Cerebrospinal fluid biomarkers for differentiating between Alzheimer's disease and Vascular dementia

Maria Bjerke

Institute of Neuroscience and Physiology

Department of Psychiatry and Neurochemistry

The Sahlgrenska Academy

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Institute of Neuroscience and Physiology

Gothenburg University

Sweden

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To my family

ABSTRACT

Patients suffering from mild cognitive impairment (MCI) run a higher risk of developing dementia, with Alzheimer's disease (AD) being the most common form. Vascular dementia (VaD) is proposed to be the second most common dementia entity, and it includes the clinically relatively homogenous subgroup of subcortical vascular dementia (SVD). Varying degrees of concomitant vascular lesions represent a link between AD and VaD, comprising a state of mixed dementia (MD). Biochemical markers provide important information which may contribute to differentiating between dementias of different etiologies, and in combination with the clinical assessment may improve diagnostic accuracy. The overall aim of this thesis is to provide for better separation between patients suffering from SVD and AD with the aid of biochemical markers.

The cerebrospinal fluid (CSF) biomarkers T-tau, P-tau$_{181}$, and Aβ_{1-42}, have proven useful in distinguishing MCI patients who ultimately develop AD (MCI-AD) at follow-up from those who remain stable. However, less is known about the biomarker pattern in MCI patients who develop SVD (MCI-SVD). An elevated baseline level of NF-L was found in MCI-SVD patients compared with stable MCI patients, while MCI-AD had decreased levels of Aβ_{1-42} and increased levels of T-tau and P-tau$_{181}$ compared with MCI-SVD patients and stable MCI patients.

The biomarkers NF-L, MBP, MMPs and TIMPs together with T-tau, P-tau$_{181}$, HFABP, and Aβ_{1-42} were assessed with the aim of improving discrimination between patients with SVD and AD as well as controls. Biochemical fingerprints representative of subcortical (NF-L, MBP and TIMP-1) and cortical alterations (T-tau, P-tau$_{181}$ and Aβ_{1-42}) provided for high discrimination between patients with SVD and AD, respectively, and between patients and healthy controls.

Enzymatic processing of the amyloid precursor protein (APP) was investigated on the basis of possible divergences in CSF APP metabolites in patients with SVD, MD, and AD as well as controls. A correlation between the levels of the soluble APP metabolite cleaved at the β site and the activity of an as yet unknown β-site cleaving metalloproteinase was found in all examined groups indicating similarities in processing pathways but dissimilarities in pathological mechanisms.

A multicentre study could be an important step to verify these results. However, high inter-centre variability is a problem for both Tau and Aβ_{1-42} measurements making such an enterprise difficult. Confounding factors affecting the stability of Aβ measurements were investigated and a major contributing factor seems to be assay specific, due to variation in antibodies and standards.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Demens är ett sjukdomssyndrom med en påtaglig nedsättning av den kognitiva förmågan. De två vanligaste demenssjukdomarna är Alzheimers sjukdom (AD) och vaskulär demens (VaD). Den vanligaste formen av VaD är subkortikal vaskulär demens (SVD) vilken är en småkärlssjukdom som ger upphov till lakunära infarkter och ischemiska vitsubstansskador i hjärnas centrala delar. AD och VaD är ofta överlappande sjukdomar och man talar då om att dessa patienter har drabbats av blanddemens (BD). Manifest demenssjukdom föregås vanligen av ett stadium av lindrig kognitiv störning (MCI). Alla patienter med MCI utvecklar emellertid inte demens.

Cerebrospinalvätska (likvor, ryggvätska) står i direkt kontakt med hjärnan och dess molekylära sammansättning antas avspegla hjärnans metabola processer. Många studier har påvisat förändringar i likvor av amyloid β (Aβ) och tau hos patienter med AD gentemot kontroller. Fokus har på senare år flyttats till MCI för att kunna särskilja dem som kommer att utveckla AD från dem som förblir stabila. Målet med avhandlingen är att undersöka potentiella markörer för småkärlssjukdom (=vitsubstansmarkörer) och jämföra dem med de mer väletablerade AD-markörerna hos patienter med SVD, BD och AD. Likaså är syftet att finna potentiella markörer för hur de olika skadetyperna uppkommer i hjärnan.

Flera studier har visat avvikelser av Aβ och tau i likvor hos patienter med MCI som senare utvecklar AD. Patienter med manifest VaD har förändringar i Aβ, men resultaten varierar för tau. Förhöjning av neurofilament (NF-L), som representerar subkortikal axonal skada, har påvisats hos patienter med SVD. Föga är emellertid känt om förändringar i MCI stadiet hos dem som senare utvecklar SVD. I den aktuella studien påvisades att MCI patienter som senare utvecklar SVD har en annan likvorprofil med förhöjning av NF-L och övervägande normala AD-markörer än de som senare utvecklar BD eller AD.

Då mätvärden avseende Aβ i likvor skiljer sig åt mellan forskningscentra gjordes en analys av möjliga felkällor. Vanligt förekommande kommersiella immunokemiska metoder testades. Preanalytisk behandling av prover och eventuella faktorer i likvor som kan påverka åtkomsten av analyten undersöktes. Största källan till variation visad sig ligga i ”mätmetodsmässiga” förhållanden, inbegripande antikroppar och buffertar.

I syfte att undersöka eventuella biokemiska skillnader mellan SVD och AD analyserades hjärnregionala markörer (tau, NF-L och myelin basiskt protein

(MBP)) i kombination med s.k. matrixmodulerande enzymer (MMP-1,-2,-3,-9 & -10) och dess hämmare (TIMP-1 & -2) liksom Aβ och heart fatty acid binding protein med hjälp av immunokemiska metoder. Med s.k. multivariat statistik kunde konstateras att MBP, TIMP-1, NFL, tau, MMP-9 och Aβ bidrog till att separera SVD från AD med hög sensitivitet (89%) och specificitet (90%).

Skillnader i nivåer av lösligt APPβ och Aβ, vilka båda klyvs ut med hjälp av enzymet β-sekretas, har påträffats i likvor från AD och SVD patienter. BACE-1 är ett β-sekretas som man tror står för denna processning hos patienter med AD. Enzymet har ett surt pH-optimum och tros klyva ut Aβ intracellulärt i en sur vesikelmiljö. Hur klyvningen tillgår hos patienter med vaskulär patologi är inte känt men man kan anta att den sker i den mer basiska miljön extracellulärt. Därför testades likvor vid ett mer basiskt pH med en framtagen substratassay som bygger på den vildtypssekvens som spänner över klyvningsstället för β-sekretas. Sänkta nivåer av lösligt APPβ och enzymaktivitet skiljde SVD patienter åt från kontroller, BD och AD. Den uppmätta aktiviteten för detta okända β-sekretas samvarierade med sAPPβ nivåerna i alla fyra grupperna, liksom med Aβ i AD gruppen. Fyndet talar för förekomsten av en ny klyvningsmekanism av APP/Aβ, vilken förmodligen har betydelse för sjukdomsprocessen vid SVD och AD.

Studierna visar att biokemiska förändringar i likvor som speglar olika sjukdomsprocesser i hjärnan sker tidigt innan de kliniska symptomen behöver vara påtagliga. De påvisade förändringarna talar också för att det är möjligt att särskilja SVD från AD med neurokemisk metodik. Resultaten har betydelse för hur man diagnostiserar de vanligaste åldersrelaterade kognitiva sjukdomarna. Därutöver är fyndet av en ny klyvningsmekanism för APP/Aβ betydelsefullt för förståelsen av en del av sjukdomsprocesserna vid utvecklandet av demenssjukdom.

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LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their roman numerals:

I. Bjerke, M; Andreasson, U; Rolstad, S; Nordlund, A; Lind, K; Zetterberg, H; Edman, Å; Blennow, K; Wallin, A. **Subcortical Vascular Dementia biomarker pattern in Mild Cognitive Impairment.** *Dement Geriatr Cogn Disord*. 28(4): 348-356, 2009

II. Bjerke, M; Portelius, E; Minthon, L; Wallin, A; Anckarsäter, H; Anckarsäter, R; Andreasen, N; Zetterberg, H;Andreasson, U; Blennow, K. **Confounding factors influencing amyloid beta concentration in cerebrospinal fluid.** *Int J Alzheimers Dis*. 15:1-11, 2010

III. Bjerke, M; Zetterberg, H; Edman, Å; Blennow, K; Wallin, A; Andreasson, U. **Cerebrospinal fluid matrix metalloproteinases in combination with markers reflecting subcortical and cortical alterations differentiate between Vascular dementia and Alzheimer's disease.** Submitted

IV. Bjerke, M; Zetterberg, H; Edman, Å; Blennow, K; Wallin, A; Andreasson, U. **A novel β-secretase activity correlates with amyloid precursor protein metabolites in cerebrospinal fluid of Vascular dementia and Alzheimer's disease.** Manuscript

ABBREVIATIONS

Aβ	Amyloid-β
AD	Alzheimer's disease
ADAM	A Disintegrin And Metalloproteinase
AICD	APP intracellular domain
APLP	APP-like protein
APOE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	β-site APP cleaving enzyme 1
BBB	Blood-brain barrier
CAA	Cerebral amyloid angiopathy
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CNS	Central nervous system
CSF	Cerebrospinal fluid
CVD	Cerebrovascular disease
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbant assay
ESI	Electrospray ionization
FRET	Fluorescence resonance energy transfer
FTICR	Fourier transform ion cyclotron resonance
IEC	Ion exchange chromatography
MAP	Microtubule-associated protein
MBP	Myelin basic protein

MCI	Mild cognitive impairment
MD	Mixed dementia
MMP	Matrix metalloproteinases
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MT	Microtubule
NF(-L)	Neurofilament (light)
NFT	Neurofibrillary tangles
KPI	Kunitz protease inhibitor
LC	Liquid chromatography
LQIT	Linear quadrupole ion trap
OPLS-DA	Orthogonal projection to latent structures discriminant analysis
ROC	Receiver operating characteristic
RP	Reversed phase
sAPPα	Soluble N-terminal APP cleaved at the α-site
sAPPβ	Soluble N-terminal APP cleaved at the β -site
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SVD	Subcortical vascular dementia
TGN	Trans Golgi network
TIMP	Tissue inhibitor of metalloproteinases
VaD	Vascular dementia
WML	White matter lesion

INTRODUCTION

1 The central nervous system

The central nervous system (CNS) consists of the spinal cord and the brain. The human CNS is made up of around a hundred billion neurons and glia and their innumerable connections are intertwined by a complex network of blood vessels [1, 2]. In the adult brain the major part of the cells originate from the glial lineage, which includes astrocytes, microglia and oligodendrocytes. Glia should not only be considered as connective tissue, as the name implies (Greek: *"glia",* glue), but as highly functional units. The glia provides the basis for appropriate development, function and repair of the neuronal network. This is possible through continuous cross-talk between the glia and neurons mediated by neurotransmitters, cytokines and trophic factor secretion [3-7].

The oligodendrocytes are responsible for the axonal integrity where the myelinating sheaths insulate electrical signals travelling down the axon. Microglia scavange the brain for cellular debris and play a part in the inflammatory process [8], while the astrocytes constitute the majority of the glial cells and are involved in homeostasis of the brain microenvironment, regulate metabolic support of neurons and contribute to the maintenance and development of the blood-brain barrier (BBB) [9]. Astrocytes also establish the connections between neurons and blood vessels. The endothelial cells of the blood-brain barrier protect the CNS from the vascular system and support it with nutrients.

The vascular system is thus the provider of vital oxygen and nutrients for the CNS, a process regulated through dynamic communications with neurons and glia [10, 11] including modulation of blood vessel dilation and constriction [12, 13], as well as homeostatic regulation of the BBB [14, 15]. At the cellular and molecular levels, communication between the circulatory system and the CNS occurs within integrated, multicellular structures, termed neurovascular units [16]. However, the information processing of the brain is believed to be performed by the neurons and that is why the main focus is usually directed towards this brain constituent.

The neuron cell body, or soma, is connected to two types of processes (figure 1): the dendrites and the axon. Dendrites receive signals from other neurons and transfer them to the soma. In addition, the soma also receives direct input. Upon sufficient membrane depolarization, an action potential is initiated at the axon

hillock, i.e., the received signal is conducted through the axon as an electrical impulse (action potential) and is further transmitted to following neurons via the synapses. The isolating myelin sheath surrounding the axon is not continuous, but interrupted by gaps called nodes of Ranvier, a circumstance which increases the electrical transmission speed by saltatory conduction. The axon can split into several axon collaterals which divide into terminal buttons forming the synaptic region. When the electrical signal reaches the synapse it causes a release from the presynaptic terminal of chemical substances, called neurotransmitters, that traverse the synaptic cleft to initiate a new signalling cascade at the postsynaptic terminal of the next neuron.

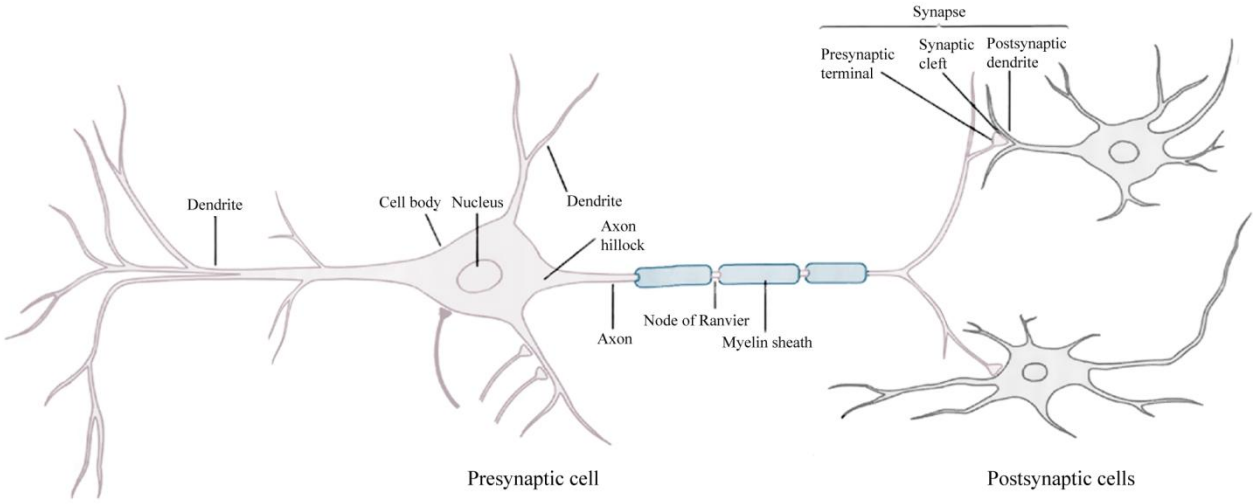

Figure 1. Schematic structure of a pyramidal neuron.

2 Central nervous system disease causing cognitive impairment

The concept of dementia as an age-dependent cognitive decline (Latin: "*de mens*", without mind) was already described thousands of years ago by Greek and Roman philosophers and physicians [17]. It refers to a state where cognitive function as well as the ability to perform the tasks of everyday life are impaired [18]. Mild cognitive impairment (MCI) is a state wherein the cognitive functions are mildly impaired, as the name implies, while the ability to perform everyday tasks is virtually intact. However, MCI is recognized as a risk factor for the development of dementia, though it is not inevitable [19]. The main risk factor for MCI and dementia is high age and several diseases and conditions may lead to dementia of which the most common ones are Alzheimer's disease (AD) and vascular dementia (VaD) [20]. Interestingly, cerebrovascular disorder is found not only in VaD, but in quite many cases of AD. Cases where Alzheimer encephalopathy and cerebrovascular disease are both present to a considerable degree, are often referred to as mixed dementia (MD) [21].

2.1 Mild cognitive impairment

MCI is a heterogeneous condition of cognitive impairment formerly classified as a transitional state between normal aging and dementia [19], but has recently been redefined as a risk factor. In 2004 a consensus report based on progress within the MCI research field by the international working group on mild cognitive impairment proposed the following criteria for MCI: (i) the patient has neither normal cognition nor dementia; (ii) there is evidence of cognitive deterioration shown by either objectively measured decline over time or subjective report of decline by self and/or informant in conjunction with objective cognitive deficits; and (iii) activities of daily living are preserved and complex instrumental functions are either intact or minimally impaired [22]. The heterogeneity of the MCI population is reflected by the various follow-up outcomes such as patients reverting to normal, remaining stable in their MCI during follow-ups or deteriorating to overt dementia. The annual conversion rate into dementia in a clinical MCI study was shown to be 5-10 percent, however it was also shown that more than 50 percent of the MCI patients did not convert even after 10 years of follow-up [23]. The aetiology of MCI is multifactorial and neuropathological studies have shown a relation to both AD pathology and cerebral infarctions [24].

2.2 Alzheimer's disease

In 1907 Alois Alzheimer published a case report on a 56 year old woman, who was suffering from progressive memory loss, disorientation, and hallucinations with neuropathological findings of senile plaques and neurofibrillary tangles at postmortem examination. These findings gave cause for Kraepelin, one of the foremost psychiatrists in Germany at that time and a colleague of Alzheimer, to later name the disease after Alzheimer.

2.2.1 Diagnostic criteria and clinical manifestation

AD is regarded as the most common form of dementia [20] and is characterized by an insidious onset with a slow progressive course with impairment of memory, language, and visuospatial functions ultimately resulting in global cognitive impairment [25]. The diagnosis of dementia is commonly based on the DSM-III-R criteria [18] and the diagnosis of AD is specifically based on the criteria of NINCDS-ADRDA [26] together with ICD-10 [27].

2.2.2 Neuropathology

Pathological studies have shown that neuronal degeneration, reflected by neuronal and synapse loss, in posterior cortical association brain regions is considerable in AD whereas only limited in the aging brain [28-34]. The most vulnerable neuronal circuits are those of the limbic structure, such as the perforant path which connects the entorhinal cortex with the hippocampus, and the long projecting corticocortical pathways linking the association areas with the prefrontal cortex [31, 35, 36]. The microscopic hallmarks of AD are dystrophic neurites, extracellular senile plaques and intracellular neurofibrillary tangles (NFT) [37]. However, both plaques and NFTs can be seen in the normal aging brain but to a lesser extent and appear to have no significant effect on cognition [38-41]. In AD however, it is believed that plaques and NFTs have detrimental effects on neuronal function and synapses leading to extensive neuronal loss compared with age-matched controls [28, 42, 43]. Senile plaques are mainly composed of amyloid-β (Aβ) peptides [44], whereas neurofibrillary tangles are assemblies of the hyperphosphorylated form of the micro-tubule associated protein tau [45].

2.2.3 Familial Alzheimer's disease

The discovery of AD cases arising from inherited autosomal dominant gene mutations which affect the amyloid precursor protein (APP) metabolism and leads to an early onset of the disease (between the fourth and the sixth decade) spurred the hypothesis that Aβ was the culprit in the disease pathology. All known familial forms of AD (FAD), accounting for less than 1 percent [46] of AD cases, are due to either mutations in the gene encoding APP or in the genes of APP cleaving enzymes (presenilin-1 and -2) [47-49]. Much effort has been focused on understanding the effects of APP and its metabolites as well as the APP cleaving enzymes and the connection to the pathology of sporadic AD, for which the currently known main risk factors are increased age and the presence of the Apolipoprotein E (*APOE*) ε4 allele [50].

2.2.4 Amyloid precursor protein function and processing

One of the earliest findings giving rise to the amyloid cascade hypothesis, stating that the mismetabolism of APP giving rise to the accumulation of Aβ seen in the AD brain, was the discovery of Aβ as a core constituent of cerebrovascular amyloid [44, 51]. Soon thereafter the gene encoding the parent protein, APP, was identified [52-55] and the APP gene was found to be located on chromosome 21[55]. Individuals with trisomy 21 (Down syndrome) who live beyond middle age

develop brain neuropathology identical to that observed in AD [56, 57], possibly due to the triplication of the APP gene. Furthermore, APP was found to be evolutionary highly conserved and two homologous mammalian proteins, APP-like protein-1 and -2 (APLP1 and APLP2), have been identified [58, 59]. The APP family proteins are intriguing with many different suggested functions such as signal receptors and/or adhesion molecules or physiological functions mediated by shedding of soluble fragments. It seems that the APP family has somewhat overlapping functions [60].

The APP is a type I transmembrane protein [53] whose transcript can be alternatively spliced resulting in three protein isoforms; APP695, APP751 and APP770 containing the same number of amino acids as the designations imply. The two longer forms contain the Kunitz Protease Inhibitor (KPI) domain (as does APLP2) and are expressed in most tissues, while the shortest form that lacks this domain is predominantly expressed in neurons [61, 62]. The full-length APP is processed by three major proteases termed α-, β- (extracellular or luminal cleavage) and γ-secretases (transmembrane cleavage) by two distinct pathways (figure 2). Sequential cleavage by α- and γ-secretase, in the so called non-amyloidogenic pathway, generates a soluble N-terminal ectodomain from the α-cleavage (sAPPα) and a fragment termed p3 by concomitant cleavage by γ-secretase. This pathway precludes the formation of Aβ, which is generated by the amyloidogenic pathway wherein sequential cleavage by β- and γ-secretase gives rise to the Aβ peptide as well as the N-terminal soluble β-cleaved fragment (sAPPβ). In addition, the γ-cleavage generates a C-terminal cytoplasmic fragment termed APP intracellular domain (AICD), which has been suggested to act as a transcription factor. However, the role of AICD in AD pathogenesis is elusive and remains to be firmly established [63].

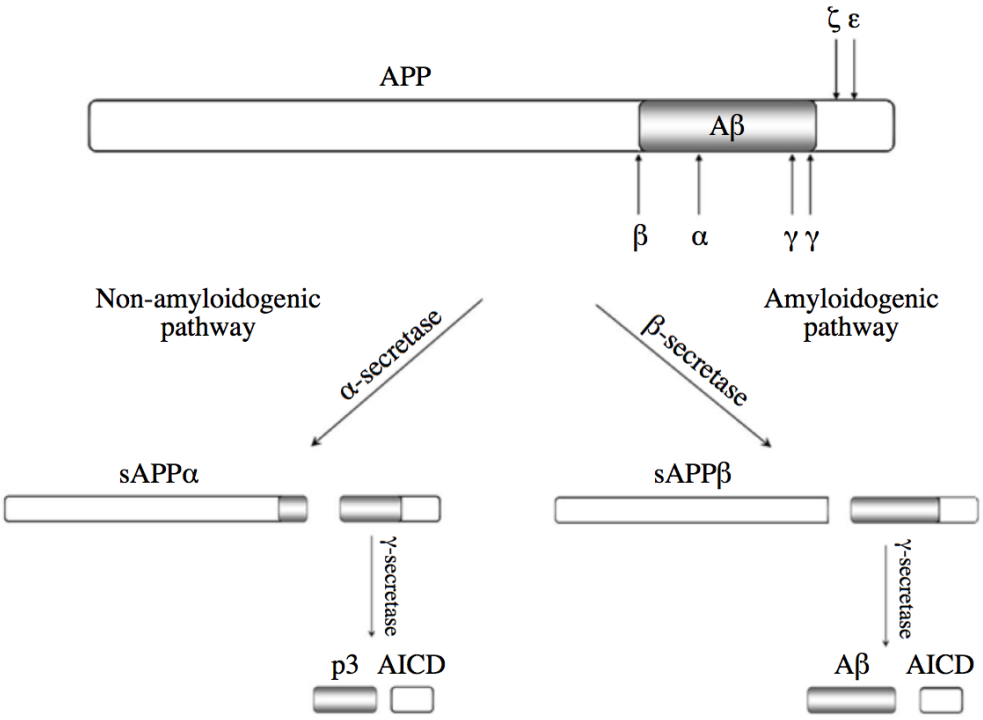

Figure 2. Non-amyloidogenic and amyloidogenic processing of APP by α- and β-secretase, respectively, in combination with gamma-secretase.

The APP is trafficked from the endoplasmic reticulum through the Golgi apparatus and the trans-Golgi-network (TGN) via secretory vesicles to the plasma membrane. Most APP is located in the Golgi and TGN. The APP ectodomain is either shed at the cellsurface or APP is re-internalized by the endosomal/lysosomal pathway and a fraction of endocytosed molecules is recycled to the cell surface. Measurable amounts of internalized APP also undergo degradation in the lysosome. The generation of Aβ has been proposed to take place either in the Golgi/TGN or in the endosomal/lysosomal system, while sAPPα is generated at the cellsurface [64]. The γ-secretase activity has been localized to several compartments including the Golgi, TGN, endosomes, and plasma membrane [65].

Though the processing of the APP family has been extensively studied, the picture is still incomplete. Since the identities of the enzymes giving rise to the different APP fragments were formerly unknown, they were simply referred to as α-, β- and γ-secretase due to the cleavage sites in APP. Since then, more detailed knowledge about their identities has been revealed.

2.2.4.1 α-Secretase

The cleavage of APP by α-secretase is presumed to preclude the formation of Aβ and generate the soluble sAPPα ectodomain. This cleavage is suggested to take place at the plasma membrane [64] and by using inhibitor profiling it was concluded that an integral membrane metalloendopeptidase gave rise to the α-cleavage [66], more specifically members of the A Disintegrin And Metalloproteinase (ADAM) family. At present, the two most established α-secretase candidates are considered to be ADAM10 and ADAM17, the latter also known as TACE (tumor necrosis factor-α converting enzyme) [67, 68]. Constitutive α-secretase cleavage of APP is attributed to ADAM10, while regulated α-cleavage is thought to be due to ADAM17 activity [69-71]. Additional metalloproteinases, belonging to either the ADAM or the Matrix MetalloProteinase (MMP) family, have been suggested as potential α-secretases contributing to the regulated shedding, however their role remains to be clarified [72]. sAPPα has been shown to have neurotrophic and neuroprotective properties [73-75]. Furthermore, ADAM10 has been reported to shed over 30 membrane proteins including Notch, which is also implicated in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [76-78]. Loss-of-function mutations in ADAM10 have been reported in families with late onset AD [79] and a reduced expression of ADAM10 in CNS neurons of sporadic AD [80]. However, whether the disease in these families is caused by an increase in Aβ and/or a concomitant decrease in sAPPα or due to functional abnormalities of other ADAM substrates remains to be elucidated.

2.2.4.2 β-Secretase

The β-secretase activity gives rise to shedding of the sAPPβ ectodomain but is also the first step in generating the Aβ peptide. At the end of the 20th century several groups identified the β-site APP cleaving enzyme 1 (BACE1) or memapsin-2, as the major β-site cleaving enzyme [81-83]. BACE1 is a transmembrane aspartyl protease believed to reside mainly within the TGN and the endosomes to perform β-site cleavage, however, it has also been found at the cell surface [65]. Several studies have investigated the effect on Aβ production in BACE1 knockout mice which have abolished Aβ formation [84-86], supporting the role of BACE as a β-secretase. However, this finding does not preclude the presence of other enzymes acting on the β-secretase site and since BACE1 show low activity against the wild type β-secretase, the search for other β-secretase candidates is a continuous endeavor of many groups. One study showed that the cysteine protease cathepsin B

colocalize with Aβ in the regulated secretory vesicle and cleaves wild type APP at the β-secretase site efficiently [87]. It was also shown, in both *in vitro* and *in vivo* models, that inhibition or knockdown of cathepsin B leads to reduced Aβ levels [87-90] and that cathepsin B only acts on the wild type β-secretase sequence and not the Swedish mutant sequence [91], which BACE1 cleaves more efficiently than the wild type sequence[92, 93]. This has implications in models built upon this mutant sequence as to preclude the contribution of cathepsin B on the β-site cleavage. However, these findings do not contradict each other but rather, as in the case of the α-secretase, suggest that BACE1 might work as a constitutive β-secretase while cathepsin B is active in the regulated secretory pathway. In addition, other substrates have been proposed for BACE1. One such substrate is neuregulin, where the abolished cleavage could lead to hypomyelination of neurons during their development as well as delayed remyelination of adult neurons [94-96]. Thus, simply targeting BACE1 as a therapeutic treatment to lower Aβ production could possibly lead to medical complications.

2.2.4.3 γ-Secretase

Presenilin-1 and -2, which are associated with familial early onset AD, are the catalytic part of the γ-secretase complex which is an aspartyl protease multiprotein complex consisting of four components: presenilin-1 or -2, nicastrin, anterior pharynx defective-1 and presenilin enhancer-2 [97, 98]. The γ-secretase complex resides primarily within the endoplasmic reticulum, Golgi/TGN, and endocytic compartments and the catalytic activity towards APP seems to be directed to the TGN and the early endosomes [99-102]. The remaining α- and β-cleaved APP C-terminal fragments are subsequently cleaved by γ-secretase to generate either the non-amyloidogeneic p3 fragment or the Aβ peptide, respectively. The γ-secretase has low sequence specificity, meaning that it can generate fragments ending at either amino acid 40 or 42 (γ-cleavage) within the Aβ sequence and recent data also indicate cleavage at the ζ-site ($Aβ_{46}$) and at the ε-site ($Aβ_{49}$) performed by γ-secretase [103-105]. It has also been suggested that γ-secretase is, directly or indirectly, responsible for the generation of Aβ fragments found in cerebrospinal fluid (CSF) ranging from amino acid 17 to 42 [106]. In addition, cleavage by γ-secretase also gives rise to the release of the AICD which has been implicated as a transcription factor [107]. Moreover, γ-secretase has many other substrates besides APP and APLP; several of them have been associated with AD and VaD, e.g., LRP1, RAGE, Neuregulin, and Notch [108].

2.2.5 Neurofibrillary tangles

Microtubules (MT) constitute one of three filament families making up the mammalian cytoskeleton, the other two being intermediate filaments and microfilaments. These polymers do not only maintain the cellular shape and mechanics of the cell, (for comprehensive review see ref. [109]), e.g., MTs are involved in processes such as mitosis, cytokinesis, and vesicular transport. MT integrity depends on the microtubule-associated proteins (MAPs) that bind to the filament in order to stabilize its structure [110]. Tau belongs to the MAP family and its primary transcript can be alternatively spliced into six different isoforms in the adult brain [111]. The isoforms contain two different domains: a projection domain containing the amino-terminal two-thirds of the molecule and a MT binding domain. Some proposed functions of the projection domain are to regulate the spacing between axonal microtubules [112] and to interact with other cytoskeletal proteins [113]. The isoforms differ in the microtubule-binding domain in that they contain three (3R) or four repeats (4R) of a MT binding motif. The 3R and 4R containing isoforms are under normal conditions expressed in a one-to-one ratio in the adult brain and an imbalance in this ratio seems to have implications in some tauopathies [114] since the amount of repeats affect the binding affinity of tau to the MT [115]. Furthermore, several post-translational modifications have been described for tau and the most extensively studied is phosphorylation. The longest tau isoform in the central nervous system has 79 putative serine or threonine phosphorylation sites. The phosphorylation of tau normally decreases with age but increases under certain pathological conditions such as AD [45]. The increased phosphorylation of tau leads to a decrease in affinity for microtubules and a subsequent destabilization of the MT network [116]. Some findings also indicate that the phosphorylation of tau promotes its self-assembly [117], which could give rise to the AD characteristic NFTs. In addition, phosphorylated tau is more resistant to degradation than non-phosphorylated tau [118].

2.3 Vascular dementia

In 1894 Otto Binswanger claimed that vascular insufficiency could cause dementia through white matter atrophy. Binswanger described a patient who suffered from slow progression of dementia with subcortical white matter atrophy, enlarged ventricles and aphasia and named the disease ‘*encephalitis subcorticalis chronica progressiva’*. In 1902 Alzheimer reexamined Binswanger’s work and conducted his own studies, the results of which supported Binswanger’s ideas, and the disease was subsequently renamed after him.

2.3.1 Diagnostic criteria and clinical manifestation

VaD is regarded as the second most common dementia disease [20] and the heterogeneous clinical presentation is described as being less insidious in onset compared with AD showing a stepwise decline in cognitive abilities [119, 120]. Sub-classes of VaD include: Large vessel disease, ischemic-hypoperfusive VaD, haemorrhagic VaD and small vessel disease [121]. A major form of VaD, and possibly the most common subtype in the elderly, is small vessel disease or more specifically subcortical ischemic VaD (SVD) [122, 123]. SVD is characterized by early neurological deficits such as gait impairment and mental slowing, impairment of executive functions and personality changes (anterior brain syndrome) [121, 124]. The diagnosis of SVD is most often based on the Erkinjuntti criteria [125]. SVD is regarded as the most homogenous subtype of VaD and is the main focus of this thesis.

2.3.2 Neuropathology in SVD

The heterogeneity of VaD is reflected by the multitude of possible symptoms gives rise to, reflecting size and numbers of lesions, as well as type of tissue damage and lesion location. Subcortical white matter disease visualized by magnetic resonance imaging (MRI) in VaD patients is thought to indirectly be causative of cognitive dysfunction [126], possibly by disconnecting the subcortical regions from the cortical regions of the brain. In support of this notion is that a disturbance in the thalamus and cortical connections is critical to the onset of cognitive dysfunction [127]. A pathological hallmark of small vessel disease is the findings of hyaline, lipid and fibrotic material in the tunica media replacing the smooth muscle cells, causing constriction of the lumen and stiffened vessel walls leading to impaired ability to regulate lumen diameter. This could cause ischemic hypoxic damage to surrounding tissue as well as blood brain barrier dysfunction with subsequent disruption in fluid circulation [123, 128, 129]. In a postmortem study it was shown that VaD patients had a pronounced loss of myelin lipids [130], which could be explained by the fact that white matter changes have been associated with disturbances in fluid circulation [131, 132] and that oligodendrocytes are vulnerable to ischemia [133, 134].

2.3.3 Familial small vessel disease

CADASIL is a dominantly inherited small artery disease which leads to disability and dementia in mid-life is caused by mutations in the *NOTCH3* gene which is located on chromosome 19 [78, 135]. Extensive white matter changes visualized by MRI are always seen in patients with CADASIL [136]. Notch is a family of type I transmembrane receptors that becomes subject to cleavage when engaged with its ligand and as with APP an intracellular domain is released subsequent to cleavage by γ-secretase. The pathway affected by Notch signalling modulates cell-fate decisions [137, 138] and in the case of the vasculature changes in signalling could lead to abnormal development [139-141]. Specifically, Notch-1 and -4 are present in the endothelium, while Notch-1 and -3 are predominant in smooth muscle cells [139, 140].

2.3.4 White matter lesions

The pathogenesis of white matter lesions (WMLs) is unclear but the prevalence of subcortical WMLs increases with age and the lesions are often visualized in elderly people undergoing CT or MRI investigation [129]. Also, WMLs frequently coincide with cerebrovascular risk factors such as hypertension and atherosclerosis [142] and WMLs are the pathological hallmark of SVD [143]. WMLs are associated with progression of MCI to dementia [143], and progressive WMLs are related to a parallel decline in cognitive function [144] and WMLs can predict cognitive decline and VaD among non-disabled elderly [145]. In a recent pathological study on human brain tissue from VaD and AD patients it was proposed that myelin loss, which was less prominent in AD compared with VaD, evolves by different mechanisms such as primary hypoxic/ischemic damage to oligodendrocytes in VaD, whereas secondary to axonal degeneration in AD [146].

2.3.4.1 Neurofilament light

Neurofilament (NF) belongs to the intermediate filament family one of three filament families making up the mammalian cytoskeleton, the other two being microtubules and actin-containing microfilaments. Intermediate filaments constitute up to 85% of the total protein content of a neuronal cell [147] and are important for the maintenance of structural integrity. There are three neurofilament chains which are named according to their molecular size, NF-L (light), NF-M (medium), and NF-H (high), when run on SDS-PAGE [148, 149]. The most

common of the three filament chains is the NF-L, with a molar ratio of 4:2:1 (NF-L: NF-M: NF-H) [150], which forms the backbone of the NF fibre onto which the heavier chains can co-polymerize [148, 151-154]. Since NF-L constitutes only a small part of the cytoskeletal components of the neuronal cell body and dendrites relative to axons [155], changes in its concentration in CSF is believed to mainly represent the integrity of the axonal compartment. Furthermore, the NF content is important for the calibre of the axons and large calibre myelinated axons outweigh small calibre unmyelinated axons in their NF content. Thus NF is important for conduction velocity of nerve impulses since axon calibre is a determinant thereof [156-161]. High levels of CSF NF-H correlate with abnormalities in both myelin basic protein and MRI in the demyelinating disease multiple sclerosis [162].

2.3.4.2 Myelin basic protein

Myelin basic protein (MBP) is a major structural constituent of the myelin sheath produced by the oligodendrocytes [163]. It accounts for approximately 30% of the total CNS myelin protein and there are four alternatively spliced isoforms with masses of 17.3, 18.5, 20.2 and 21.5 kDa of which the 18.5 kDa protein is the most abundant in mature myelin [164]. The function of MBP is to maintain the myelin sheath construction through electrostatic interaction between the positively charged basic amino acid residues of arginine and lysine within the MBP, and the negatively charged phosphate groups of the lipids in the membrane [165, 166]. Moreover, another myelin constituent, the myelin-associated glucoprotein, has been suggested to regulate the axon calibre by phosphorylation of the NF-H and NF-M side-arms and thereby increase the NF interspace and subsequent calibre [167, 168]. However, the molecular cascade remains unclear. Whether the rarefaction of white matter, one of the hallmarks of SVD, is due to nerve fibre degeneration, gliosis, or demyelination or a combination of all three remains elusive. However, myelin degeneration in CADASIL and SVD has been verified by postmortem staining of MBP [169]. Significantly elevated levels of MBP have also been found in CSF of patients with stroke with subcortical infarcts affecting the white matter as opposed to stroke with cortical infarcts [170] and thus indicate its potential as a regional marker of infarction as well as a marker of WMLs.

2.4 Mixed dementia

Mixed dementia (MD) is, as the name implies, caused by more than one disease process in the brain. The most common cause is a combination of AD pathology and vascular disease, the latter constituting cerebrovascular disease (CVD),

ischemia/hypoxia caused by small vessel disease, small infarctions, or stroke [171]. A MD diagnosis requires clinical evidence of neurodegenerative dementia in combination with CVD or a typical neurodegenerative symptomatology in addition to significant ischemic lesions assessed by neuroimaging [172, 173]. There is still intense debate about the contribution of degenerative processes in VaD, and vice versa. Some investigators question the diagnosis of VaD due to the fact that many of these patients show some signs of AD pathology at autopsy; the converse standpoint argues that the impact of CVD in the AD process since CVD is common at autopsy in patients with AD [174, 175]. Some even go so far as to say that the AD pathology might be secondary to CVD [176-178]. Another approach is to question the strict dichotomy between AD and VaD and some investigators believe that MD is one of the most common forms of dementia, since both AD pathology and cerebrovascular pathology increase with age [171, 179, 180]. There is no agreement in the literature regarding the prevalence and incidence of MD [181].

Traditionally the focus of brain research has mainly been on the cortex and less attention has been paid to the subcortical white matter. However, findings such as those reported by Brun and Englund, that white matter changes found in AD resembled those found in Binswanger's disease but was still distinct enough to define its own label of "white matter disorder", spurred the interest in the role of WMLs in VaD and AD [182].

3 Common and divergent pathological features of AD and VaD

3.1 Cerebral amyloid angiopathy in AD and VaD

Sporadic cerebral amyloid angiopathy (CAA), i.e., the deposition of Aβ in the cerebral and leptomeningeal vessel walls, is a common denominator of AD and VaD [183] and ischemia has been proposed as an initiator of the pathology. However, it has also been proposed that primary CAA induces injuries on the vasculature which give cause to hypertension and subsequent ischemia [184]. CAA is presumed to start in leptomeningeal or parenchymal blood vessels in the neocortex, followed by allocortex and cerebellum and finally within the deep grey nuclei and occasionally in the white matter and brainstem [185]. Although $A\beta_{1-40}$ is the predominant peptide deposited in the cerebrovasculature, as opposed to $A\beta_{1-42}$ which is mainly seen in parenchymal plaques, $A\beta_{1-42}$ is in fact also enriched in the capillaries [186-188]. CAA has been strongly associated with AD pathology and over 80% of all AD patients have CAA, but it is also seen in VaD [189-191].

Experimental models suggest that CAA may exert a functional effect on cerebral microvasculature, leading to alterations in vessel tone and reactivity [192] and the severity of CAA has also been related to vessel wall destruction [193]. Other clinicopathological features of CAA are angiitis, intracerebral haemorrhage, and cerebral infarction [194-196]. It has also been shown that mice overexpressing APP are more sensitive to ischemia than wild type mice [197] and that ischemia experimentally can lead to CAA by inducing amyloid dysmetabolism and deposition [198]. Other mechanisms such as default clearance of Aβ as well as ApoE along the perivascular interstitial fluid pathways of the brain parenchyma and leptomeninges, under pathological conditions leads to CAA [199, 200] and this would explain the association of CAA with the APOE ε4 allele [201].

3.2 Matrix metalloproteinases

Modification of the extracellular matrix (ECM) of the adult brain is a major task of the serine protease tissue plasminogen activator/plasmin system and the matrix metalloproteinases (MMPs). MMPs belong to a family of zink-dependent peptidases known to modify substrates including collagens, gelatin, laminin, fibronectin, elastin, myelin basic protein, growth factors, and cytokines [202, 203]. The MMP family members have three structural domains in common: the pro-peptide domain containing a cysteine residue that binds to the zink ion in the catalytic domain, to maintain the inactivity of the zymogen, and the hemopexin-like C-terminal domain which mediates substrate and inhibitor interaction (matrilysins lack this domain) [202, 204]. MMPs are mainly secreted as zymogens that are activated through a mechanism called the “cysteine-switch”, a disruption of the cysteine-zink interaction, which allows the Zn^{2+} to interact with water that is needed for catalytic activity. The disruption of the interaction can be proteolytically initiated by the removal of the pro-peptide [205, 206] by other activated MMPs or plasmin [207, 208] or through chemical modification by mercurial compounds, sulfhydryl reagents and reactive oxygen species [209-211]. The activity of MMPs is further regulated by tissue inhibitors of metalloproteinases (TIMPs 1-4) that either bind to the zymogen to prevent the “cysteine-switch” or interact with the catalytic site of the enzyme causing its inactivation [202, 212].

The MMP family consists of collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10 and -11), matrilysins (MMP-7 and -26), membrane-type (MT) MMP (MMP-14, -15, -16, -17, -24 and -25) and other MMPs (MMP-12, -19, -20, -21, -23, -27 and -28). Together they degrade most

components of the ECM and a wide array of bioactive molecules [213]. They are known to be involved in the cleavage of cell surface receptors, the release of apoptotic ligands, and regulation of chemokine/cytokine activity [214]. MMPs are also thought to play a major role in cell behaviour such as cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defence.

3.2.1 Matrix metalloproteinases in AD and VaD

White matter gliosis and increased localization of inflammatory cells in the white matter around blood vessels and in the vicinity of demyelination are pathological hallmarks of SVD or Binswanger disease [215]. The astrogliosis is associated with fibrohyalinosis of the blood vessels which are also surrounded by activated microglia/macrophages showing up-regulated markers of inflammation along with extravasated proteins, suggesting disruption of the BBB [216, 217]. The reactive glia secretes various potentially damaging substances, including proteinases, free radicals and cytokines. MMPs are associated with inflammation and are increased in reactive glia in VaD [218]. A crucial function of the inflammatory system is to remove tissue debris from a site of injury, as well as participating in repair processes such as remodelling of the ECM. As a consequence of this repair process, proteinases may be released in the vicinity of the myelin. Several proteinases, including the MMPs and serine proteases, have been shown to be involved in not only demyelination [219, 220] and BBB opening [221] but also in the repair process of angiogenesis and neurogenesis [222-224]. However, the reactive gliosis that initially may protect the injured brain might subsequently lead to inhibition of neuronal regeneration through glial scar formation.

Arteriosclerosis, affecting both microvessels and cerebral arteries, promoted by chronically elevated blood pressure, dyslipoproteinemia or diabetes mellitus can lead to complete stenosis by rupture of atheromatous plaques and subsequent infarctions of the surrounding cerebral areas [225]. Cerebral ischemia/stroke has been shown not only to increase the risk of VaD but also to increase the risk of AD [226]. An increased expression of MMP-3 has been demonstrated in human atheroma [227], together with an increase in expression of MMP-1, -2 and -9 in macrophages, smooth muscle cells and endothelial cells [228]. Furthermore, an increased activity of MMP-2 and MMP-9 was found in human brain tissue after focal ischemia [229] in addition to an up-regulation of MMP-1, -2, -3, -8, -9, -10, and -13, and TIMP-1 in human brain tissue after stroke [230]. Experimental studies

have implicated a complex role of MMP in atherosclerosis. On the one hand, MMP-1, -2, and -9 have been proposed to be responsible for plaque destabilization and rupture [231, 232]. On the other hand, overexpression of MMP-1 in APOE knockout mice resulted in less advanced atherosclerosis, suggesting a protective role for MMP-1 in atherosclerosis [233]. An inactivation of the MMP-3 gene showed no effect on plaque density but reduced the prevalence of aneurysm [234]. Moreover, MMP-3 has also been implicated as an intracellular mediator of neuronal apoptosis [235] and neurons undergoing apoptosis release the active form of MMP-3 [236]. There seems to be converging data on the pattern of induction of MMP-2 and MMP-9 during hypoxia/ischemia showing an early transient increase in MMP-2 and reversible BBB opening. This is followed by an increase in MMP-9 leading to a more extensive BBB damage which coincides with an elevation of interleukin-1β. The knockout of MMP-9, but not MMP-2, was shown to attenuate BBB opening as well as reduce infarction in a model of focal cerebral ischemia [237, 238]. However, other studies suggest that MMP-3, and MMP-9 could rather play a protective role in atherosclerosis due to an exacerbated unstable plaque phenotype observed in these knockout mice [239]. The diverse roles of MMP might explain the lack of long-term benefit of broad spectrum inhibition, which results in interference of angiogenesis and neurogenesis and thus hampers recovery [224]. Another effects attributed to MMP-2, -3 and -9 is the breakdown of MBP in brain tissue which might explain the demyelination observed in the brain of vascular cognitively impaired patients [240]. Furthermore, it has been shown that the expression of MMP-3 and MMP-9 are elevated in the human brain and co-localized with amyloid plaques and neurofibrillary tangles [241, 242] and the expression of MMP by astrocytes and neurons has been shown to be induced by Aβ [243-246]. In addition, MMP-2, -3 and -9 are all able to degrade Aβ *in vitro* [247, 248] and it has therefore been suggested that MMPs are a part of the Aβ clearance system in the brain.

3.2.2 Tissue inhibitors of metalloproteinases

TIMPs are the major physiological inhibitors of MMPs. However, TIMPs also have distinct functions separated from those connected to the MMP activity. When the cDNA for TIMP-1 was first cloned [249], it was found to be identical to a factor that has erythroid potentiating activity [250]. Later, TIMP-1 was shown to have cell growth promoting activity on various cell types including keratinocytes and fibroblasts [251, 252]. These cell promoting activities have also been shown for TIMP-2 [253, 254]. The effects are independent of MMP inhibition, because

TIMPs that lack MMP inhibitory activity, either by mutations or reduction/alkylation, retained cell growth promoting activity [255] and they were not produced by synthetic MMP inhibitors. In an investigation of the cellular events associated with TIMP-induced cell growth, Wang et al. [256] found that both TIMP-1 and TIMP-2 increased the level of Ras-GTP, but utilize different signalling pathways: TIMP-1 activates the tyrosine kinase/mitogen activated protein kinase pathway, whereas TIMP-2 signaling is mediated by protein kinase A activation which is directly involved in Ras/phosphoinositide 3-kinase complex formation. This suggests that TIMP-1 and TIMP-2 have distinct receptors. Recent studies have shown that TIMP-1 binds to CD63 [257] and TIMP-2 to α3β1 integrin [253], and these interactions have been found to inhibit apoptosis and arrest cell growth, respectively. The binding of TIMP-2 to α3β1 integrin was shown inhibit endothelial cell proliferation through vascular endothelial cell growth factor or fibroblast growth factor stimulation [253]. It has also been shown after cerebral ischemia in rat that the expression of MMP-9 and TIMP-1 was enhanced in cerebral blood vessel smooth muscle cells and in microvessels within the ischemic region [258] and both markers have been associated with WMLs in human brain ischemia [259].

4 The Cerebrospinal fluid

The CSF surrounds the central nervous system by occupying the subarachnoid space and further fills the intra-cerebral space of the ventricular system and the spinal cord. It is generally believed that the majority of CSF is formed by the modified ependymal cells of the choroid plexus in the ventricles walls by filtration of blood and the remaining CSF is derived from the ependymal lining of the ventricular walls [260] and the extracellular fluid of the brain. The total volume of CSF is approximately 165 ml and about 80 percent of the proteins originate from serum, while nearly 20 percent of the CSF proteins are brain derived; however, less than 1 percent are brain specific [261]. The main exit pathway for CSF is the through the arachnoid villi. The CSF is in continuum with the interstitial fluid surrounding the various CNS cells and the release of cellular constituents into the extracellular space during degeneration or acute damage should thus be reflect in the biochemical composition of the CSF. The CSF provides the brain with mechanical support in terms of buoyancy and protection and delivers nutrients, electrolytes, and signalling molecules to the brain parenchyma, but also functions to clears the brain from metabolic waste and is important in the maintenance of physiological pH [262].

Early diagnoses of degenerative brain diseases are of obvious importance for possible medical intervention. The content of the CSF may reflect an ongoing degeneration or the magnitude of acute damages. At present, however, only few biochemical variables are well established components of the clinical diagnostic procedure, with regard to degenerative brain disorders.

4.1 Cerebrospinal fluid biomarkers for AD and VaD

A biomarker is a substance which reflects physiological alterations that can be measured in biological samples such as fluids, tissues or cells. The main contribution of biomarkers is in the field of diagnostics, prognostics as well as in monitoring treatment of a disease. Biomarkers can also provide insight into pathophysiological alterations such as in the case of tau, which indicates degeneration of cortical axons in both Creutzfeldt-Jakob disease and AD [263]. Tau has proven useful in identifying AD patients from healthy controls [264]; however, there is a slight overlap with other neurodegenerative diseases such as Lewy body dementia, frontotemporal dementia and VaD [265]**.** By combining it with biomarkers reflecting other AD pathological hallmarks, such as P-tau reflecting the neurofibrillary tangles [45] and $A\beta_{1\text{-}42}$ reflecting the amyloid plaques [44], further specificity can be gained [264, 266]. These three biomarkers have also proven useful in identifying patients with MCI who will progress into overt dementia of AD aetiology [267, 268].

Markers reflecting CVD might further aid in the separation of AD and VaD. WMLs have been shown to correlate with the CSF concentration of NF-L [269] and an increase in NF-L protein concentrations have been found in patients with VaD [270]. However, a slight increase has also been found in AD [271]. Furthermore, increased levels of MBP in CSF has been shown to be related to subcortical stroke as opposed to cortical stroke [170] and thus both markers seem suited for detection of WMLs in CSF. However, whether the changes in both markers will be seen at an early stage of MCI and whether they show divergent patterns due to differences in pathological mechanisms in AD and VaD remain to be investigated.

MMPs are elevated in CSF in various neuroinflammatory conditions including infections, acute stroke and multiple sclerosis [272]. In cerebrovascular disease, MMPs are induced by hypoxic hypoperfusion of the white matter and an increased concentration of MMP-9 has been reported in CSF of SVD patients compared with

AD, while MMP-2, TIMP-1 and TIMP-2 were found to be unchanged in both groups [273]. Reduced levels of MMP-2 and MMP-3 have been found in AD patients with significantly reduced $A\beta_{1-42}$ levels, possibly reflecting a disturbed clearance leading to subsequent plaque formation [274]. Assessment of CSF MMP and TIMP changes in combination with markers reflecting cell specific alterations could possibly provide for valuable knowledge connected to disease specific pathophysiological mechanisms.

Biomarkers are being increasingly used to answer specific clinical questions, to provide answers for critical decision making regarding drug-targeted interactions and to support difficult clinical diagnostic decisions.

5 Material, Methods and Statistical analyses

5.1 Patient material

The Gothenburg MCI Study is an ongoing study that started in 1999 with the purpose of identifying neurodegenerative, vascular and stress-related disorders at an early stage before the development of overt dementia [275]. The patients included in this longitudinal study undergo biannual clinical examinations including neurological, psychiatric, and cognitive assessments, neuropsychological testing as well as MRI, SPECT (not the healthy controls), EEG, and sampling of blood and CSF. The diagnoses of MCI and (subsequent) dementia are founded on the validation of somatic anamnesis, clinical neuropsychiatric assessment and MRI, the clinician being blinded to results from biochemical analyses, APOE genotyping, SPECT, EEG and neuropsychological evaluations. All patients and controls give informed consent to their participation in the Gothenburg MCI study, which is conducted according to the provisions of the Helsinki Declaration and approved by the Ethics Committee of Gothenburg University, Sweden (diary number: L091-99, date: 990521).

5.1.1 MCI classification

Classification of MCI is founded on the validation of somatic anamnesis and the following checklists for cognitive function: Stepwise comparative status analysis [276] validates basic cognitive symptoms that reflect deterioration of certain brain regions; I-Flex, a shorter version of the executive interview [277] validates executive symptoms; Mini-Mental State Examination [278] validates mental status by measuring cognitive functions; and the Clinical Dementia Rating scale [279] provides global measures of cognitive ability to function. Taking all of the clinical test results into account, the Global Deterioration Scale [280] is used clinically as an instrument for the overall assessment of the severity of cognitive impairment upon which the MCI diagnosis is based. Subjective and objective verification of progressive cognitive impairment of more than six months, together with one positive outcome on either of the above mentioned checklists is needed for the inclusion in the MCI study.

5.1.2 Dementia diagnostic criteria

The dementia diagnosis is founded on anamnesis, somatic examination, neuropsychiatric evaluation and MRI. The diagnosis of dementia is based on the DSM-III-R criteria [18] together with the criteria of NINCDS-ADRDA [26] and ICD-10 [27] with regard to AD, Erkinjuntti criteria [125] with regard to SVD, and ICD-10 with regard to MD (AD with cerebrovascular lesions). Other dementia diagnoses, such as Lewy body dementia, frontotemporal dementia, and dementia non ultra descriptum, will not be covered by this thesis.

5.1.3 Healthy controls

Healthy controls are mainly recruited from senior citizens' organizations, while a few are spouses of study patients. Controls are not included if they had subjective or objective signs of a cognitive disorder as assessed according to the procedure described above.

Patients and controls afflicted with acute/instable somatic disease, severe psychiatric disorder (major depressive disorder according to DSM-III-R criteria, psychotic disorder and bipolar affective disorder), substance abuse, or confusion caused by drugs, are not included in the study.

5.2 Experimental Methods

CSF is obtained by lumbar puncture through the L3/L4 or L4/L5 interspace. The lumbar punctures are performed in the morning to avoid any influence on the result from possible diurnal fluctuations in biomarker levels. The CSF, collected in polypropylene tubes, is submitted to centrifugation at 2,000 x g at +4˚C for 10 min. The ensuing supernatant is aliquoted into screw-cap polypropylene tubes and stored at -80^{o}C pending biochemical analyses.

5.2.1 Enzyme linked immunosorbent assays

The enzyme-linked immunosorbant assay (ELISA) method was developed in the 1960s and 1970s by several scientists as a replacement for the radioimmunoassay, which involve the use of radioactive antigens (standards). Since then it has become an important diagnostic tool due to its ability to produce simultaneous rapid quantification of a large number of samples. The sensitivity and specificity of a

particular ELISA depends on the incorporated antibodies, but is still considered highly sensitive. Both monoclonal and polyclonal antibodies are used for ELISA. A monoclonal antibody, i.e., an antibody produced by a single clone of hybridoma cells, is preferabe either as a capture or a detection antibody since it is pure and highly specific.

The purpose of an ELISA is to determine the presence of and quantify a substance of interest. This can either be done by direct immobilization (direct ELISA) of the sample containing the antigen onto a solid support, or through immobilization of the antigen through specific binding to a capture antibody that has been immobilized. The latter is called a sandwich ELISA and is more specific due to the epitope recognition of the antibody rather than unspecific binding to the support, usually a polystyrene microtiter plate containing 96 wells, by adsorption. The next step involves the specific binding of a detection antibody to the antigen. This antibody is either conjugated directly to an enzyme or to a molecule such as biotin that can bind to another molecule such as streptavidin which in turn is coupled to the enzyme. Biotin-streptavidin is an enhancement step leading to improved detection. The enzyme is allowed to react with a chromogen which will produce a coloured product, thus the reaction system is known as colorimetric detection. The detection antibody can also be coupled to a fluorophore allowing for direct detection without the need of an enzymatic reaction step.

5.2.1.1 Fluorescent bead based technology

Multiplex bead assay platforms, such as the xMAP technology developed by Luminex Corporation (Luminex Corporation, Austin, Texas, USA), is advantageous compared to a regular ELISA due to its ability to simultaneously analyze multiple antigens within one reaction, rather than measuring one antigen per reaction. The xMAP technology utilizes beads or microspheres that are internally dyed with a unique fluorescent colour code and each set of beads are coated with a distinct set of antibodies targeting a specific analyte permittting identification. Different sets of microspheres can then be mixed into one sample. The detection antibodies are conjugated to biotin which will bind to the added streptavidin conjugated to the fluorescent dye phycoerythrin which allows for quantification. The samples are excited by two different lasers during flow cytometry revealing the identity of each bead as it passes through as well as the quantity of antigen revealed by the conjugated detection antibody. With this

technique one can obtain more information from less sample volume, thus saving valuable patient material, and it is less time consuming.

5.2.1.2 Electrochemiluminescent technology

Another refined method based on the ELISA principle is the electrochemiluminescent technology developed by Meso Scale Discovery (Meso Scale Discovery, Gaithersburg, Maryland, USA). This technique utilizes carbon electrodes incorporated into the bottom of the plate. The capacity of carbon to bind biological reagents, without affecting the biological activity, by passive adsorption is greater than for polystyrene. The Meso Scale Discovery assays use SULFO-TAG™ which is an electrochemi-luminescent label that emits light upon electrochemical stimulation. The detection process is initiated by the electrodes and only labels in the vicinity of the electrode are excited and detected. The co-reactants (tripropylamine, TPA) in the read buffer are also stimulated when in proximity of the electrodes allowing the chemical reaction between the reactive TPA and the SULFO-TAG to take place whereupon light is emitted. Furthermore, multiple excitation cycles of each label permit signal amplification and thus increase the assay sensitivity.

5.2.2 Fluorescent enzymatic activity assay

Fluorescence Resonance Energy Transfer (FRET) is a physical phenomenon that relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule. FRET is used to investigate molecular interactions due to its dependence on distance. The donor molecule is a chromophore that absorbs the light energy being emitted from a light source and the acceptor is the chromophore to which the energy is subsequently transferred from the donor. This so called resonance interaction occurs over a distance that is greater than, that which is typical between atoms within a molecule. While the distance between the donor and the acceptor is greater than the inter-atomic distance, they must be in close proximity to each other (typically 10-100 Å) to allow for energy transfer. The emission spectrum of the donor must overlap the excitation spectrum of the acceptor in order for FRET to occur (Figure 3).

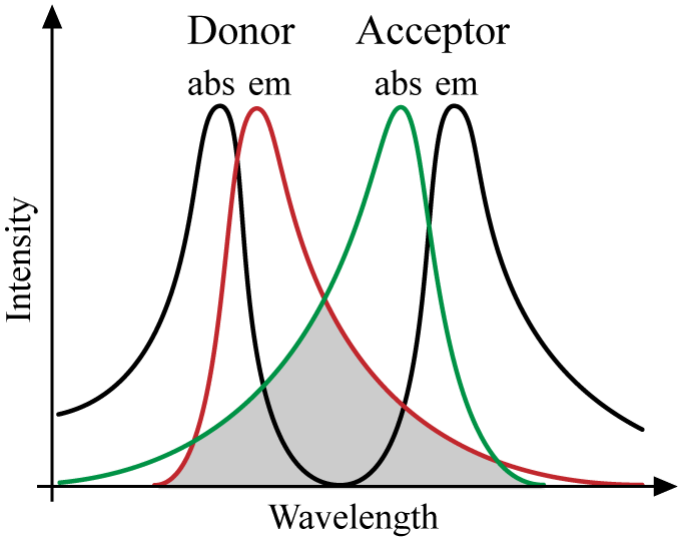

Figure 3

The emission spectrum (red) of the donor overlaps with the acceptor excitation spectrum (green) which allows FRET to take place.

The donor molecule is always a fluorophore and its electrons jump from the ground state to a higher energy level when appropriately excited by a photon. Electrons in atoms and molecules can change energy levels by absorbing or emitting a photon whose energy is equal to the energy difference between the two levels. The exited electrons decay to the lowest energy level through vibrational relaxation and eventually decay back to the ground state, whereupon a photon is emitted. When conditions are met for FRET to occur then the photon is *not* emitted, but instead the energy is transferred to the acceptor molecule. The acceptor electrons in turn become excited, as in the case for the donor molecule, and subsequently return to the ground state while emitting light. A characteristic of FRET is the property of light absorption at a particular wavelength and subsequent emission of light of a longer wavelength.

An acceptor, on the other hand, does not have to be a fluorophore but can also be used to quench fluorescence. One such example is the substrate molecule in a protease assay where a peptide containing the protease cleavage sequence keeps the fluorescent moiety of the donor in one terminus and the quenching molecule in the other terminus in close proximity. In this case, when the fluorescent donor molecule comes in close proximity to such an acceptor, also called a quencher, it will result in a loss of signal. By contrast, if the close proximity of a fluorescent donor and a quencher is disrupted the result would be an increase in fluorescence.

5.2.3 Proteomic Methods

5.2.3.1 Ammonium sulfate precipitation

Ammonium sulfate precipitation, or salting out, is a method used to purify proteins by altering their solubility. This technique is useful to quickly remove large amounts of contaminant proteins, as a first step in a purification protocol and to concentrate the protein of interest from a dilute solution.

The principle of salting out is based on the solubility properties of proteins due to the ionic strength of a solution. The process can be divided into two phases: First, there is an increase at low salt concentrations in protein solubility with an increasing salt concentration of the solution. This is called salting in. Secondly, when the salt concentration is further increased an opposite effect will occur, with a decrease in protein solubility and subsequent precipitation. This is called salting out. Ammonium sulfate is an excellent choice of salt since it is highly water soluble and has no negative effects on enzymatic activity.

The protein fractions are usually withdrawn from the solution by a step-wise increase in ammonium sulfate concentration with a recovery of the precipitate at each step by centrifugation. Solid ammonium sulfate is added to the supernatant from the previous step to increase the salt concentration in order to precipitate more proteins. The precipitates are individually dissolved in a buffer of choice. The aim is to find the precipitate containing the highest amount of the desired protein, whilst leaving most of the undesired protein still in solution or vice versa.

5.2.3.2 Size exclusion chromatography

Size exclusion chromatography (SEC), is a technique that separates molecules based on their size. The sample to be fractionated will pass through a gel filtration medium packed into a column. This medium, or stationary phase, is made of carbohydrate polymeric beads and the mobile phase goes through the stationary phase at a different speed depending upon the size of the molecule. The buffer or organic solvents used as the mobile phase are chosen based on the chemical and physical properties of the specific protein sample. The advantage of gel-filtration chromatography is that the medium can be varied to suit the properties of a sample for further purifications. The high resolution fractionation of biomolecules can be

used for isolation of monomers from aggregates, and to determine molecular weight. If a given gel filtration column is calibrated with several proteins of known molecular mass, the mass of an unknown protein can be estimated by its retention time.

5.2.3.3 Ion exchange chromatography

Ion exchange chromatography (IEC) separates molecules based on their net charge which depends on the mobile phase. The functional groups of the proteins, which contain positive and negative charges, interact with the stationary phase usually made of agarose or cellulose beads covalently attached to charged functional groups. The proteins can then be eluted by the addition of a buffer with increasing ionic strength (gradient) leading to a displacement of the proteins by similarly charged species. Elution can also be done by adjusting the pH of the mobile phase.

5.2.3.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), is a technique used to separate proteins according to their size in an electric field. The sample of interest is mixed with SDS, an anionic detergent that denatures secondary and non–disulfide–linked tertiary structures, which applies an identical negative charge to the protein in proportion to its mass resulting in fractionation by size. Heating the samples allows SDS to bind in the hydrophobic regions and complete the denaturation. The disulfide bonds, which are not disrupted by SDS, may intentionally be disrupted by heating the protein in the presence of a reducing agent such as dithiothreitol.

The samples are loaded onto a crosslinked polymer gel and an electric field is applied across it, which causes the negatively-charged proteins to migrate across the gel towards the positive anode. Depending on its size, each protein will move differently through the gel matrix: short proteins will more easily fit through the polymer pores, while larger ones will encounter more resistance. The gel is subsequently stained and the protein bands are excised. The cystein residues in the proteins are reduced and alkylated in order to improve recovery but also to minimize bond formation and chain modification. The proteins are then digested, by for example trypsin, and subsequently extracted from the gel for subsequent submission to the mass spectrometer.

5.2.3.5 Reversed phase liquid chromatography

Reversed phase (RP) liquid chromatography (LC) is usually employed as a final enrichment and desalting step prior to mass spectrometry (MS). The stationary phase of a RP column is generally made up of hydrophobic alkylic chains (-CH_2-CH_2-CH_2-CH_3) which interact with the analyte. There are three common chain lengths, C4, C8, and C18. C4 is generally used for proteins while C18 is mostly used to capture peptides or small molecules. A larger protein molecule will be likely to have more hydrophobic moieties to interact with the column stationary phase, while peptides that are smaller need the more hydrophobic longer chain lengths to be captured, so C8 and C18 are used for peptides or small molecules. The analytes stick to reverse phase columns in an aqueous mobile phase and are eluted with a gradient of organic solvent in aqueous mobile phase order to separate the analytes based on their hydrophobic character.

5.2.3.6 Electrospray ionization linear quadrupole ion trap Fourier transform ion cyclotron resonance mass spectrometry

MS provides mass measurement, or the mass-to-charge ratio (*m/z*), of charged proteins, peptides and peptide fragments. A mass spectrometer consists of three major components: an ion source, a mass analyzer and a detector. The sample is introduced into the ion source, where the analyte is transferred into gas-phase and ionized. The mass analyzer separates the ions according to their *m/z* registered by the detector and a mass spectrum is obtained depicting the ion intensity against the *m/z*. The instrument used in this work is a hybrid linear quadrupole ion trap (LQIT) Fourier transform ion cyclotron resonance (FTICR) mass spectrometer.

Electrospray ionization (ESI) is one type of ion source that produces gaseous ions from liquid solution. In ESI the eluate from the chromatographer is sprayed from an emitter and enters the mass spectrometer through an orifice and the gas-phase ions are transported to the analyzer. For the LQIT, the ions are trapped in an electrical quadrupole field. The ions can now either be directly detected by the ion trap detector, which is faster and more sensitive, or transferred to the ICR cell for high mass accuracy measurement. In the ICR cell ions are trapped in a strong magnetic field and detected by image current induction. The recorded signal is then Fourier transformed, yielding a mass spectrum.

Tandem mass spectrometry (MS/MS) can be performed by isolation and subsequent fragmentation of desired species. The most common fragmentation technique is so-called collision induced dissociation, where the selected ions are forced to collide repeatedly with helium which is present in the LQIT. The obtained fragment ions can then in turn either be detected by the ion trap detector or transferred to the ICR cell. The standard procedure is to detect intact tryptic peptides in the ICR cell for high mass accuracy and the fragment ions in the LQIT for high sensitivity.

5.2.4 Protein identification

The identification of proteins is made possible by matching the experimental mass spectrometric data obtained with theoretical protein sequence data contained in existing databases.

5.2.4.1 Identification by MS/MS analysis

Protein digestion prior to MS/MS analyses is performed in order to obtain specific peptide cleavage patterns representative of the protein combined with the enzyme of choice to facilitate the database analysis. A commonly used enzyme is trypsin, which will generate C-terminally truncated peptides ending at either arginine or lysine. The m/z values detected, representing the peptides from a certain protein, is usually referred to as peptide mass fingerprints. The experimentally obtained values are typically submitted to a database search to match all existing proteins within that database that have been theoretically cleaved by the same enzyme. Thus the cleavage by for instance trypsin will narrow down the possible peptide fragments and focus the search. The search will then generate a list of proteins starting with the one that has been matched to the largest amount of experimental peptides matching the theoretical peptides of that protein.

5.3 Statistical analyses

Non-parametric statistical methods have been used for the statistical assessment of demographic, clinical and CSF variables. Comparisons across groups were performed using the Kruskal-Wallis test. Univariate pairwise comparisons have been assessed by the Mann-Whitney U test for continuous variables between groups, while the non-parametric $\chi 2$ was used for dichotomous variables. The

nonparametric Friedman's or Wilcoxon tests were used for pairwise comparisons between two related samples. Correlation analyses were performed using the Spearman rank correlation; the values are presented by the Spearman's rank correlation coefficient (ρ). Receiver operating characteristic (ROC) analysis was performed to evaluate biomarkers discriminating ability between groups as well as the performance of different immunological assays.

Multivariate discriminant analysis (DA) was performed using the orthogonal projection to latent structures (OPLS) algorithm implemented in the software SIMCA P+ (v. 12, Umetrics, Umeå, Sweden). The algorithm finds the direction in the multivariate orthogonal space spanned by the different variables, e.g., P-tau$_{181}$, T-tau, and Aβ_{1-42}, that best separates the predefined groups, e.g., AD and controls. This direction is represented by a so called score vector. A corresponding loading vector carries the information on how the different variables contribute to the separation. Subsequent receiver operating characteristic (ROC) analysis can be performed on the values after projection onto the score vector to evaluate the discriminating power of the model.

OBJECTIVES

The overall study objective is to improve the possibility to differentiate between patients with AD and SVD by the use of CSF biomarkers. The specific objectives are:

- To examine the discriminating ability of T-tau, P-tau$_{181}$, and Aβ_{1-42} together with NF-L at baseline in MCI patients converting into AD, MD and SVD.

- To examine confounding factors affecting measurements of Aβ_{1-42} in CSF.

- To compare the ability of commercial assays for Aβ to discriminate between AD patients and controls, and to examine whether CSF denaturation can improve the assay's ability to discriminate between the groups.

- To examine whether MBP could add further information to the above mentioned biomarkers with regard to regional pathology of AD, MD and SVD.

- To examine whether MMPs, TIMPs and HFABP together with the five mentioned biomarkers, Aβ_{1-42}, T-tau, P-tau$_{181}$, NF-L and MBP, could discriminate between patients with WML and controls as well as AD patients.

- To examine divergences in APP metabolism, through enzymatic assays, in AD, MD, SVD and controls and assess the related APP metabolites in CSF.

RESULTS AND DISCUSSION

6 Paper I

The main finding of this longitudinal study, *Subcortical Vascular Dementia Biomarker Pattern in Mild Cognitive Impairment*, was the significantly elevated baseline level of NF-L in those patients with MCI who developed SVD (MCI-SVD) at follow-up compared with the stable MCI (MCI-MCI) patients and controls. Furthermore, MCI patients who progressed into AD (MCI-AD) as well as patients who progressed into MD (MCI-MD) had decreased baseline levels of $A\beta_{1-42}$ and increased levels of T-tau and P-tau_{181} compared with patients with stable MCI and controls, which has previously been shown by others [267], but also compared with MCI-SVD patients (figure 4).

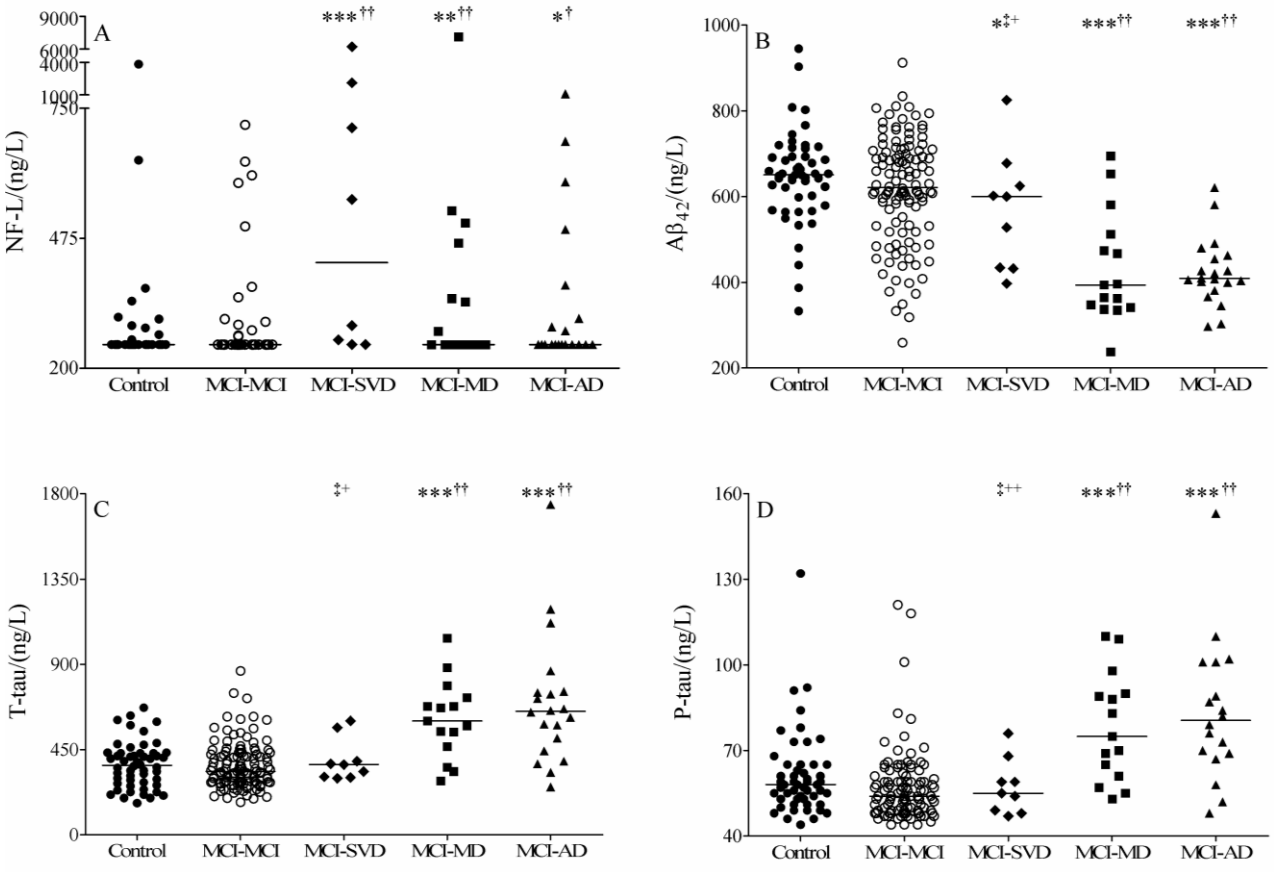

Figure 4 Comparisons of NF-L, $A\beta_{1-42}$, T-tau, and P-tau_{181} levels in patient groups based on follow-up diagnosis. Significance levels:* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. controls, † $p < 0.001$, †† $p < 0.001$ vs. MCI-MCI; ‡ $p < 0.005$ versus MCI-AD; + $p < 0.05$, ++ $p < 0.005$ vs. MCI-MD.

There was a slight decrease in the $A\beta_{1-42}$ levels in the MCI-SVD patients compared with controls, but no difference was found when compared to MCI stable patients. There are at least two possible explanations for this, one being that there are still patients included in the MCI stable group that will progress into dementia at future follow-ups, however it has also been shown that less than half of the patients with MCI will convert to dementia even after 10 years of follow-up [23]. Nevertheless, MCI patients with a pathological biomarker pattern seem more prone to convert to dementia than those without such a pattern [281]. Another possible explanation is that different primary disease mechanisms ultimately converge into the same pathological findings of decreased Aβ; however, with a less pronounced decrease in the patients primarily affected by WML.

The present results also indicated that the elevated NF-L in the MCI-SVD group appears to be the most important variable in separating patients with ongoing vascular lesions compared with those who remain stable, while P-tau_{181}, T-tau and $A\beta_{1-42}$ did not contribute to the discrimination between these groups (figure 5 A and D). However, a combination of the four biomarkers seems to work well in separating the patients with MCI-SVD from patients with MCI-MD (figure 5 B and E) and MCI-AD (figure 5 C and F), a finding which could be of considerable importance in clinical practice and possibly also in future drug trials. Furthermore, the biomarker pattern provides an insight into the localization of brain damage as reflected by the increase in tau in disorders mainly affecting the cortex such as Creutzfeldt-Jakob disease and AD, as opposed to SVD with its WMLs which are biochemically detected by an increase in NF-L. Finally, it appears that early changes in the CSF levels of NF-L confirm the very mild changes visualized in the AD by MRI, and it is possible that the diagnosis of MD will be more frequently used in clinical practice with increasing sensitivity of MRI and that this will actually result in the identification of a clinically purer AD group.

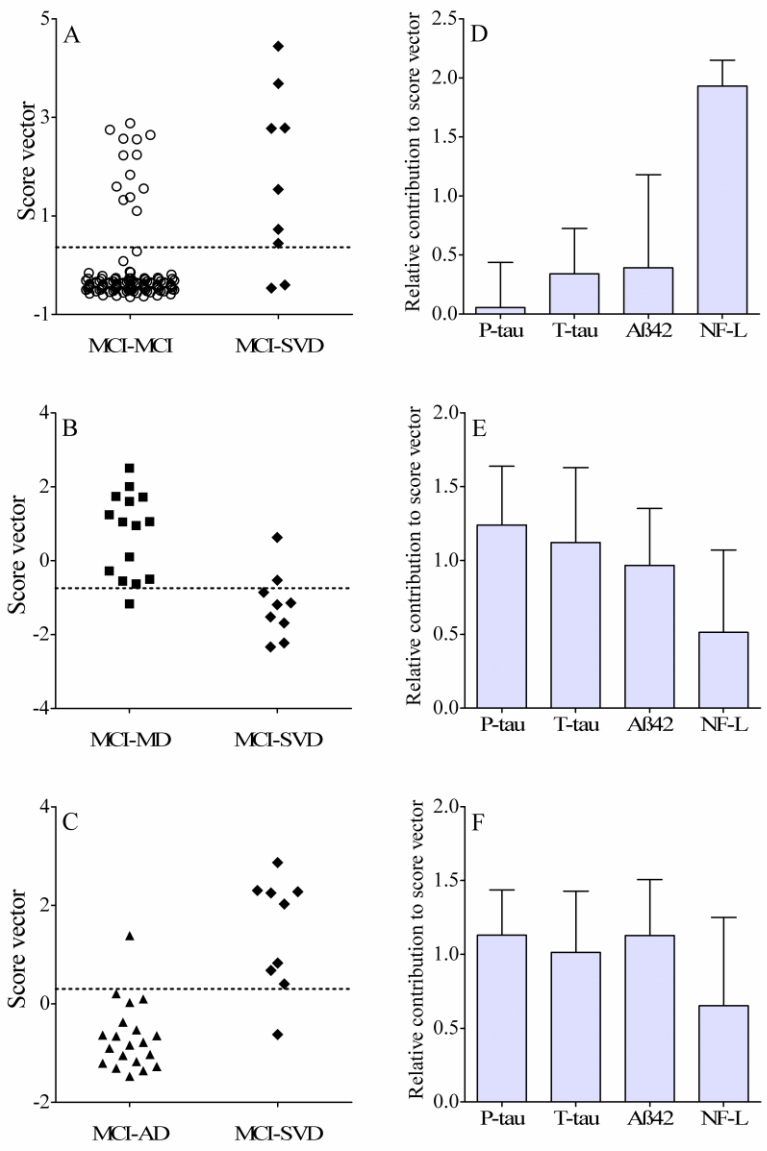

Figure 5 Pairwise group comparisons using multi-variate analysis. The score vector (y-axis in the scatter plot) is a line through the orthogonal 4-dimensional space spanned by the 4 CSF markers. The direction of this vector, along which the best separation between the different groups can be found, is calculated by the OPLS-DA algorithm. Cutoff values from ROC analyses are shown as dotted lines in the scatter plots and the sensitivity (Se), specificity (Sp), and AUC for the comparisons are: A) MCI-MCI versus MCI-SVD Se = 78%; Sp = 89%; AUC = 0.83 B) MCI-MD vs MCI-SVD Se = 78%; Sp = 93%; AUC = 0.92 C) MCI-AD vs MCI-SVD Se = 89%; Sp = 95%; AUC = 0.95

7 Paper II

High inter-center discrepancies have been reported for concentrations of $A\beta_{1-42}$, T-tau and P-tau [282] leading to different cut-off values between various centers with the highest variability shown for $A\beta_{1-42}$ [283]. This creates problems when research centers try to merge data in order to conduct larger more reliable investigations. Not only does the variation in CSF biomarker levels complicate multicenter research studies, it also precludes the introduction of generally applicable cut-off levels in clinical routine. The inter-center variability of analytical results may be due to differences in pre-analytical procedures for CSF collection and sample processing, analytical procedures and techniques and batch-to-batch variation of biomarker assays. Due to the high inter-center variability of reported $A\beta_{1-42}$ levels in CSF, possible pre-analytical and analytical factors were investigated in *Confounding Factors Influencing Amyloid Beta Concentration in Cerebrospinal Fluid.*

The confounding factors found to influence the $A\beta_{1-42}$ concentration in CSF are summarized below.

Preanalytical Factors

(i) An increase in $A\beta_{1-42}$ concentration was found in noncentrifuged CSF samples possibly due to a release of the analyte caused by cell lysis, thus it is very important to centrifuge the CSF within a short standardized time interval after LP.

(ii) A decrease in $A\beta_{1-42}$ levels due to the adsorption of the analyte to different types of test tubes was found. Thus standardization of test tubes used for CSF sampling should be undertaken. Polypropylene has so far been shown to be the most suitable but there may be differences among polypropylene tubes as well.

(iii) Pretreatment of CSF with detergent-containing buffers or heat denaturation leads to an increase in $A\beta_{1-42}$ levels which is probably due to dissociation of Aβ bound to proteins or release of Aβ from oligomers, also assay specific effects should be considered. For these reasons a standardization of dilution factors, buffer additives and sample processing is necessary prior to analysis.

(iv) The CSF $A\beta_{1-42}$ concentration decreased when plasma was added at a concentration corresponding to a CSF/serum albumin ratio of 11-55, which is probably due to the binding of free Aβ to plasma proteins.

Analytical Factors

(i) Different immuno-assays employing various antibodies and possibly dissimilar sources for the calibrator peptides lead to divergences in the absolute $A\beta_{1-42}$ concentration (figure 6), however no assay appears to perform much better than the other when concerned with diagnostic accuracy. Therefore, it is not possible to make inter-center comparisons when using different assays and when no international Aβ golden standard is available.

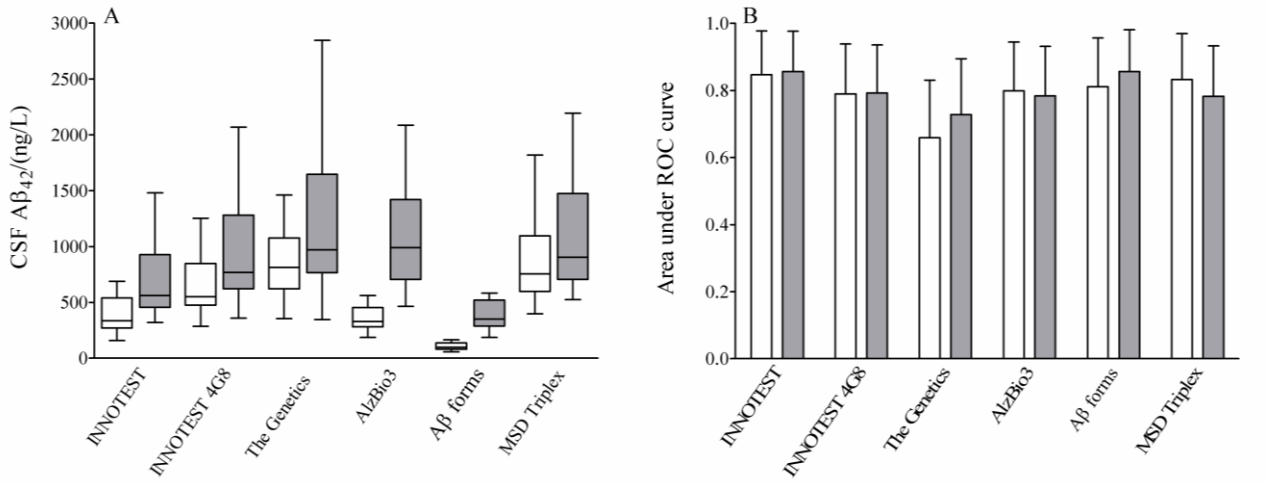

Figure 6 Pretreatment of neat CSF (white boxes) with detergent-containing buffers (grey boxes) lead to an increase in $A\beta_{1-42}$ levels possibly due to dissociation of Aβ bound to proteins or release of Aβ from oligomers, also assay specific effects should be considered. However, all assays seem to perform equally well when concerned with prognostic accuracy after detergent treatment.

Even though the CSF concentration of $A\beta_{1-42}$ does not seem to be affected by a spinal cord gradient, circadian rhythms, blood contamination or storage/thawing conditions other proteins may be affected. It is thus necessary to use a standardized protocol to allow for inter-center comparisons. A quality control (QC) program has been initialized in order to further investigate the issue of the biomarker variability with an aim to standardize CSF biomarker measurements. The QC program is run by the Clinical Neurochemistry Laboratory in Gothenburg, in conjunction with the Alzheimer's Association.

However, when different centers employ the same ELISA from the same manufacturer divergences often still remain [284], as exemplified below (figure 7) were samples from center 1 were re-analyzed at center 2 (figure 7B) in order to verify if the divergence in $A\beta_{1-42}$ concentration (figure 7A) was due to differences in study population or to biomarker analysis. This issue was unfortunately not addressed in the above article. Whether these divergences were due to inter-

technician variability, instrument calibration or batch-to-batch inconsistencies remain to be elucidated.

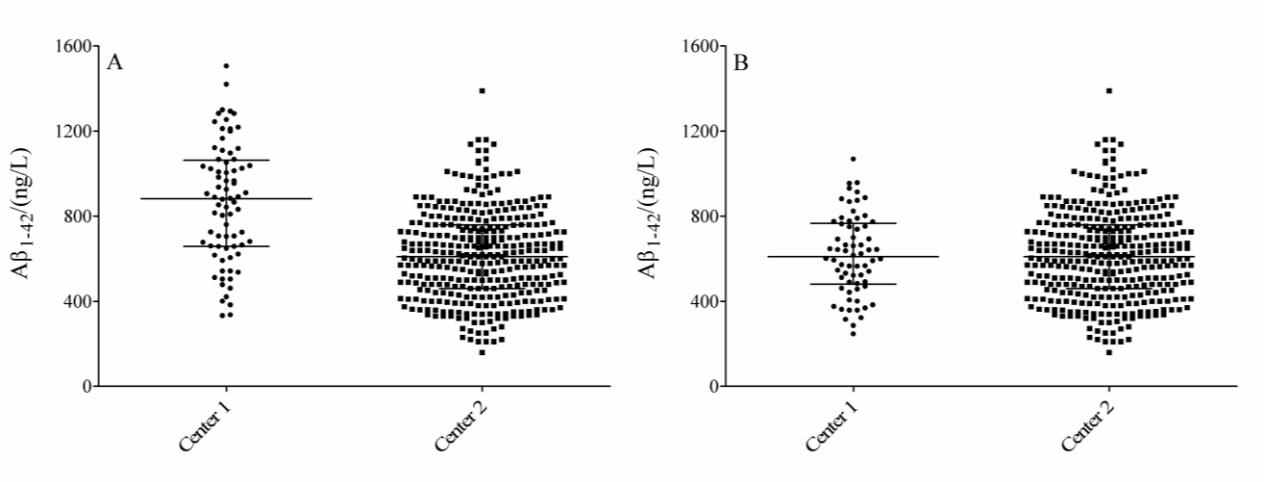

Figure 7 A) The $A\beta_{1-42}$ biomarker levels measured at each center. B) The $A\beta_{1-42}$ biomarker concentration of the re-analyzed CSF samples from center 1 performed at center 2

8 Paper III

The aim of study III was to further improve the separation of patients with VaD and MD with subcortical WML (herein termed SVD) from patients with AD, based on biomarker profiles. In other words, the focus was to elucidate if the patients with VaD and MD who had WMLs in common share a biochemical profile representative thereof, even though the MD patients also share the biomarker profile of the AD patient group due to the overlap in cortical pathology. The main findings of the present study were divergent biochemical profiles reflecting subcortical and cortical alterations affecting patients with SVD and AD, respectively. The elevated levels of MBP, TIMP-1, NF-L and MMP-9 seem to reflect a subcortical profile, while P-tau$_{181}$, T-tau and Aβ_{1-42} mainly represent the profile of AD with cortical alterations (figure 8). Another important finding was the ability of the biomarkers to separate the SVD patients from controls.

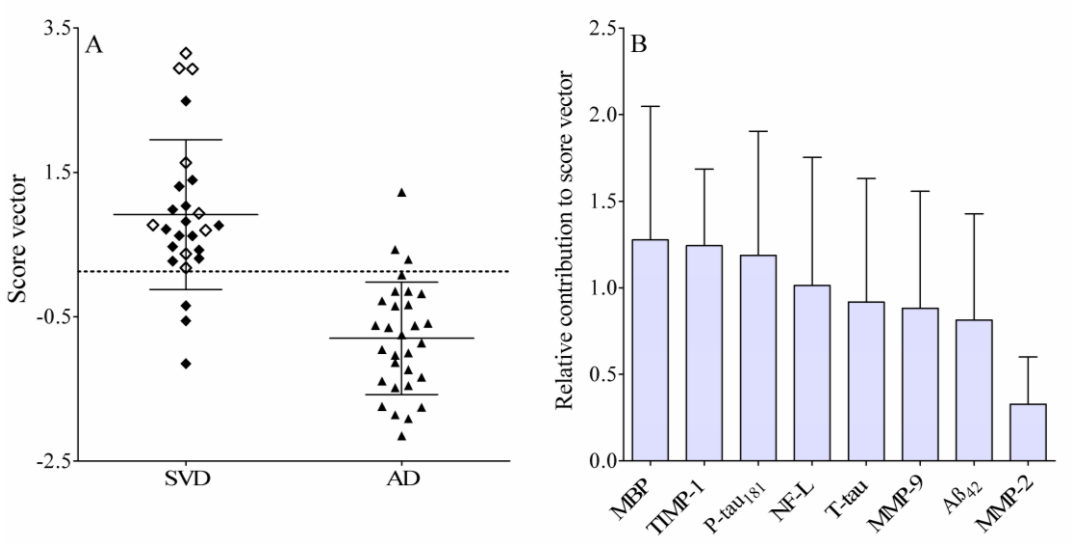

Figure 8 A) The separation between SVD and AD, with a sensitivity of 89% and a specificity of 90% (AUC=0.92). B) Relative contribution of biomarkers to the separation between SVD and AD

The biomarkers were selected for their potential to give a CSF profile to the WMLs. The NF-L was once again analyzed to represent white matter axonal pathology, with a new improved ELISA method, and MBP with the potential to reflect myelin sheath damage. Furthermore, MMP and TIMP were chosen primarily due to their involvement in tissue remodeling, demyelination and degeneration of the BBB, but also because of their possible role in Aβ metabolism. The AD biomarkers, Aβ_{1-42}, T-tau and P-tau$_{181}$, were selected to fulfill the cortical counter-profile and HFABP which could possibly reflect an overall neurodegeneration.

Since the WMLs are frequent pathological hallmarks seen in VaD patients and are in part thought to be caused by small vessel disease [128, 285], the expected finding would be that the NF-L protein and MBP markers could contribute to discrimination between the patient groups. However, cerebral ischemia/stroke has been shown to not only increase the risk of VaD but also to increase the risk of AD [226], therefore we did not know to what extent the biomarkers would contribute. Increased levels of NF-L have previously been reported in both AD and VaD [286], while the T-tau and P-tau$_{181}$ levels have been found to be unchanged in SVD [287] which was also seen in the pure VaD patients in the present study. Our findings of increased CSF levels of NF-L and MBP in patients with WML support the suggested ability of these biomarkers to reflect ongoing axonal damage and demyelination. Furthermore, they were not only important markers for separating the dementia groups, but also in separating SVD and controls (figure 9).

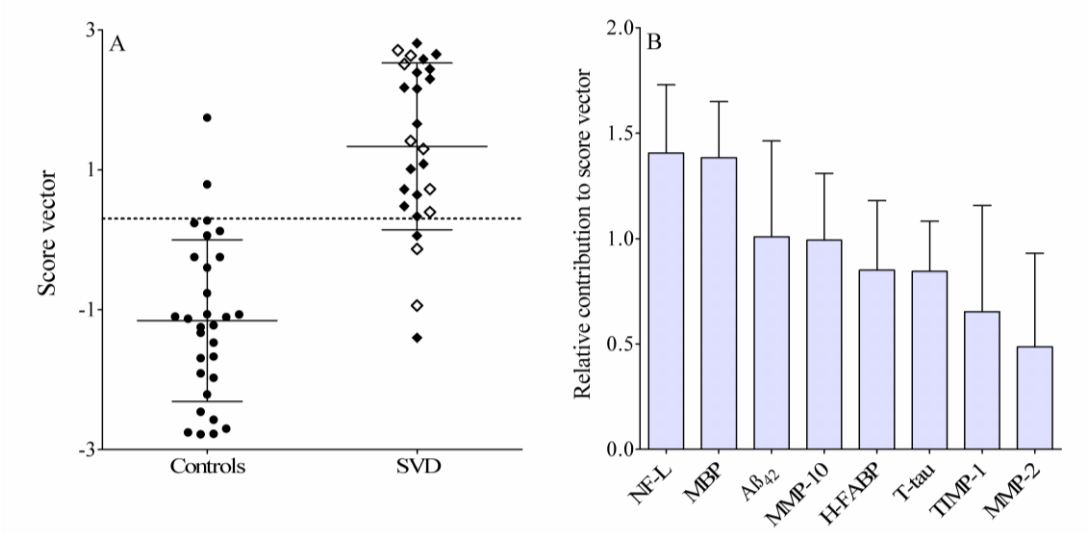

Figure 9 A) The separation of SVD and controls, with a sensitivity of 85% and a specificity of 93% (AUC=0.93). B) Relative contribution of biomarkers to the separation between SVD and AD

It was also shown that patients with AD exhibited an increase in NF-L as well as a slight increase in MBP compared with controls, which could reflect AD patients with concomitant white matter pathology not yet significant enough on MRI to warrant the diagnosis of MD. Large cerebral infarctions, cortical microinfarctions and WMLs are not only seen in VaD but also in AD and depending on the vascular burden are recognized as MD [173, 288]. These findings could indicate an important role for early detection of WMLs by these biomarkers. In a recent pathological study on human brain tissue from VaD and AD patients it was proposed that myelin loss, which was less prominent in AD compared with VaD,

evolves through different mechanisms such as primary hypoxic damage to oligodendrocytes in VaD whereas secondary to degeneration of axons in AD [146]. It could be speculated that such pathogenetic patterns are reflected by the slightly higher increase of NF-L in the AD patient compared with MBP (figure 10), which would point to an early degeneration of the cell body with subsequent demyelination occurring later on. While in the case of SVD demyelination possibly occurs at an earlier stage with concomitant axon degeneration. It could also be hypothesized that this divergence in pathology would be even more pronounced in the earlier stages of MCI.

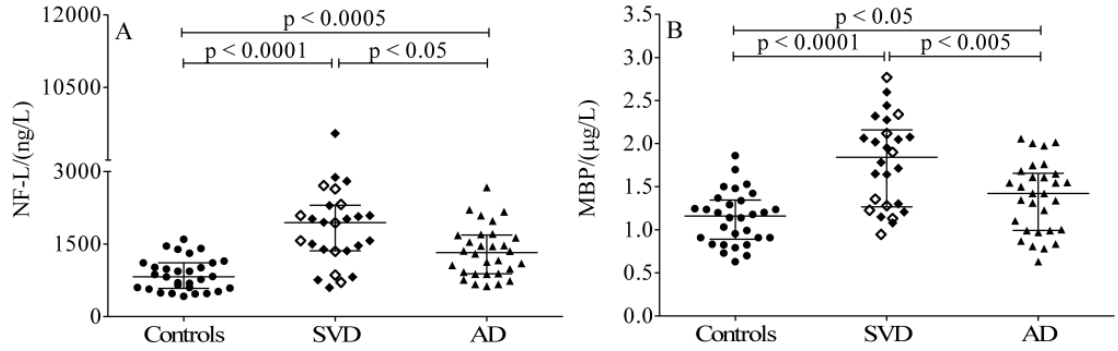

Figure 10 A) A significant increase in NF-L in SVD and AD patients compared with controls. The overlap between SVD and AD is slightly more pronounced for NF-L than for MBP. B) MBP is increased in both SVD and AD compared with controls, but the AD group show a larger overlap with controls than for NF-L.

A common denominator for VaD and AD, that could explain the altered amyloid metabolism previously reported in both VaD and AD patients [289], might be cerebral ischemia which has been associated with amyloid angiopathy [290]. The distribution of plaques in the AD brain mirrors to a great extent the pathologically altered cerebral microvasculature [291] and it has been shown that hypoxic factors can contribute to increased BACE1 gene transcription resulting in elevated activity and subsequent increased Aβ production [292]. The findings of decreased $A\beta_{1\text{-}42}$ in patients with WMLs and AD support our findings in study I and could point to a common vascular aetiology. However, it could also be that different primary disease mechanisms ultimately converge into the same pathological findings.

Interestingly, there was a correlation between MMP-10, H-FABP and T-tau in the AD patient group, while a correlation in the SVD group was found between MMP-10 and H-FABP, T-tau, NF-L and MBP. Thus the brain specific alterations, as

visualized by the biomarkers, seem to converge with some general features of neurodegeneration. Consequently, it appears that MMP-10 and H-FABP can be of use in discriminating between the dementia groups and controls, but not between the dementia groups.

Another finding was that both MMP-9 and TIMP-1 were increased in SVD patients compared with AD patients and controls, making them an integral part of the discriminate model between the dementia groups. However, their exact pathological mechanism remains to be determined. The activity of MMP-9 has been found to be elevated in human brain tissue after focal ischemia [229] and experimental models thereof have shown an attenuation of neuronal damage when MMP-9 is knocked-out [293]. Furthermore, BBB permeability and neuronal apoptosis was increased in TIMP-$1^{-/-}$ ischemic mice possibly due to a failure in the suppression of MMP-9 activity [294]. Thus the balance between MMP-9 and TIMP-1 in neurodegeneration seems important. However, none of the assessed MMPs correlated to BBB dysfunction, with the exception of TIMP-1 possibly due to an overall regulation of several MMPs potentially connected to the breakdown of the BBB. Furthermore, TIMP-1 seems to contribute more than the investigated MMPs in the separation of SVD and AD, and is also of importance in discriminating between SVD and controls possibly due to its overall MMP regulatory function.

9 Paper IV

In paper I and paper III it was shown that patients with WMLs and patients with AD exhibited an overlap with regard to decreased levels of $A\beta_{1-42}$. This finding, which has previously been shown by others, spurred the investigation of the amyloid metabolism in the present study. The main finding of this study was that a novel β-secretase activity in CSF correlates with the concentration of the sAPPβ fragment, released from APP by enzymatic cleavage at the β-site, in AD, MD, SVD and controls (figure 11).

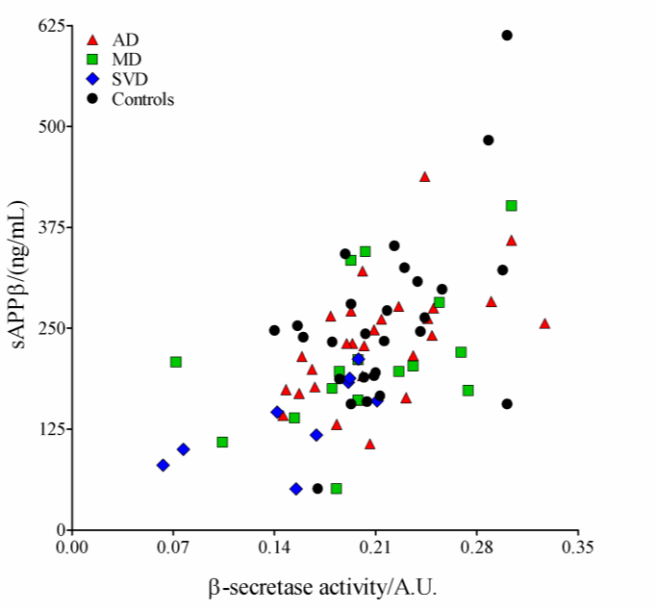

Figure 11 The correlation between sAPPβ levels and β-secretase activity in CSF. The activity correlated with sAPPβ in the controls (ρ=0.40, p=0.04), in SVD (ρ=0.77, p=0.016), in MD (ρ=0.51, p=0.046) as well as in AD (ρ=0.57, p=0.001)

More specifically, the enzyme activity and the concentration of the sAPPβ were decreased in the SVD patient group compared with MD and AD patients (figure 12). There was also a correlation between the β-secretase activity and $A\beta_{1-40}$ in the controls (ρ=0.62, p<0.0005) and AD patients (ρ=0.53, p<0.005), but not in the SVD and MD patient groups. The latter finding could possibly be explained by the small study material or differences in pathology since the Aβ accumulation is a feature found in both AD and VaD, however, with somewhat different compositions as well as topology, i.e. parenchymal ($A\beta_{1-42}$> $A\beta_{1-40}$) and cerebrovascular ($A\beta_{1-40}$> $A\beta_{1-42}$) depositions, respectively. Studies addressing these differences in pathology of the human brain between VaD and AD are so far few and inconclusive. There is however increasing interest regarding differences in the mechanisms leading to the accumulation of Aβ. One theory involves cerebrovascular disease as a common denominator, which triggers a cascade of events leading to combined amyloid mismetabolism and inflammation initiated through different pathways [295].

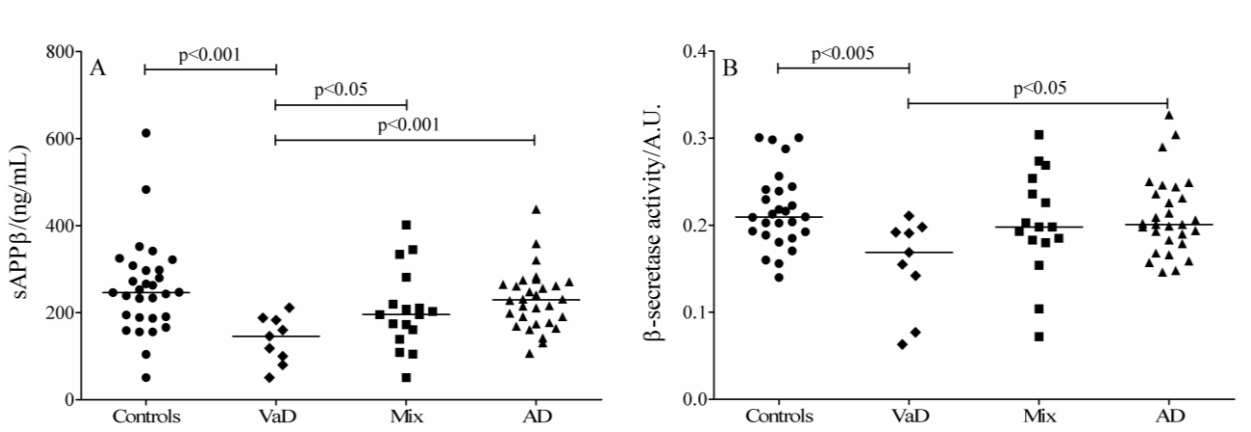

Figure 12 A) The sAPPβ fragment is significantly decreased in the CSF of SVD patients compared with all the other groups. B) The β-secretase activity is significantly decreased in CSF of SVD patients compared with controls and AD patients.

The involvement of inflammation is in this case particularly interesting, since the findings in study III suggest that patients with WMLs exhibit differences in the pattern of MMPs and TIMPs, since the inflammatory system and MMP/TIMP system are closely connected. Furthermore, ADAM-10 is also inhibited by various TIMPs, thus possibly connecting the α-secretase activity to the MMP/TIMP system and several MMPs have in turn been shown to be able to cleave the Aβ peptide. Moreover, it was concluded in the present study, through inhibitory studies utilizing the developed FRET enzymatic activity assay based upon the wild type APP sequence covering the β-site, that the enzyme exhibits metal preference. It was also verified that the activity was taking place at a near physiological pH and that inhibitors known to affect BACE1 and Cathepsin B, which are enzymes also known to cleave at the β-site, did not influence the CSF activity at all. The substrate cleavage site at the β-site was verified by ESI-QIT-FTICR MS/MS coupled in line with reversed phase liquid chromatography. However, there were also cleavage sites both upstream and downstream of the β-site, many of which could give rise to the N-terminally truncated Aβ isoforms that have been found in the human brain [296, 297]. The $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides have been the center of attention due to their high abundance in the AD brain. However, other N-terminally truncated Aβ peptides have also been reported to be highly abundant such as Aβ peptides starting at amino acids three and four [296, 298], and both cleavage sites were identified in CSF by the enzyme assay using the substrate spanning the β-site.

Naturally-occurring axonal pruning and neuronal cell death help sculpt neuronal connections during development, but their mechanistic basis remains poorly understood. Several experimental studies have indicated that the soluble N-terminal

part of APP conveys neurotrophic effects (residues 96-110 and 319-335) [73, 299] and it was recently shown that sAPPα and sAPPβ both exhibit an inducing effect on axonal outgrowth [300]. In contrast, a recent study implicated that the 35 kDa N-terminal sAPP fragment (residues 1–286) is a ligand for the death receptor DR6 which triggers neuronal cell death [301]. In a study of CSF this fragment was not detected by using IP in combination with MALDI–TOFMS, LC–ESI–MS/MS or Western blot analysis on human CSF or brain. However, several N-terminal fragments all corresponding to an approximate mass of 12 kDa (all starting at aa 18 and ending at aa 119, 121, 122, 123, 124 or 126) were detected and found to be somewhat increased in CSF of AD patients [302]. The decrease in measured sAPPα and -β, as seen in the SVD patient group, could thus be due to such a cleavage since the antibody in this assay has its epitope on the N-terminal side of these cleavage sites. However, no significant difference was seen in the activity measured by a developed FRET enzymatic activity assay utilizing a substrate spanning over the amino acids giving rise to the 12 kDa fragments. The enzymatic activity appeared to be due to an aspartyl protease belonging to the Cathepsin family, as shown by enzyme inhibition and protein purification followed by LC-MS/MS (ammonium sulfate precipitation, SEC, IEC, SDS-PAGE, and RPLC-ESI QIT-FTICR MS/MS). Interestingly, one candidate for the cleavage and release of the 12kDa fragment was the lysosomal cathepsin D which has been suggested to be involved in cell growth promoting activities [303, 304] the same effect that has been observed for the sAPP fragments. However, the enzyme activity giving rise to the 12kDa fragment was not significantly changed among the patient groups and the healthy controls.

In conclusion, a trend towards a decrease in CSF $A\beta_{1\text{-}40}$ levels in SVD compared with MD, AD and controls, and significantly lower levels of sAPPα and sAPPβ in patients with SVD compared with AD patients and controls in combination with the finding of a correlation between the sAPPβ levels and the β-secretase activity suggest differences in APP metabolism. This difference might be connected to differences found in the MMP/TIMP system since altered APP metabolism, giving rise to a decrease in the levels of sAPP fragments, has been seen in other diseases involving neuroinflammation [305], which seems to be an integral part of VaD with WMLs [306].

CONCLUSIONS

One conclusion to be drawn from the present thesis is that the pathological continuum from 'pure' AD to 'pure' SVD can be visualized by differences in biomarker profiles. Biomarkers reflecting the neuropathology affecting the cortex in the AD brain, T-tau, P-tau$_{181}$ and Aβ_{1-42}, together with biomarkers reflecting the subcortical pathology of the SVD brain, NF-L, MBP, TIMP-1 and MMP-9, were the integer part of the multivariate discriminant model. The model proved its considerable ability to discriminate between AD and SVD. Furthermore, models that distinguish AD patients and SVD patients from controls with high precision were also obtained, and in both cases with a major contribution from NF-L, Aβ_{1-42}, MMP-10 combined with T-tau and MBP, respectively. Even though the biomarkers are not disease specific, their assessment can still be an important tool to distinguish between dementia conditions and to contribute to the early detection of patients with MCI who are at high risk of deteriorating into overt dementia.

Not only was there a difference in the biomarker profile between patients with 'pure' SVD and AD, but the biomarker profile of the MD group gave insight into the meaning of a mixed pathology. This might seem patently obvious; however, it is of the utmost importance when it comes to clinical trials. The treatment of patients with pure AD as opposed to MD might result in differences in responses due to divergences in activated inflammatory and MMP/TIMP systems. By using a multivariate model based on relevant biomarkers these patients will be more easily depicted.

Furthermore, the demonstration of significantly lower levels of sAPPα and sAPPβ in patients with SVD compared with AD patients and controls, in combination with the finding of a correlation between the sAPPβ levels and the β-secretase activity suggest differences in APP metabolism. Also, the trend towards a decrease in CSF Aβ_{1-40} levels in SVD compared with MD, AD and controls supports this notion. These divergences might be connected to differences found in the MMP/TIMP system, since altered APP metabolism, giving rise to a decrease in sAPP fragments has been seen in other diseases involving neuroinflammation and inflammation seems to be an integral part of VaD with WMLs.

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