%. For the analysis of clinical samples different lots of the agrin ELISA kit were used in which a larger inter-lot variation was found for two internal controls (16.5 %). Thus, agrin concentration for the samples was corrected for the internal controls used in that assay.

Statistical analysis

Statistical analyses were performed on SPSS version 20 (Chicago, IL, USA). Since data were non-normally distributed, the initial correlation analyses were performed using non-parametric Spearman's correlations. For subsequent analyses, data were log-transformed and ANCOVA was used (with age as covariate) to analyze differences between groups. The assumption of homogeneity of regression was tested ($p > 0.05$). Partial correlation analyses were also conducted to analyze associations between the different variables.

Results and discussion

In this study, we investigated the potential of agrin as a CSF biomarker for early diagnosis of AD using a specific ELISA immunoassay. Demographic data for the 60 patients are presented in Table 1. Groups significantly differed in MMSE scores as well as in the CSF concentrations of $A\beta_{42}$, t-Tau and p-Tau, but not in age or gender; which reflects the selection criteria. Correlation analysis revealed a significant interaction between agrin CSF concentration and age (Figure 1B). Strikingly, correlation analyses run within each diagnostic group revealed that the interaction between age and agrin levels was specific and strengthened in demented cases as opposed to controls (Figure 1C-E). Consequently, age was introduced as a covariate in subsequent statistical models.

Our data revealed no significant differences in agrin CSF concentration between the different clinical groups (Figure 2A). No significant associations were found between agrin CSF concentration with MMSE scores or the CSF concentration of t-Tau, p-Tau and $A\beta_{42}$, though a positive tendency was observed in the latter (Figure 2B-E). In summary, the data indicate that agrin CSF concentration cannot be used as a biomarker for early diagnosis of AD. However, our findings do reveal an interesting association between agrin and age in demented patients as well as a tendency for a correlation between agrin and $A\beta_{42.}$

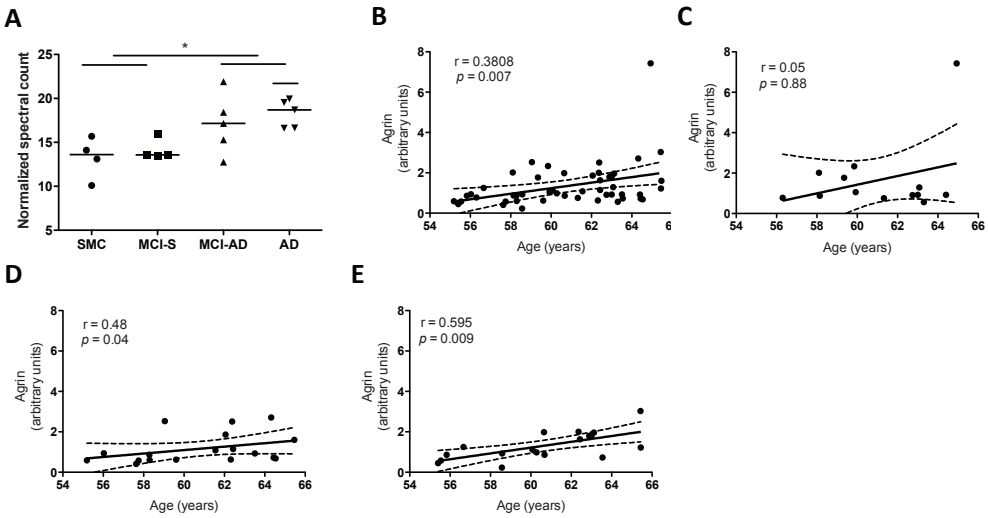

Figure 1. Agrin CSF concentration correlates with age only in Alzheimer's patients.
A, Proteomic analysis of CSF samples from SMC (n = 4), MCI-S (n = 4), MCI-AD (n = 5) and AD (n =5) cases. The spectral counts of agrin peptides in CSF increased a long with disease progression. ***B,*** Correlation analysis showed a significant association between agrin and age. When results were divided into the different clinical groups, CSF agrin concentration did not correlate with age in the SMC cases (n = 13) (**C**) but it did in the mild (n = 18) (***D***) and severe (n = 18) (***E***) AD patients. Correlation coefficients and *p* value are presented in the inserts. Regression line is shown and dot lines represent the 95% confident intervals.

In a previous hypothesis free pilot proteomics study we found that the levels of agrin were significantly increased in AD CSF compared to controls (Fig 1A, n=5/group). The discrepancy between our results and the proteomics data is likely explained by the epitope recognized by the antibody used in the current ELISA system. This antibody detects a sequence within the N-terminal 110 kDa fragment, while the vast majority of the peptides found in the proteomics study reside in the C-terminal region. In addition, the peptides sequences detected in this ELISA lie within the laminin EGF-like and Kazal-like domains of the agrin protein, which can form disulfide bonds[29,30]. Those properties may prevent the detection of relevant peptides in the CSF and consequently weaken the possibility of finding differences between the clinical groups. Similarly, the current results challenged another previous study that found increased levels of soluble agrin in the post-mortem hippocampus AD patients in early stages of the disease[14]. The discrepancy may again be attributable to the antibodies used in our ELISA system, as the previous investigation utilized an in-house ELISA kit that detected the 50 kDa C-terminal fragment of agrin. Taken together, we can conclude that the N-terminal 110 kDa fragment of agrin is not changed in AD CSF; however, whether the concentration of the 50 kDa C-terminal fragment is modified between the different patient groups remains to be further investigated.

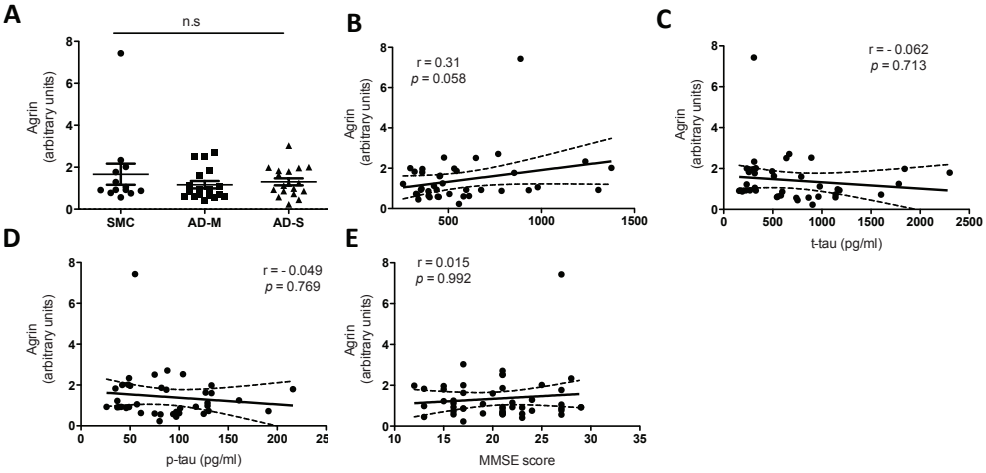

Figure 2. Agrin CSF concentration is not changed in AD and does not correlate with the classical AD markers.

A, Agrin concentration in CSF from SMC (n= 13), AD – M (n = 18) and AD - S (n = 18) cases. No significant difference was observed between groups. Partial correlation analysis of CSF concentrations of Agrin with $A\beta_{42}$ (***B***), t-Tau (***C***), p-Tau (***D***) and with MMSE scores (***E***). Correlation coefficients and *p* value are presented in the inserts. Regression line is shown and dot lines represent the 95% confident intervals. n.s: non significant

We observed a tendency for a positive correlation between the CSF levels of agrin and $A\beta_{42}$, suggesting an association of agrin with AD pathology, and more specifically with Aβ plaque formation. This agrees with previous studies which showed that agrin was associated with senile plaques in human AD brain tissue[16,18,19]. Moreover, agrin was able to bind Aβ accelerating the fibrillation process[19] and thus, suggesting a role in plaque formation. Taken together, previous data indicate that agrin expression is changed in AD tissue and may play an important role in the development of this disorder[10,15]. However, according to the current results, those changes are not clearly reflected in CSF.

Although we did not observe a difference in the CSF levels of N-terminal agrin between AD and non-demented subjects, the strong correlation with age only in the demented cases was striking. These results suggest that age may produce greater and detectable changes in the levels of agrin CSF when comparing AD patients and control cases, which might be prompted by the higher heterogeneity of AD pathology in older patients. The influence of age on agrin CSF concentration can be a relevant concern, since the incidence of AD increases in older populations, with prevalence reaching 19% in patients aged 75-84 and 44% in patients over the age of 84[31]. Thus, it would be interesting to investigate agrin's potential as a CSF biomarker in older groups. Whether the correlation between age and agrin CSF concentration reflects an underlying pathological process of aging and dementia remains to be investigated.

In summary, the results of the current study showed no difference in the concentration of agrin in CSF between controls and AD patients, indicating that agrin cannot be used as an early biomarker for AD diagnosis using the current assay. However, it remains to be investigated whether changes in agrin CSF concentrations are detectable with a different ELISA assay. In addition, the positive correlation of agrin and age in demented cases suggests that changes in CSF agrin concentration might be visible in older patients with more heterogeneous pathology.

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Disclosure statement

Dr. Teunissen serves on the advisory board of Fujirebio and Roche, received research consumables from Euroimmun, IBL, Fujirebio, Invitrogen and Mesoscale Discovery. Dr Scheltens serves/has served on the advisory boards of: Genentech, Novartis, Pfizer, Roche, Danone, Nutricia, Jansen AI, Baxter and Lundbeck. He has been a speaker at symposia organized by Lundbeck, Lilly, Merz, Pfizer, Jansen AI, Danone, Novartis, Roche and Genentech. He serves on the editorial board of Alzheimer's Research & Therapy and Alzheimer's Disease and Associated Disorders, is a member of the scientific advisory board of the EU Joint Programming Initiative and the French National Plan Alzheimer. The Alzheimer Center receives unrestricted funding from various sources through the VUmc Fonds. Dr Scheltens receives no personal compensation for the activities mentioned above.
None of the other authors has any competing interest.

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Chapter 10

Summary and general discussion
Future perspectives: what's next?

DISCUSSION

Advancing age is the greatest risk factor of multiple neurodegenerative disorders such as Alzheimer's disease (AD), the most common form of dementia. As life span increases, AD will pose a huge challenge in the social and economical burden with more than 100 million of individuals predicted to develop AD by 2050[1]. Thus, there is a great interest not only in the development of more specific, sensitive and practical tools to diagnose AD in its earliest possible phase, but also in the development of effective treatment. Scientific advances have provided a good overview of the main pathological hallmarks on AD, which are the basis for the development of novel biomarkers and potential disease modifying therapies[2]. Unfortunately, there is still no test available to efficiently predict the development of AD[3] and the clinical trials performed with potential disease-modifying therapies have been so far ineffective[4]. This can be partially attributed to the limited knowledge about the etiological factors underlying the pathology of AD. Thus, there is an urgent need to identify novel proteins and pathways involved AD pathogenesis, which will likely promote the development of novel alternative therapeutic strategies. Global protein profiling by mass spectrometry-based proteomics has evolved as a new hypothesis-free avenue to optimally unravel new candidates involved in different diseases including neurodegenerative disorders[5]. Using proteomics we have identified novel early biomarkers and potential players in AD, including BRI2 and agrin.

Thus, the main focus of this thesis was to explore the involvement of BRI2 and related proteins in different stages of AD pathology as well as the potential of both BRI2 and agrin as early biomarkers for AD diagnosis. To this end, the following aims were formulated:

- Extensively investigate the expression of BRI2 and its processing enzymes in post-mortem tissue at different stages of the disease.
- Study the effects of abnormal BRI2 forms in cellular processes involved in AD pathogenesis.
- Investigate the potential of BRI2 and agrin as early biomarkers for AD.

In this section we will discus the potential causes and consequences of the increase detected in AD of BRI2 (loss and/or gain of protein function) and the subsequent identified BRI2-processing enzyme, SPPL2b, which suggest the contribution of a novel pathway in AD pathogenesis (Fig. 1). In addition, we will examine also the potential of the different protein changes (BRI2, agrin and SPPL2b) as biomarker tools for AD.

BRI2 in AD tissue: loss of BRI2 function?

In the last decade, several cellular and mice studies revealed an association between BRI2 and several proteins critically involved in the amyloid cascade hypothesis (i.e. amyloid precursor protein (APP), Aβ or insulin degrading enzyme (IDE))[6–8]. Interestingly, mutations in BRI2 are responsible for the development of familial British and Danish dementias (FBD and FDD), which share striking clinical and pathological similarities with AD. In chapter 2 we discussed the literature available not only for BRI2 but also regarding the links of BRI2 with known pathological pathways in AD. Based on the published data, it was suggested that lack or reduced functionality of BRI2 could affect the production, fibrillation and clearance of Aβ as well as the stability of tau, highlighting the potential of this protein as a pathological contributor to AD.

Our extensive analysis of BRI2 in post-mortem tissue (chapter 3) revealed that an abnormal, larger form of BRI2 of approximately 45 kDa was significantly increased in the hippocampus of AD patients, the main area involved in learning and memory. This was further supported by the immunohistochemical characterization of BRI2 in AD hippocampus, which revealed a deposition of the BRI2 ectodomain associated with senile plaques. Those results were in agreement with the BRI2 staining previously observed in the temporal cortex of one AD case[9]. The correlation detected between the 45 kDa BRI2-positive bands and the BRI2 staining suggests that BRI2 deposits represent the larger 45 kDa BRI2 form. Despite the expected molecular weight of BRI2 is 30 kDa, several studies have also reported higher molecular weights ranging between 40 and 50 kDa[10–13]. Interestingly, the analysis of the recombinant BRI2 ectodomain (76-266, $rBRI2_{76-266}$) showed bands not only at 25 kDa but also at higher molecular weights including 50 kDa. Indeed, large complexes of $rBRI2_{76-266}$ larger than 600 kDa were detected by size exclusion chromatography (chapter 2 and 3). Thus, it is possible that strong BRI2 aggregates are formed in AD, leading to the larger BRI2 forms, as is the case for other proteins such as Aβ[14] or α-synuclein[15].

Protein aggregation can lead to a loss of protein function as it has been described for other proteins including parkin and DJ-1, involved in the pathogenesis of Parkinson's disease (PD)[16]. Thus, the aggregation/deposition of BRI2 may also lead to a loss/reduced BRI2 function (Fig. 1). Previous studies have indicated a role of BRI2 in cognitive performance since reduced levels and loss of wild-type BRI2 function caused memory loss in FBD and FDD mice models[17,18]. Interestingly, we observed that the accumulation of BRI2 ectodomain started in stages in which cognitive symptoms are becoming to be noticeable (Braak III-IV for NFT[19–21]), suggesting a relationship between BRI2 deposition and memory impairment (chapter 3). Thus, it is tempting to hypothesize that the increase deposition/aggregation of BRI2 in AD may reflect a change on BRI2 functionality ultimately leading to the memory decline observed in early stages of AD (i.e. cognitive impairment) via as yet unknown

pathways. Further support between BRI2 aggregation and subsequent loss of function comes from the analysis of BRI2-APP complexes in human brain tissue. Previous studies indicated that BRI2 works as an APP binding protein downregulating the production of Aβ[22–28]. We have detected APP-BRI2 complexes also in human brain hippocampus. However, we observed a remarkable decrease of those complexes in the hippocampus of AD patients, supporting a loss of BRI2 functionality in AD (chapter 3). Nevertheless, this analysis needs to be extended to a larger number of patients in order to confirm the results. An additional BRI2 function that might be altered due to its aggregation state is the secretion of IDE, which influences the degradation of Aβ[29,30]. We detected a significant inverse correlation between the activity of IDE and the increase of BRI2 45-kDa form and BRI2 deposits in AD hippocampus (chapter 4), indicating that changes in IDE protein concentration and activity parallels the increase of abnormal BRI2 forms. Although correlation does not imply causation, those data further suggest that the deposition or the formation of larger BRI2 forms in AD can reduce the BRI2 function. A relationship between BRI2 aggregation and subsequent loss of function is also sustained by the *in vitro* experiments performed with the recombinant BRI2 ectodomain, $rBRI2_{76\text{-}266}$ (chapter 5). As discussed in chapter 2, BRI2 ectodomain contains a BRICHOS domain, which is able to efficiently delay Aβ aggregation[31,32]. However, $rBRI2_{76\text{-}266,}$ which contains the BRICHOS domain, failed to delay Aβ fibrillation as efficient as the BRI2-BRICHOS domain alone ($BRI2_{113\text{-}231}$), indicating that the formation of large aggregates may influence BRI2 activity. Noteworthy, both BRI2 (90-236) and BRI2 BRICHOS domain (residues 113-231) can also form different aggregates but they still active in delaying Aβ fibril formation. Thus it is important to unravel which specific BRI2 oligomeric state can influence BRI2 activity.

BRI2 aggregates: gain of toxic function?

Regulated intramembrane proteolysis (RIP) is a tightly regulated mechanism essential for a wide variety of signaling processes and the maintenance of cell proteostasis[33]. Deregulation of RIP has already been linked to different disorders including AD[34]. Abnormal RIP of APP due to the mutations found in familial AD can increase the presence of Aβ variants that are more prone to aggregate[34]. Similar to APP, BRI2 undergoes RIP in which it is cleaved by furin, ADAM10 and SPPL2b leading to the release of short BRI2 variants[10,11]. Interestingly, we observed that while the levels of both furin and ADAM10 were decreased in AD hippocampus, the levels of SPPL2b were drastically increased. Those data suggest that in sporadic AD there is a deregulation of BRI2 RIP (Fig. 1), halting the formation of the shorter BRI2 fragments and enhancing the released of the whole BRI2 ectodomain (residues 76-266) (chapter 3). According to the analysis of the $rBRI2_{76\text{-}266}$, BRI2 ectodomain is an aggregation prone BRI2 variant (chapter 3 and 5) and thus it might be the basis

leading to the BRI2 deposits and larger BRI2 complexes observed in early stages of AD (chapter 3).

Compelling evidence has shown that protein misfolding and aggregation (e.g. Aβ, α-synuclein, prion protein) leads to a gain of toxic function promoting synaptic impairment and neuronal apoptosis[35–40]. Similarly to aggregated proteins such as Aβ or α-synuclein, the presence of large BRI2 complexes in AD may lead to a gain of toxic function and have a negative effect on cell homeostasis (Fig. 1)[35–37,41]. Thus in chapter 5 we investigated *in vitro* the effects of $rBRI2_{76-266}$ on important molecular pathways involved in AD pathogenesis. We found that the incubation with $rBRI2_{76-266}$ for 24 hours led to a clear activation of the apoptotic response by decreasing the levels of the survival proteins Bcl-2 and increasing the levels pro-apoptotic protein Bax. The increased activity of caspases 3 and 9, downstream effectors in the apoptosis cascade[42], further confirmed the pro-apoptotic effects of exposure to rBRI2 ectodomain. However, the strong changes observed in the different survival/apoptotic proteins analyzed were not reflected in the cell viability assay, since incubation with $rBRI2_{76-266}$ lead only to a 10% cell death. Since the activation of caspases by toxic stimuli (i.e. Aβ) can occurs long before severe cell death[43,44], it is possible that the changes observed in the apoptotic pathway after incubation with $rBRI2_{76-266}$ are still in an early stage, which cannot be fully recognized by the MTT assay. In addition, the activation of compensatory mechanisms that ultimately may prevent neuronal loss can not be excluded, as we did not test those. Thus, additional studies using longer time exposures or analyzing potential protective mechanisms are needed. Nevertheless, the data suggest that the presence of BRI2 complexes may participate in the neurodegenerative processes via apoptosis pathways.

But how can $rBRI2_{76-266}$ trigger apoptosis? One important mechanism that can induce an apoptotic response and is closely related to the neurodegenerative process in protein-misfolding disorders is the unfolded protein response (UPR)[45]. UPR is activated in order to restore cell proteostasis when neurons are subjected to ER stress due to misfolded proteins or extracellular stimulus. However, an apoptotic signaling cascade will be activated if ER stress remains unmitigated[46,47]. UPR markers have been detected in early stages of AD and PD[48,49] and its inhibition prevented neurodegeneration independently of the primary pathogen in a mouse model of prion disease[50], indicating that the UPR is an important response and a causative contributor to the pathogenesis of protein misfolding disorders[51]. Nevertheless, incubation with $rBRI2_{76-266}$ did not significantly modify the classical UPR markers indicating that UPR does not specifically contribute to the apoptosis induced by $rBRI2_{76-266.}$ Interestingly, we observed a slight up-regulation of BiP mRNA levels indicating that $rBRI2_{76-266}$ can induce a mild ER stress. This mechanism can contribute to cell death via Ca^{2+} release and activation of GSK3β[52,53], which was activated after incubation with $rBRI2_{76-266.}$

GSK3β is an enzyme that is also involved in the hyperphosphorylation of tau[54] and thus it can contribute to the formation of NFT. The correlation observed between BRI2 deposits and NFTs in AD hippocampus suggests a relationship between these two characteristics (chapter 3). In addition, the double transgenic tg-FDD-tau mouse showed accumulation of wild-type BRI2 as well as an enhance phosphorylation and truncation of tau before amyloid deposition[55], which also supports that BRI2 deposits could participate in NFT formation. Although incubation of neuronal cells with $rBRI2_{76-266}$ activated GSK3β, it did not modify the levels of p-tau at positions 181 or 231. However, we detected an increase in the levels of truncated tau at D421, probably caused by the increased in caspase 3 activity (chapter 5). Truncation of tau at D421 has been observed in AD[56,57] and several lines of evidence indicate that it likely plays an important early role in the formation of NFT[58–62] similarly to hyperphosphorylated tau. Thus, the presence of larger BRI2 complexes in AD may participate in tau pathogenesis through the truncation of tau. In addition, this truncated form can induce cell death[63], suggesting another signaling pathway by which $rBRI2_{76-266}$ may induce apoptosis.

Formation of aberrant protein complexes and deposits is a common denominator in the pathogenesis of multiple neurodegenerative disorders. The different studies performed in the last decades using genetics and protein approaches in diverse models have unraveled that protein misfolding can induce a pathogenic process via loss of physiological function, gain of toxic function or combination of both. Taken together, it is tempting to speculate that formation of BRI2 complexes in early stages of AD (chapter 3) may lead not only to a loss of BRI2 function (Aβ imbalance and cognitive performance) but also to a gain of toxic function (tau truncation and apoptosis) (Fig. 1). Nevertheless several aspects need to be further investigated, including the specific conformational characterization and sequencing of the BRI2 deposits in AD tissue and a thorough analysis of the effects of aggregated BRI2 on AD related mechanisms.

Disassembling BRI2 pathway: SPPL2b as a novel AD hallmark?

The high aggregation capacities of $rBRI2_{76-266}$ led us to hypothesize that an abnormal processing of BRI2 could be involved in the formation of larger BRI2 complexes observed in early stages of AD. The analysis of the enzymes involved in BRI2 cleavage not only suggests that in fact that could be the case but it additionally unraveled an outstanding increase in the levels of SPPL2b in AD hippocampus (chapter 3). SPPL2b is a relatively novel protease involved in the RIP not only of BRI2 but also of the transferrin receptor-1 (TfR1)[64] and tumor necrosis factor α (TNFα)[65,66] (Fig. 1). Interestingly, an imbalance in iron metabolism[67] as well as increased levels TNFα[68,69] have been also reported in early stages

of AD. In addition, recent studies indicate that SPPL2b may play a role in learning and memory since its expression was specially pronounced in the murine hippocampus[70]. Thus, in chapter 6 we hypothesized that SPPL2b may play a yet unanticipated role in AD pathogenesis and aimed to extensively characterize the drastic increase observed in AD patients. In agreement with the results of chapter 3, immunohistochemical characterization showed a strong SPPL2b reactivity in the hippocampus of AD patients, which was absent or remarkably reduced in control cases; this is unlike Aβ deposition, which can also be detected in non-demented cases[71,72]. These results indicate that SPPL2b might be a novel pathological hallmark of AD with a magnitude comparable to that observed for Aβ plaques or NFT, highlighting the importance that this protein may have not only in AD pathogenesis but also as a potential marker for AD pathology.

The correlation found between the presence of BRI2 deposits and the SPPL2b staining support the hypothesis that SPPL2b changes contribute to the formation of large BRI2 complexes. Further analysis revealed that the SPPL2b increase was detectable at early stages of AD coinciding with the appearance of NFTs in the hippocampus (Braak II-III for NFT[19]). Moreover, strong changes were observed between Braak stages III and IV, the latter being related to memory impairment[20,21]. These results, together with the predominant expression of SPPL2b in AD hippocampus compared to other brain areas, suggest a close relationship between SPPL2b, NFT formation and cognitive decline in AD. In fact, recent studies have shown that the TNFα-ICD, which is released after SPPL2b cleavage, can play a critical role regulating not only the levels of Aβ but also memory performance via the interleukin 12 pathway[66,73,74]. Whether the BRI2-ICD released after SPPL2b processing also plays a role in AD needs yet to be elucidated since its physiological function remains unknown[75].

Interestingly, a lower but noticeable SPPL2b expression was also detected in the most affected brain areas of other protein misfolding dementias such as PD or frontotemporal lobar degeneration (FTLD). Interestingly, SPPL2b staining pattern was associated with the main pathological characteristic of those disorders including α-synuclein inclusions and Lewy bodies in PD and tau aggregates, Pick's bodies and oligodendroglial coiled bodies in FTLD types[76], suggesting a novel role of SPPL2b in protein aggregation mechanisms. This is further supported by the intracellular location of SPPL2b in the endosomes/lysosomes[77], where it may participate in the ubiquitin and autophagy-lysosome pathways[78] (Fig. 1). However, it is conceivable to hypothesize that SPPL2b plays a more relevant role in the pathogenesis of AD considering not only that stronger SPPL2b changes are observed in AD compared to other protein-misfolded dementias (chapter 6), but also the higher SPPL2b physiological expression specifically within the hippocampal areas[70]. The data of chapter 6 additionally revealed that SPPL2b is a novel tau binding protein as judged by the double immunohistochemetry analysis and the immunoprecipitation experiments.

Although those analyses need to be extended to a larger number of patients, the data suggest that SPPL2b-tau complexes were specially observed in the tissue of AD patients but not in control cases. Whether SPPL2b binding contributes to tau aggregation or to halt tau misfolding remains to be elucidated.

Taken together, the results of chapter 6 reveal that SPPL2b is a novel pathological hallmark of AD associated with tau pathology. Taking into account that SPPL2b is involved in the RIP of proteins that are already related to early stages of AD and can contribute to the development of the classical AD hallmarks, it is tentative to speculate that SPPL2b is a potential novel etiological factor of AD that contributes to the initiation of the pathological cascade. On the other hand and similarly to the UPR, SPPL2b expression might be triggered as a protective mechanism against an already on-going pathological process trying to restore cell homeostasis. In addition, the potential of SPPL2b as a pathological marker should be explored further considering the strong divergence on SPPL2b expression between controls and AD patients.

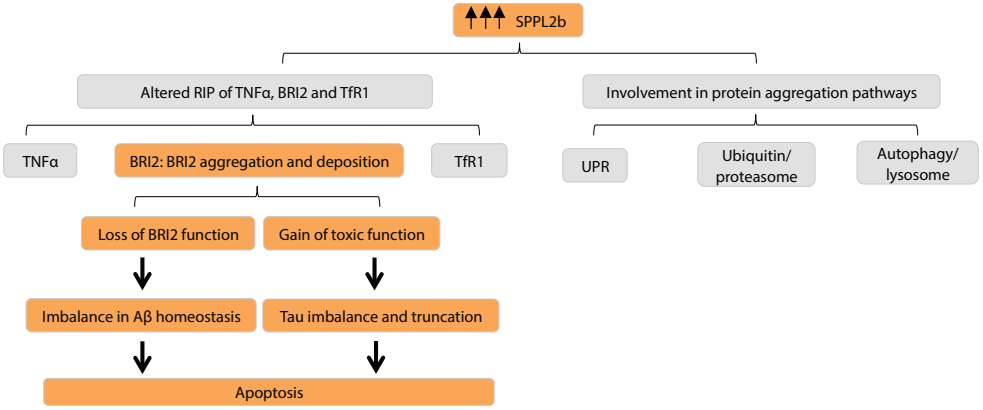

Fig. 1. Hypothetical contribution of the identified protein changes (SPPL2b and BRI2) to AD pathogenesis. The strong increase observed in SPPL2b may alter the regulated intramembrane proteolysis (RIP) of TNFα, TfR1 and BRI2. Abnormal RIP of BRI2 may explain the aggregation and deposition of BRI2 observed in AD as proposed in the discussion. BRI2 changes can induce a loss and a gain of toxic BRI2 function, which can influence different molecular pathways of AD such as Aβ metabolism (production, aggregation and clearance), tau truncation and apoptosis. In the other hand, SPPL2b may also participate in protein aggregation pathways, which are critical in the development of protein misfolding disorders. Orange squares represent the changes identified in this thesis

Novel AD protein hallmarks as potential CSF biomarkers.

One of the greatest challenges in AD research is to discover novel biomarkers able to predict the development of AD before extensive neuronal damage has taken place. Failure of current treatment paradigms is at least partly explained by their late administration. Thus, early diagnosis will likely increase the efficiency of potential disease modifying therapies. In addition, besides the important role of biomarkers as diagnostic tools, they can also be useful for prognosis, biological read-out for therapy development, monitoring disease progression and treatment efficiency.

It is known that the pathological changes leading ultimately to AD start decades before clinical symptoms[79] and thus, biomarkers reflecting the underlying pathological process are ideal potential tools for early diagnosis. Cerebrospinal fluid (CSF) is one of the main sources for biomarker discovery of the central nervous system since it is in direct contact with the brain and thus it can reflect the biochemical alterations occurring in it[80]. The current core AD CSF biomarkers, which reflect the classical pathological hallmarks ($A\beta_{42}$, t-Tau and p-Tau)[81–84], have been included in the new updated criteria for AD diagnosis (NIA-AA) to increase/decrease the confidence that dementia is due to AD[85]. One limitation of the use of the current biomarkers as diagnostic tool is the great variability found in the biomarker measurements between different studies as well as the diagnostic accuracy of those measurements[86]. One of the major sources of this variability is the lack of standardized protocols including preanalytical factors such as length of sample storage or freeze/thaw cycles[87,88]. Thus in chapter 7 we merged previous guidelines in CSF biobanking to create a new updated recommendation to standardize the preanalytical cofounding factors that can influence not only the analysis of the core AD and PD biomarkers but also for the analysis of potential novel CSF biomarkers in AD, PD and other neurodegenerative disorders. The quantification of the current core AD CSF biomarkers can accurately discriminate between AD and healthy controls and they can also reasonably predict the transition of mild cognitive impairment (MCI) to AD[89–91]. Nevertheless, the interest on biomarkers able to predict AD before the development of clinical symptoms is still ongoing.

BRI2 in CSF

The early increased expression of BRI2 in post-mortem human AD hippocampus (chapter 3) together with the increase in MCI converters in CSF shown by proteomics (chapter 8) suggested that BRI2 is a potential early biomarker for AD. This is further supported by an independent proteomics study which also detected an increase of BRI2 in AD CSF patients compared to controls[92]. Thus, we developed a specific enzyme-linked immunosorbent

assay (ELISA) to measure BRI2 levels in CSF and a preliminary validation of the assay was performed (chapter 8.1). Then we performed a case-control study, in which we determined the levels of BRI2 in CSF samples from non-demented control (subjected memory complaints, SMC) and AD patients (chapter 8.2), as well as a longitudinal approach in which samples from patients with MCI that after follow-up either remain stable (MCI-S) or converted to AD (MCI-AD) were also included (Chapter 8.3). No difference in the concentration of BRI2 CSF was found between the different clinical groups, suggesting that the neuropathological changes of BRI2 were not reflected in CSF and that BRI2 cannot be used as a biomarker using this ELISA. The discrepancy found between the proteomics data and the ELISA results might be explained by the BRI2 epitopes recognized by the latter, which may not exclusively detect the specific BRI2 fragments changed in AD leading to a loss of sensitivity. However, BRI2 CSF levels positively correlated with the levels of t-Tau and p-Tau, which reflect axonal neurodegeneration and NFT formation respectively[81,82,84]. These results not only support our previous *in vitro* findings in which $rBRI2_{76\text{-}266}$ promoted a neuronal apoptotic response and the truncation of tau (chapter 5) but also are in agreement with our neuropathological examination in which BRI2 changes correlated with NFT formation (chapter 3).

Interestingly BRI2 CSF levels correlated with the levels of $A\beta_{40}$ but not $A\beta_{42,}$ suggesting a relationship of BRI2 with Aβ production and not with Aβ plaque formation. Taken into account that BRI2 can regulate Aβ load[22–29], the correlation of BRI2 with $A\beta_{40}$ might not be surprising. Considering that BRI2 influences the aggregation and fibrillation of Aβ[31,32], the question is how the lack of correlation between BRI2 and $A\beta_{42}$ can be explained. Recent studies suggest that BRICHOS interferes with the generation of toxic and likely oligomeric $A\beta_{42}$ species rather than mature fibrils[93]. Changes in $A\beta_{42}$ reflects, however, plaque formation and thus likely the formation of insoluble Aβ fibrils in which BRI2 BRICHOS does not have any effect. The association of BRI2 BRICHOS with Aβ oligomers in an earliest phase but not with amyloid fibrils may hamper the detection of any correlation between BRI2 and $A\beta_{42}$. Thus, it would be interesting to analyze whether the levels of BRI2 in CSF correlate with the levels of oligomeric Aβ. Nevertheless, other unknown causes may explain the lack of correlation between $A\beta_{42}$ and BRI2 in CSF, as it happens for the enigmatic poor correlation observed between $A\beta_{42}$ and disease severity. Taken together, the BRI2 CSF results indicate that BRI2 cannot be used as an AD biomarker but it further supports an involvement of BRI2 in the underlying pathological pathways leading to AD.

Agrin in CSF

Agrin is a proteoglycan that had also been identified as a potential AD CSF biomarker in the proteomics study (chapter 9). Interestingly, agrin is a well-known Aβ associated

protein[94,95], and it was found to be increased in post-mortem hippocampus and prefrontal cortex of AD patients. Moreover, hippocampal soluble agrin was increased in early stages of AD[96]. However, to the best of our knowledge, the levels of agrin in CSF have never been measured. The results of chapter 9 revealed no difference in the levels of agrin CSF between the different clinical groups, nor a correlation with any of the classical AD CSF biomarkers, indicating that agrin changes are not reflected in CSF and thus it can not be used as an AD biomarker using the current assay. Noteworthy, the analysis of agrin in post-mortem tissue was performed using an in-house ELISA, which detected a specific agrin fragment that is not detectable with the commercial assay used in our study. Similarly, most of the agrin peptides identified in the proteomics study could not be recognized by the ELISA used in our study, based on sequence and epitope analysis. Strikingly, we found a strong association between the levels of agrin CSF and age only in the demented cases, suggesting that age may produce greater and detectable changes in the levels of agrin between AD patients and control cases. In summary, although the levels of agrin in CSF cannot be used as a diagnostic marker using the current immunoassay, it would be interesting to analyze the levels of agrin using the alternative ELISA available in an older cohort of patients.

SPPL2b in CSF

One of the novel pathological hallmarks identified in this thesis was SPPL2b (chapter 6). Interestingly, unlike Aβ deposition[71], SPPL2b staining was nearly absent in the hippocampus of control cases, highlighting the importance that this protein may have as a potential diagnostic marker of AD pathology. Since SPPL2b is a multi-pass transmembrane protein, its detection in CSF is challenging. However, a recent study was able to detect the integral membrane protein presinilin 1 in CSF[97] by western blot using a specific pre-treatment of CSF. Applying the same methodology we were able to detect in the CSF three different forms of SPPL2b at 150, 100 and 60 kDa, the latter being the expected molecular weight. Previous animal models have shown that SPPL2b can also run around 100 kDa[70]. Interestingly the presence of SDS-resistant dimers have been previously reported not only for SPP protein but also for SPPL3[98], which are from the same family as SPPL2b. Therefore, the higher molecular weight forms of SPPL2b may represent SPPL2b dimers or trimers. Alternative, glycosylation could also account for the higher forms of SPPL2b, as previously observed in a transfected cell line[66]. But how can a highly hydrophobic protein reach the CSF? Although the exact mechanisms by which neuronal proteins reach the CSF remains unclear, its conceivable that the CSF acts as lymphatic vasculature to drain and clear the different protein fragments (i.e. Aβ, BRI2, agrin) from the brain interstitium[99]. Importantly, brain interstitial fluid also contains extracellular vesicles (EV) and in fact, the presence

EV in CSF has also been reported[100]. One of the most well-characterized type of EV are exosomes, which can play an important role in neurodegenerative disorders[101,102]. Thus, taken into account that SPPL2b is found in the plasma membrane and endosomes, it is conceivable that SPPL2b, similarly to APP or different solute carriers[103], may reach the CSF via extracellular vesicles (i.e. exosomes).

Interestingly, in chapter 6 we detected that the 60 kDa form was significantly decreased in AD CSF compare to non-demented controls, as occurs with other AD-related proteins such as $A\beta_{42}$[104]. Although the mechanisms leading to this inverse expression patterns between brain tissue and CSF remains elusive, the reduced levels of $A\beta_{42}$ in CSF is attributed to the higher aggregation capacities of $A\beta_{42,}$ which leads to higher deposition of this protein in the brain. Taking into account that the function or involvement of SPPL2b in AD remains elusive, it is difficult to give a plausible explanation to the observed SPPL2b decrease in CSF. However, the close relationship of SPPL2b with protein aggregates may indicate that SPPL2b is trapped in aggregated structures leading to the decreased levels in CSF, and that by this interaction even influences the aggregation process.

Strikingly, while SPPL2b correlated with the $A\beta_{42}$ concentration and MMSE scores, it did not correlate with the levels of either t-Tau or p-Tau. The correlation between CSF SPPL2b with both the $A\beta_{42}$ concentration and MMSE scores supports the idea that changes in SPPL2b take place in very early stages of AD and are associated with cognitive decline. However, how can the lack of correlation between SPPL2b and tau be explained considering the strong association between those proteins in brain tissue? Previous models of the temporal evolution of AD biomarker situated Aβ as the first biomarker to become abnormal[105]. Nevertheless, due to the high pathological complexity of sporadic AD those models are expected to be different when compering early- and late-onset AD patients. Thus, a new model has suggested that in late-onset AD patients neurodegeneration due to tauopathy occurs before Aβ abnormalities, but changes are under the detection limit of the current biomarkers for neurodegeneration[106]. Thus, the detection of tau changes in CSF only in advanced stages together with the early changes on SPPL2b CSF may explain the lack of correlation between SPPL2b CSF and the concentration of t-Tau or p-Tau in CSF. Taken together, the CSF results call for the development of more sensitive techniques for the specific detection of SPPL2b *in vivo,* in either CSF or brain tissue (i.e. immunoassays and imaging approaches), to permit further investigation of the potential of SPPL2b as a biomarker for AD.

CONCLUDING REMARKS.

The great scientific efforts in understanding AD etiology during the last century have revealed that we are facing a highly complex and heterogeneous disorder with devastating consequences to society. The further molecular understanding of AD pathogenesis opens new insights on the development not only of disease modifying therapies but also of novel biomarkers that may help to predict AD in the earliest possible stage. In this thesis, we aimed to unravel novel hallmarks of AD with a potential involvement in the development of this disorder using proteomics candidates as starting point. In addition, we evaluated whether the novel AD signatures were reflected in CSF and thus, their potential as early biomarkers. Several studies in the last years have unraveled multiple potential AD biomarkers that still need to be validated[107,108]. Given the complexity of this disorder, it is conceivable that a combination of multiple markers including neurochemical molecules and imaging patterns will likely help to accurately predict AD, and thus a synergistic collaboration between scientists from different disciplines is needed. In addition, the development of more sensitive techniques together with the standardization of protocols and the definition and classification of the non-demented control groups (are subjective memory complaints cases optimal controls?) will likely promote the development of predictive diagnostic tools.

The most important findings of this thesis are:

- BRI2 is increased in early stages of AD associated with amyloid plaques (chapter 3).
- BRI2 processing enzymes are changed in AD (chapter 3).
- The BRI2-APP binding is present in human tissue and reduced in AD patients. (chapter 3).
- BRI2 changes correlated with decreased concentration and activity of IDE, an enzyme involved in Aβ degradation (chapter 4)
- Full length BRI2 ectodomain is less efficient on delaying Aβ fibrillation than BRI2 BRICHOS domain (chapter 5).
- Full length BRI2 ectodomain can induce an apoptotic response and promote tau truncation (chapter 5).
- SPPL2b is present in AD post-mortem tissue but absent or remarkably lower in control cases (chapter 6).
- SPPL2b increase started in very early stages of AD and is associated with the classical protein aggregates in AD and other misfolding dementias (chapter 6).
- SPPL2b is a novel tau binding protein (chapter 6).

- SPPL2b was decreased in AD CSF and correlated with the concentration of $A\beta_{42}$ and cognitive decline (chapter 6).
- BRI2 CSF cannot differentiate AD from healthy controls but correlates with the concentration of t-Tau, p-Tau, $A\beta_{40}$ and specific inflammatory and vascular markers (chapter 8).
- Increase of agrin in AD human tissue is not reflected in CSF but it is influenced by age in the demented cases (chapter 9)

FUTURE PERSPECTIVES: WHAT'S NEXT?

SPPL2b and BRI2 as potential etiological factors of AD.

In this thesis we have extensively analyzed the expression of both BRI2 and SPPL2b in post-mortem tissue unraveling the presence of two new characteristics in AD. However, it would be important to further explore the human AD neuropathology and perform a thorough characterization of the specific BRI2 and SPPL2b forms that are modified in AD. Knowing the specific characteristics of those proteins in AD (i.e. glycosylation, aggregation, sequence) will help for the development of sensitive techniques accurately detecting the pathological forms *in vivo*. In addition, taking into account the consequences that SPPL2b activity may have on its substrates, it is important to develop tools able to determine whether the increase on SPPL2b reflects also an increase of SPPL2b activity. Those tools can be then also used to perform a high throughput screening of pharmacological compounds able to bind/inhibit SPPL2b.
Although the data outlined in this thesis revealed that both BRI2 and SPPL2b are promising novel potential contributors to the pathogenesis of AD, we cannot ignore that the studies performed are primarily observational (with the exception of chapter 5). Thus, the current results set the basis for future *in vitro* and *in vivo* experiments, which will give mechanistic insights into the consequences of the SPPL2b increase and BRI2 changes. In this sense, SPPL2b overexpressing cell lines are already available and a mouse model overexpressing SPPL2b is currently being developed. The combination of human material, cell and animal models will help to unravel whether BRI2 and SPPL2b play indeed an important role in AD pathogenesis.

On the road to early AD diagnosis.

The SPPL2b changes found in CSF and tissue support the potential of this protein as an early AD biomarker, but more sensitive and specific techniques are needed. Noteworthy, the pathological changes occurring within the brain are not always reflected into the CSF

as seen not only in this thesis but also in numerous previous studies (i.e. serum amyloid P[109] or C-reactive protein[110] among others). Therefore, considering that SPPL2b reactivity was nearly absent in non-demented controls, it would be also interesting to study the potential of imaging techniques such as positron emission tomography (PET) to measure SPPL2b changes *in vivo*.

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APPENDIX

NEDERLANDSE SAMENVATTING

Gedurende de afgelopen eeuw heeft men zich enorme wetenschappelijke inspanningen getroost om het ontstaan en het verloop van de ziekte van Alzheimer (Alzheimer's Disease, AD) beter te kunnen begrijpen. Dit onderzoek heeft ons geleerd dat het hier een zeer complexe en heterogene aandoening betreft, met een verwoestende uitwerking, niet alleen op de patiënt en zijn omgeving, maar ook op de maatschappij als geheel. Een verder begrip van het AD-ziekteproces op moleculair niveau kan leiden tot een beter inzicht in de mogelijkheden voor het ontwikkelen van alternatieve therapieën die de ziekte kunnen remmen. Bovendien kan een groter inzicht mogelijk licht werpen op moleculen die als nieuwe 'biomarkers' kunnen worden ingezet bij het zo vroeg mogelijk voorspellen van het optreden van de ziekte.

Het is daarom van het grootste belang om te komen tot een identificatie van eiwitmoleculen die een rol spelen bij de AD-pathogenese, en een opheldering van hun functionele verbanden (in 'moleculaire paden': cellulaire signaal- of werkingsroutes van onderling interagerende eiwitten). In het laatste decennium heeft het bepalen van zeer gedetailleerde, weefsel- of celspecifieke eiwitprofielen ('proteomen') met behulp van hoogwaardige massaspectrometers een hoge vlucht genomen. Als geen ander stelt deze 'proteomics'-techniek de onderzoeker in staat om zonder hypothese vooraf verschillende situaties met elkaar te vergelijken, en afwijkende (eiwit)patronen te onderkennen bij tal van ziekten (zoals neurodegeneratieve aandoeningen)- afwijkingen die mogelijk in het geheel niet werden geanticipeerd. Met behulp van 'proteomics' hebben we inmiddels een reeks nieuwe kandidaat-biomarkers voor vroege AD-diagnose geïdentificeerd die mogelijk ook een causale rol spelen. Tot deze groep behoren de eiwitten BRI2 (BRICHOS Domain Containing 2B, ook bekend als ITM2B, integral membrane protein 2B) en AGRN (agrine).

Het doel van het in dit proefschrift beschreven onderzoek was om nieuwe, mogelijk bij het ontstaan van AD betrokken kenmerken van AD te onthullen, uitgaande van bovengenoemde kandidaateiwitten. Daarnaast werd gekeken of de via 'proteomics' geïdentificeerde groep van eiwitten die in AD afwijkt en tezamen een zogenaamde 'signature' voor AD vormen ook kan worden teruggevonden in ruggemergvocht (cerebrospinal fluid, CSF). Dergelijke eiwitten zouden bij uitstek dienst kunnen doen als 'biomarkers' voor vroege AD-diagnose.
Het eerste deel van dit proefschrift richt zich op het belang van een disfunctioneren van het BRI2-eiwit voor de belangrijkste moleculaire paden die zijn betrokken bij AD. Dit kan

leiden tot een aangepaste hypothese omtrent het ontstaan van de ziekte (**hoofdstuk 2**).In overeenstemming met deze hypothese ontdekten we in postmortem weefsel van AD-patiënten in verschillende ziektestadia veranderingen in het niveau van zowel het BRI2-eiwit zelf als van eiwitten die betrokken zijn bij het bewerken van BRI2 tot een bio-actief molecuul. Bovendien ontdekten we dat de vorming van BRI2-APP-complexen, al eerder beschreven in cel- en muizenmodellen, ook optreedt in menselijk hersenweefsel, maar in mindere mate in AD-patiënten ten opzichte van gezonde controles (**hoofdstuk 3**). Opmerkelijk genoeg vonden we dat de veranderingen in het BRI2-niveau in postmortem weefsel is gecorreleerd met veranderingen in de expressie en de activiteit van het eiwit IDE (Insulin-Degrading Enzyme), een enzym dat ondermeer is betrokken bij de afbraak van bèta-amyloid (Aβ) peptides die worden gevonden in plaques binnen het brein van AD-patiënten (**hoofdstuk 4**).

De resultaten beschreven in hoofdstuk 3 suggereerden het bestaan van grotere BRI2-bevattende structuren in AD-weefsel en vormden de basis voor een vervolgstudie waarin we het effect onderzochten van de aanwezigheid van grote recombinante BRI2-structuren op moleculaire paden geassocieerd met AD-pathogenese, zoals apoptose (geprogrammeerde celdood) en de 'unfolded protein response' (UPR, cellulaire reactie op niet goed gevouwen eiwitten), of op de afknotting of fosforylering van het van 'tangles' bekende tau-eiwit (**hoofdstuk 5**).

Een ander resultaat beschreven in hoofdstuk 3 betreft een dramatisch veranderd niveau van één van de BRI2-bewerkende enzymen, SPPL2B (Signal Peptide Peptidase Like 2B) in hersenweefsel van AD-patiënten. Het SPPL2B-eiwit is een relatief nieuw ontdekt transmembraanprotease waarvan de relatie met AD niet was geanticipeerd. Daarom hebben we de expressie van SPPL2B uitgebreid bestudeerd in postmortem weefsel van zowel AD-patiënten en gezonde controles als patiënten die leden aan andere ziektes geassocieerd met defecte eiwitvouwing, zoals frontotemporale dementie en de ziekte van Parkinson (**hoofstuk 6**). We ontdekten ook een associatie van de SPPL2B-expressie met die van het tau-eiwit in verschillende tau-gerelateerde aandoeningen ('tauopathies'). Een eerste evaluatie van SPPL2B in CSF leerde ons dat het niveau van dit eiwit niet alleen lager is in AD-patiënten ten opzichte van controles zonder dementie, maar ook correleert met het niveau van Aβ-peptides en het optreden van cognitieve stoornissen.
In het laatste deel van dit proefschrift richten we ons op onderzoek naar het potentieel dat eiwitten zoals BRI2 en agrine hebben als in CSF te detecteren 'biomarkers' voor vroege diagnose van AD. Dit was aannemelijk aangezien veranderingen in het niveau van beide eiwitten al eerder zijn gerapporteerd voor hersenweefsel[127] en CSF[128] van AD-patiënten. Om een dergelijk potentieel te kunnen onderzoeken is een standaardprocedure (SOP,

Standard Operating Procedure) voor het hanteren en in een biobank opslaan van grote verzamelingen (cohorts) CSF absoluut vereist om accurate resultaten te kunnen genereren die ook door andere onderzoeksgroepen kunnen worden verkregen in een vergelijkbare studie. Als onderdeel van het BIOMARKAPD-consortium dat is gericht op het formuleren van internationaal geldende SOP's hebben we eerdere aanbevelingen voor de CSF-gebaseerde analyse van 'biomarkers' voor AD en de ziekte van Parkinson bijgewerkt en samengevoegd (**hoofdstuk 7**). Vervolgens hebben we eerst een nieuwe, specifieke immuunbepaling voor het BRI2-niveau in CSF-monsters ontwikkeld (**hoofdstuk 8**). Met deze bepaling is het niveau van BRI2 geanalyseerd in een 'case-control' studie met CSF van AD-patiënten en controles zonder dementie, en in een longitudinale studie waarin naast deze eerste twee groepen ook twee groepen waren opgenomen met een milde cognitieve stoornis (MCI-S, stabiel; MCI-AD, op weg naar ontwikkeling van AD). Ook werd de associatie van het BRI2-niveau met klassieke 'biomarkers' voor AD onderzocht. In het laatste hoofdstuk beschrijven we onderzoek waarin wordt geëvalueerd of de veranderingen in agrine in AD-weefsel ook tot uiting komen in CSF, en of er een relatie is met klassieke AD 'markers'. Hierbij is gebruik gemaakt van een commerciële immuunbepaling (**hoofdstuk 9**).

Samenvattend: dit proefschrift beschrijft de analyse van nieuwe eiwit-'signatures' voor vroege stadia van AD. De resultaten vormen de basis voor toekomstig onderzoek dat is gericht op het begrijpen van de rol van veranderingen in (het niveau van) BRI2 en SPPL2B in de pathogenese van AD. Dit kan leiden tot nieuwe inzichten die zouden kunnen uitmonden in nieuwe preventieve en ziekteremmende therapieën. Daarnaast vragen de door ons waargenomen sterke veranderingen in SPPL2B-niveaus in AD-patiënten ten opzichte van gezonde individuen om de ontwikkeling van veel gevoeligere technieken voor de *in vitro*- en/of *in vivo*-detectie van SPPL2B in CSF of in hersenweefsel. Hierbij valt te denken aan immuunbepalingen en imaging-technieken. Dit zal in de toekomst het potentieel duidelijk kunnen maken van SPPL2B als diagnostische 'biomarker' voor AD.

LIST OF ABBREVIATIONS

2D-LC/MS	2 dimensions liquid chromatography- mass spectrometry
ABri	British amyloid
AD	Alzheimer's disease
ADan	Danish amyloid
AICD	Amyloid precursor protein intracellular domain
ApoE	Apolipoprotein E
ApoJ	Apolipoprotein J / Clusterin
APP/AβPP	Amyloid-β precursor protein
Aβ	Amyloid-β
$Aβ_{40}$	Amyloid-β 1-40
$Aβ_{42}$	Amyloid-β 1-42
BACE1	β-secretase 1
BBB	Blood brain barrier
BiP	Binding immunoglobulin protein
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CHOP	CCAAT-enhancer-binding protein homologous protein
cNEUPRO	Clinical NEUroPROteomics of neurodegenerative diseases
CR1	Complement receptor 1
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CV	Coefficient of variation
DAB	3,3-diaminobenzidine
DMEM	Dulbecco's modified essential medium
DTT	Dithiothreitol
EEF	Eukaryotic Elongation factor
ELISA	Enzyme linked immuno-sorbent assay
ER	Endoplasmatic reticulum
EV	Extracellular vesicles
FAD	Familial Alzheimer's disease
FBD	Familial British dementia
FDD	Familal Danish dementia
FTD	Frontotemporal dementia
GSK3β	Glycogen synthase kinase-3β
HSPG	Heparin sulfate proteoglycans
ICAM-1	Intercellular adhesion molecule 1
ICD	Intracellular domain
IDE	Insulin-degrading enzyme
IL-2	Inflammatory cytokine interleukin-12
IPTG	Isopropylthiogalactoside
ITM2b/BRI2	Integral membrane protein 2B
LLOD	Lower limit of detection
LPC	Protein converstase type 7

LPH	Limulus Polyphemus Hemocyanin
LPR	Liquid permanent red
MCI	Mild cognitive impairment
MCI-AD	Mild cognitive impairment converting to AD
MCI-S	Mild cognitive impairment remaining stable
MIP1α	Macrophage inflammatory protein-1α
MMSE	Mini mental state examination
MPER	Mamalian protein extraction reagent
MRM	Multiple reaction monitoring
MS	Multiple sclerosis
MTT	(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFT	Neurofibrillary tangles
NIA-AA	National institute of aging - Alzheimer's association
NSAIDs	national institute of neurological and communicative disorders
NTF	N-terminal fragment
NUBIN	NeuroUnit Biomarkers for Inflammation and Neurodegeneration
p-Tau	Phosohorylated tau
p23WT	23-aa C-terminal peptide
PC	Protein convertase
PC6A	Protein convertase type 5 isoform A
PD	Parkinson's disease
PET	Positron emission tomography
PHF	Paired helical filaments
PiD	Pick's disease
PMI	Post-mortem interval
proSP-C	Lung surfactant protein C
PS1	Presenilin 1
PS2	Presenilin 2
PSP	Progressive supranuclear palsy
qPCR	Quantitatve realtime polymerase chain reaction
RIP	Regulated intramembrane proteolisys
sAPPβ	N-terminal-soluble APPβ
SEM	Standard error of the mean
SMC	Subjective memory complaints
SOP	Standard operating procedures
SPPL2b	Signal peptide peptidase like protein 2b
t-Tau	Total tau
TfR1	Transferrin receptor-1
ThT	Thioflavin T
TMB	Tetramethylbenzidine/Dimethylsulfoxide
TREM2	Triggering receptor expressed on myeloid cells 2
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule 1
VaD	Vascular dementia
VEGF	Vascular endothelial growth factor
Xbp-1	X-box binding protein 1

LIST OF THESES ALZHEIMER CENTER

1. L. Gootjes: Dichotic Listening, hemispheral connectivity and dementia (14-09-2004)
2. K. van Dijk: Peripheral Nerve Stimulation in Alzheimer's Disease (16-01-2005)
3. R. Goekoop: Functional MRI of cholinergic transmission (16-01-2006)
4. R. Lazeron: Cognitive aspects in Multiple Sclerosis (03-07- 2006)
5. N.S.M. Schoonenboom: CSF markers in Dementia (10-11-2006)
6. E.S.C. Korf: Medial Temporal Lobe atrophy on MRI: risk factors and predictive value (22-11-2006)
7. B. van Harten: Aspects of subcortical vascular ischemic disease (22-12-2006)
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48. C. Möller: Imaging patterns of tissue destruction – Towards a better discrimination of types of dementia (01-05-2015)
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Del Campo M, Hoozemans JJM, Zuroff L, Schröder B, Scheltens P, Fluhrer R, Teunissen CE *SPPL2b is drastically increased in early stages of Alzheimer's disease and associated with tau pathology.* Under review.

Del Campo M and Teunissen CE. *Facilitating the validation of novel biomarkers: an optimal workflow for immunoassay development.* In preparation

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CURRICULUM VITAE

Marta del Campo Milán was born on May 10th, 1986 in Madrid, Spain. She graduated high school in 2004 from the Instituto público Mirasierra in Madrid. That same year she started her studies in Biology at the Universidad Autónoma de Madrid. In 2007 she received an Erasmus Mundus grant to study one academic year at the University of Bedfordshire (United Kingdom). During this year she collaborated in a research project related to Parkinson's disease, which raised her interest in human pathophysiology and more specifically in neurodegenerative disorders. In 2009 she started her master's degree in Molecular Bioscience and Biotechnology and she participated in a project related to cardiovascular disease. In 2010, her interest in neurodegeneration and more specifically in Alzheimer's disease was raised again by the PhD projects supported by the ENC-Network. This organization aims to integrate information from multiple disciplines combining basic and clinical research to finally get a better understanding about the nervous system and its respective disorders. She did not hesitate to apply for an ENC-project at the Clinical Chemistry department and Alzheimer Center of the VUmc in Amsterdam. In September 2010, she started the research project that resulted in this thesis under the supervision of M.A. Blankenstein, Ph. Scheltens, C.R. Jimenez and C.E. Teunissen. She performed part of her PhD also at the Center for Neuroscience and Cell Biology (CNC) in Coimbra under the supervision of C. Oliveira and C. Pereira. Since December 2014 she works as a post-doc researcher at the department of Clinical Chemistry of the VUmc in Amsterdam.

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"Reserve your right to think, for even to think wrongly is better than not to think at all."

Hypatia