

**Cerebrospinal fluid biomarkers reflecting  
 $\beta$ -amyloid and axonal pathology in  
Alzheimer's disease and related conditions**

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Cover image: Alzheimer's disease butterfly, Lisa Angbäck

The butterfly (gr. psyché) symbolizes the human mind in Greek mythology

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Printed by Ineko AB, Gothenburg, Sweden, 2011

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ISBN 978-91-628-8366-9

”Declare the past, diagnose the present, foretell the future, practice these acts.”

Hippocrates



## Abstract

Cerebrospinal fluid (CSF) biomarkers may be used to identify and monitor pathological processes in the central nervous system. CSF biomarkers in Alzheimer's disease (AD) include  $\beta$ -amyloid 42 (A $\beta$ 42), total-tau (T-tau) and phosphorylated-tau (P-tau), reflecting brain amyloid, axonal and tangle pathology, respectively. This dissertation aims at defining and validating CSF biomarkers for amyloid and axonal pathology in AD and related conditions.

We found that CSF A $\beta$ 42, T-tau and P-tau identified early-stage AD patients in a uniquely large multi-center study, and achieved very high diagnostic performance in a well-controlled mono-center study, with careful standardization of clinical procedures, sample handling, and laboratory performance. The distribution of CSF A $\beta$ 42, T-tau and P-tau levels differed across age groups, likely reflecting age-dependent prevalence of AD-like pathology in cognitively stable individuals.

In the multi-center study, differences in the measured CSF biomarker levels were seen across laboratories. To monitor this, we established an external quality control program for CSF biomarkers. This program continues to grow and currently includes over 70 laboratories world-wide.

BACE1 is a key enzyme for A $\beta$  production, and therefore an attractive therapeutic target in AD. CSF biomarkers were studied to measure pharmacodynamic effects of BACE1-inhibitors. A panel of novel biomarkers was identified that may be used to track treatment effects in clinical trials.

Finally, CSF biomarkers of amyloid and axonal pathology were studied in the lysosomal disease Niemann-Pick type C and in Lyme neuroborreliosis. Both these diseases had distinctly altered markers of amyloid metabolism and axonal pathology, and the biomarkers responded to treatments.

In summary, this dissertation indicates that CSF biomarkers are useful in early AD diagnosis, identification of treatment effects and monitoring of amyloid and axonal pathology across neurological diseases. It introduces a quality control program to facilitate global biomarker implementation. With the advancement of biomarkers as components of novel diagnostic criteria, knowledge of CSF biomarker alterations in different diseases will support optimal patient management.

## Populärvetenskaplig sammanfattning

Genom att mäta olika ämnen som avspeglar biologiska processer i kroppen ("biomarkörer") kan man få kunskap om en patients hälsotillstånd. Flera biomarkörer är förändrade i ryggvätskan (likvor) vid neurologiska och psykiatriska sjukdomar beroende på sjukdomsprocesser i hjärnan. Vid Alzheimers sjukdom ansamlas proteinämnet  $\beta$ -amyloid 42 ( $A\beta_{42}$ ) i klumpar (amyloida plack) mellan hjärnans nervceller och nivåerna av  $A\beta_{42}$  i ryggvätskan sjunker. Dessutom förtvinar nervcellernas utskott (axon) och utsöndrar proteinet tau (T-tau), som ibland är förändrat med extra fosforyleringar (P-tau). Genom att mäta  $A\beta_{42}$ , T-tau och P-tau i ryggvätskan hos en patient med kognitiv störning kan man få ledtrådar om problemens orsak och komma närmare en säker diagnos.

Denna avhandling syftar till att undersöka markörer för amyloidomsättning och axonskador vid Alzheimers sjukdom och andra hjärnsjukdomar.

Vi fann att ryggvätskenivåerna av  $A\beta_{42}$ , T-tau och P-tau var förändrade vid Alzheimers sjukdom redan vid tidiga kliniska symptom. Nivåerna av  $A\beta_{42}$ , T-tau och P-tau i ryggvätskan varierade med åldern hos personer utan kognitiv svikt. Detta beror troligen på att förändringar i hjärnan kan upptäckas med biomarkörer innan de ger upphov till symptom. Biomarkörerna varierade mellan laboratorier. Vi etablerade därför ett internationellt kontrollprogram för mätningarna, som kan underlätta användning av dessa biomarkörer i framtiden.

Det saknas fortfarande effektiv behandling mot Alzheimers sjukdom. Ett enzym som är inblandat i produktionen av  $A\beta_{42}$  är BACE1. Vi identifierade biomarkörer som förändrades vid behandling med BACE1-hämmare. Dessa biomarkörer kan användas i studier av nya läkemedel mot Alzheimers sjukdom.

Vi undersökte också biomarkörer för amyloidomsättning och axonskador vid den sällsynta ärftliga sjukdomen Niemann-Pick typ C och vid borreliainfektion i centrala nervsystemet. Sammanfattningsvis fann vi att biomarkörer i ryggvätska kan vara användbara för tidig diagnos av Alzheimers sjukdom och ge information vid flera andra sjukdomstillstånd. Mer kunskap om biomarkörer kan troligen bidra till bättre vård av patienter inom neurologi och psykiatri i framtiden.

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## List of original papers

This dissertation is based on the following papers:

### Paper I

**Mattsson N**, Zetterberg H, Hansson O, Andreasen N, Parnetti L, Jonsson M, Herukka SK, van der Flier WM, Blankenstein MA, Ewers M, Rich K, Kaiser E, Verbeek M, Tsolaki M, Mulugeta E, Rosén E, Aarsland D, Visser PJ, Schröder J, Marcusson J, de Leon M, Hampel H, Scheltens P, Pirttilä T, Wallin A, Jönhagen ME, Minthon L, Winblad B, Blennow K.

*CSF biomarkers and incipient Alzheimer disease in patients with mild cognitive impairment.*

JAMA 2009; 302(4): 385-393.

### Paper II

Johansson P<sup>§</sup>, **Mattsson N**<sup>§</sup>, Hansson O, Wallin A, Johansson JO, Andreasson U, Zetterberg H, Blennow K, Svensson J.

<sup>§</sup>contributed equally

*Cerebrospinal fluid biomarkers for Alzheimer's disease – diagnostic performance in a homogeneous mono-center population.*

Journal of Alzheimer's Disease. 2011; 24(3): 537-46.

### Paper III

**Mattsson N**, Rosén E, Hansson O, Andreasen N, Parnetti L, Jonsson M, Herukka SK, van der Flier WM, Blankenstein MA, Ewers M, Rich K, Kaiser E, Verbeek M, Tsolaki M, Mulugeta E, Aarsland D, Visser PJ, Schröder J, Marcusson J, de Leon M, Hampel H, Scheltens P, Wallin A, Jönhagen ME, Minthon L, Winblad B, Blennow K, Zetterberg H.

*Age and diagnostic performance of Alzheimer's disease CSF biomarkers.*

Neurology. In press.

### Paper IV

**Mattsson N**, Andreasson U, Persson S, Arai H, Batish SD, Bernardini S, Bocchio-Chiavetto L, Blankenstein MA, Carrillo MC, Chalbot S, Coart E, Chiasserini D, Cutler N, Dahlfors G, Duller S, Fagan AM, Forlenza O, Frisoni GB, Galasko D, Galimberti D, Hampel H, Handberg A, Heneka MT, Herskovits AZ, Herukka SK, Holtzman DM, Humpel C, Hyman BT, Iqbal K, Jucker M, Kaeser SA, Kaiser E, Kapaki E, Kidd D, Klivenyi P, Knudsen CS, Kummer MP, Lui J, Lladó A, Lewczuk P, Li QX, Martins R, Masters C,

McAuliffe J, Mercken M, Moghekar A, Molinuevo JL, Montine TJ, Nowatzke W, O'Brien R, Otto M, Paraskevas GP, Parnetti L, Petersen RC, Prvulovic D, de Reus HP, Rissman RA, Scarpini E, Stefani A, Soininen H, Schröder J, Shaw LM, Skinningsrud A, Skrogstad B, Spreer A, Talib L, Teunissen C, Trojanowski JQ, Tumani H, Umek RM, Van Broeck B, Vanderstichele H, Vecsei L, Verbeek MM, Windisch M, Zhang J, Zetterberg H, Blennow K.

*The Alzheimer's Association external quality control program for CSF biomarkers.*

Alzheimer's & Dementia. 2011; 7(4): 386-395.

Paper V

**Mattsson N**, Rajendran L, Zetterberg H, Gustavsson M, Andreasson U, Olsson M, Brinkmalm G, Lundkvist J, Jacobson LH, Perrot L, Neumann U, Borghys H, Mercken M, Dhuyvetter D, Jeppsson F, Blennow K, Portelius E.

*BACE1 inhibition induces a specific cerebrospinal fluid  $\beta$ -amyloid pattern that identifies drug effects in the central nervous system.*

Manuscript.

Paper VI

**Mattsson N**, Zetterberg H, Bianconi S, Yanjanin N, Fu R, Månsson JE, Porter FD, Blennow K.

*$\gamma$ -Secretase-dependent amyloid  $\beta$  is increased in Niemann-Pick type C. A cross-sectional study.*

Neurology 2011; 76(4): 366-72.

Paper VII

**Mattsson N**, Bremell D, Anckarsäter R, Blennow K, Anckarsäter H, Zetterberg H, Hagberg L.

*Neuroinflammation in Lyme neuroborreliosis affects amyloid metabolism.*

BMC Neurology 2010; 10: 51.

## Abbreviations

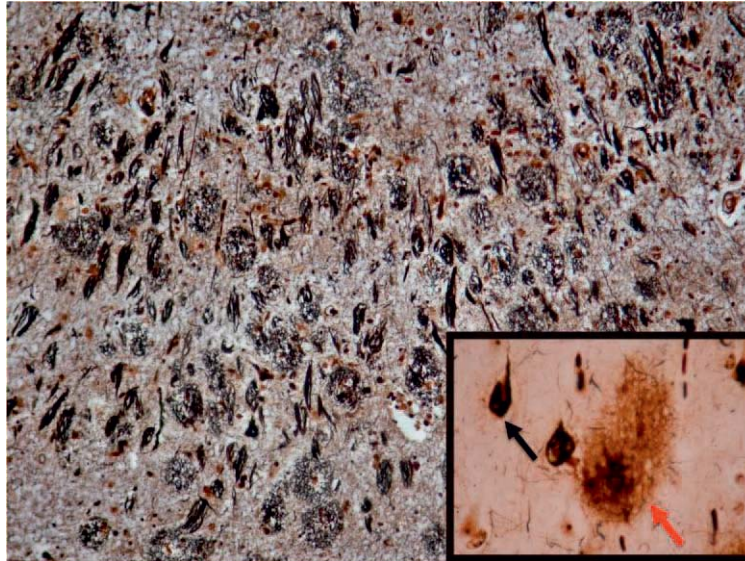
A $\beta$ ,  $\beta$  amyloid  
AD, Alzheimer's disease  
ADAM, A Disintegrin And Metalloproteinase  
APOE, Apolipoprotein E  
APP, amyloid precursor protein  
AUROC, area under the receiver operating characteristic  
BACE1,  $\beta$ -site amyloid precursor protein-cleaving enzyme 1  
CID, collision induced dissociation  
CSF, cerebrospinal fluid  
DR6, death receptor 6  
ELISA, enzyme-linked immunosorbent assay  
ESI, electrospray ionization  
FTICR, Fourier transform ion cyclotron resonance  
GFAP, glial fibrillary acidic protein  
IP, immunoprecipitation  
LC, liquid chromatography  
LQIT, linear quadrupole ion trap  
LNB, Lyme neuroborreliosis  
MALDI, matrix-assisted-laser desorption/ionization  
MCI, mild cognitive impairment  
MRI, magnetic resonance imaging  
MS, mass spectrometry  
NFT, neurofibrillary tangle  
NPC, Niemann-Pick type C disease  
P-tau, phosphorylated tau  
PET, positron emission tomography  
PHF, paired helical filaments  
PiB, Pittsburgh compound B  
PS1, presenilin-1  
PS2, presenilin-2  
sAPP, soluble amyloid precursor protein  
QC, quality control  
T-tau, total tau  
TOF, time-of-flight

## Introduction

Neurological diseases are major causes of morbidity and mortality. The most common neurodegenerative disease is Alzheimer's disease (AD), with about 25 million patients world-wide and a rapidly increasing prevalence [1]. There is no existing disease-modifying therapy or cure for AD [2]. If available in the future, such a therapy will likely be most efficient in the early stages, when a diagnosis by clinical examination is difficult or impossible to obtain [3]. Cerebrospinal fluid (CSF) biomarkers may help if used as diagnostic tools [4], but they need validation in early-stage AD and their measurements need standardization across centers [5]. CSF biomarkers may be used to study pathological processes in neurological diseases directly in patients, and as pharmacodynamic markers of drug effects in the central nervous system (CNS), and in other aspects of clinical trials, to speed up drug development [3, 6, 7]. This dissertation investigates CSF biomarkers for amyloid and axonal pathology in AD, the lysosomal disease Niemann-Pick type C (NPC) and Lyme neuroborreliosis (LNB), where different forms of amyloid and axonal pathology may be present [8-10].

### Alzheimer's disease

In 1906, the German psychiatrist and neuropathologist Alois Alzheimer (1864-1915) presented the clinical case of Auguste Deter, who developed impaired short-time memory and delusions in her late 40s, and progressed with severe disorientation and impaired long- and short-term memory [11]. Alzheimer associated these symptoms with extracellular neuritic plaques and intraneuronal neurofibrillary tangles (NFTs) that he found in her brain post mortem (Figure 1). In the 1980s, NFTs and plaques were shown to contain tau proteins and  $\beta$  amyloid ( $A\beta$ ) peptides, respectively [12-15]. The influential psychiatrist Emil Kraepelin (1856-1926) introduced the term AD to describe this early-onset dementia ( $< 65$  years of age) [16], but as similarities in brain pathology across ages were recognized, both early- and late-onset cases were eventually called AD. Most patients have sporadic AD (SAD), but a small minority ( $< 1\%$ ) have autosomal dominant familial AD (FAD) [3], which usually produces symptoms before 65 years of age and sometimes as early as the third decade of life [17]. Symptoms of AD include loss of episodic memory and language, apraxia, agnosia, impaired judgment, decision-making and orientation, and in later stages even motor system dysfunction [3]. Most AD patients die within 8-10 years after onset of symptoms [18], but the speed of decay is variable [19].



**Figure 1.** AD neuropathology. A $\beta$  plaques and NFTs in an AD brain. The insert shows plaque (red arrow) and NFT (black arrow) pathology at high magnification. Images courtesy of Dr Nenad Bogdanovic, Karolinska Institutet, Sweden, and Pfizer Limited, UK.

The gold standard for AD diagnosis is neuropathology and a clinical diagnosis can only be made to the “probable” degree of certainty [20], although it should be noted that there is variability also in neuropathological examination [21]. Since the 1990s, imaging and biochemical markers have been shown to identify AD *in vivo*, which is recognized in ongoing revisions of AD criteria [22-24].

In early symptomatic phases, AD patients may be diagnosed with mild cognitive impairment (MCI) which is a syndrome characterized by objectively verified cognitive dysfunction in memory or non-memory domains, adjusted for age and education, but not sufficiently severe to fulfill the criteria for dementia [25, 26]. About 6-15% of MCI patients (differing between epidemiological studies and referral settings) progress annually in their symptoms to reach criteria for AD dementia [27-29]. These are often referred to as MCI-AD patients in studies. If AD biomarker evidence is present at the MCI stage, patients may be designated “MCI due to AD” [23], prodromal AD [24] or incipient AD patients [4]. Other MCI patients have benign non-progressive symptoms, while some develop other forms of dementia.

### **Neuropathology of AD**

Tau proteins in NFTs are abnormally phosphorylated and are called P-tau below (see [30] for a recent review on tau pathology). Compared with normal tau, P-tau has aggregation properties and reduced capacity for binding to microtubules, which disrupts axonal transport mechanisms. It is noteworthy, that tau is extensively phosphorylated during neurodevelopment, perhaps facilitating developmental flexibility and synaptic pruning [31, 32]. In AD, NFTs are seen before the appearance of A $\beta$  deposits and develop in a highly predictable pattern throughout the brain [33]. They first emerge in the transentorhinal region, are later seen in the hippocampus, amygdala and neocortical association areas and finally appear in the primary motor and sensory areas.

Neuritic plaques contain fibrillary A $\beta$ , activated microglia and dystrophic neurites with P-tau aggregates, and are surrounded by astrocytes. The plaques have a less distinct pattern of development than the NFTs, appearing first in temporal neocortical areas and later throughout the neocortex, in deeper brain nuclei and the hippocampus [34-37].

In late-stage AD, the brain is severely atrophic, but some regions, such as the inferior frontal cortex, remain essentially spared [38]. There is also selective neuronal vulnerability, with most loss of cholinergic neurons and neurons with long, thin, unmyelinated or sparsely myelinated axons [33].

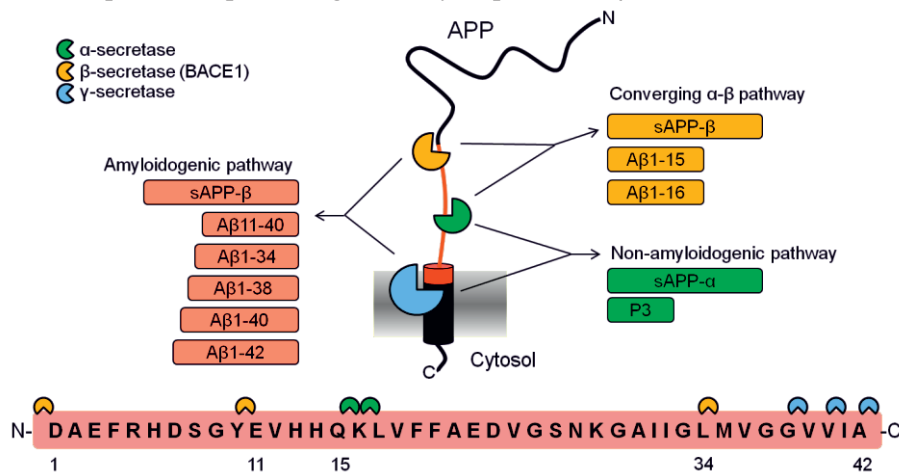
Typical neuritic plaques, with a dense core of fibrillar A $\beta$  are only seen in the AD brain, but diffuse plaques with non-fibrillary A $\beta$  can be seen in other conditions, such as traumatic brain injury, dementia pugilistica and Lewy body dementia [39], and also differ in occurrence among subgroups of AD patients [40]. NFTs are present in several dementing disorders [39], including NPC [41].

Loss of synapses is the neuropathological feature with the strongest correlation to clinical severity in AD [42]. The NFT load correlates to loss of neurons [43] and to clinical severity [44, 45], while the correlation between neuritic plaques and symptoms is weaker [46], although it has been suggested that it might be stronger for soluble A $\beta$  species [47].

Mild to moderate AD-like neuropathology is seen in many elderly without cognitive decline [48] but severe brain changes are found only in symptomatic individuals [39]. Thus, other factors, such as cognitive reserve or co-morbidities may modulate symptom onset. AD-like changes in healthy elderly suggest that there might be a long lag phase between the first brain changes and symptom onset, which is similar to other common pathologies in the elderly, such as atherosclerosis or neoplastic changes in the prostate [49].

## APP and A $\beta$ metabolism

A $\beta$  is cleaved from the transmembraneous protein amyloid precursor protein (APP) which is encoded by the *APP* gene on chromosome 21 [50-52]. Complex mechanisms of proteolytic enzymes degrade APP in different pathways (Figure 2) (see [6, 53, 54] for recent reviews). In the amyloidogenic pathway, APP is believed to be transported to the plasma membrane, endocytosed in recycling endosomes, and cleaved at the N-terminal end of A $\beta$  in an acidic environment by the  $\beta$ -secretase active enzyme BACE1 ( $\beta$ -site APP cleaving enzyme 1, reviewed in [55]). However, several cellular pathways for APP processing have been proposed, emphasizing metabolism in synaptic clear vesicles in the constitutive pathway or in dense core vesicles in the regulated secretory pathway [56].  $\beta$ -secretase-mediated APP cleavage liberates a soluble N-terminal ectodomain (sAPP- $\beta$ ), and the remaining C-terminal APP stub ( $\beta$ -CTF, C99) is processed by the membrane-bound protein complex  $\gamma$ -secretase to A $\beta$  peptides and the APP intracellular domain (AICD).  $\gamma$ -Secretase may produce different A $\beta$  isoforms, including A $\beta$ 1-42 (A $\beta$ 42), A $\beta$ 1-40, A $\beta$ 1-38 and species down to A $\beta$ 1-17 [57]. The production of these peptides is believed to occur in a stepwise fashion [7]. In a non-amyloidogenic pathway, APP is cleaved within the A $\beta$  sequence by enzymes with  $\alpha$ -secretase activity, such as ADAM10 [58]. The  $\alpha$ - and  $\beta$ -secretase pathways may also converge to release short A $\beta$  peptides, including A $\beta$ 1-14, A $\beta$ 1-15 and A $\beta$ 1-16 [57], and several other enzymes may cleave APP at different positions, producing a variety of possible A $\beta$  isoforms [59].

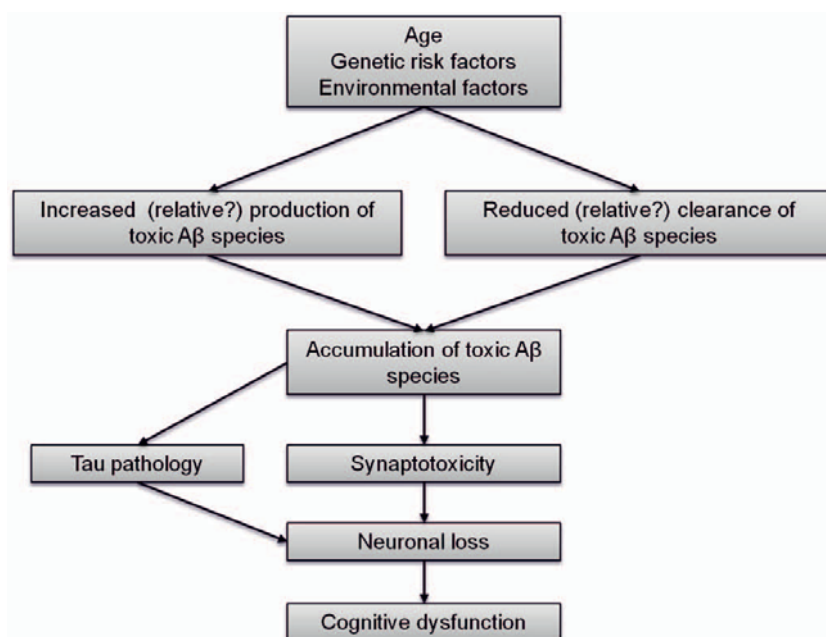


**Figure 2.** Schematic figure of major APP degradation pathways, highlighting selected peptides. A $\beta$ 1-42 is indicated by the red section within the APP molecule. The lower part of the figure shows the amino acid sequence of A $\beta$ 1-42, including selected cleavage sites that depend on the activities of  $\alpha$ -secretase, BACE1 and  $\gamma$ -secretase.



### The amyloid cascade hypothesis

The precise relations between A $\beta$ , tau and clinical disease are key issues in AD research. According to a dominating theoretical framework, the amyloid cascade hypothesis [60, 61], a relative increase in toxic A $\beta$  species, resulting from increased production or decreased clearance [62], triggers tau pathology, synaptic dysfunction and neuronal loss [7, 63] (Figure 3). Originally, the hypothesis was based on the accumulation of A $\beta$  in AD brains, the findings of disease-causing mutations in genes involved in A $\beta$  metabolism, and the observation that trisomy 21 (Down's syndrome) leads to early-onset dementia, which might be related to the extra gene copy of *APP* in the disease. In experimental paradigms A $\beta$  is synaptotoxic [64-66], neurotoxic [67], disrupts cellular membranes [49], interferes with mitochondrial function [68], or activates damaging microglia [69, 70], but the precise mechanisms of importance *in vivo* in humans are unclear, especially in SAD. Currently there is much focus on the potentially toxic effects mediated by oligomeric A $\beta$  in AD [49].



**Figure 3.** The amyloid cascade hypothesis. Possible relations between risk factors, A $\beta$  pathology, tau pathology and cognitive dysfunction.

Modifications of the amyloid cascade hypothesis emphasize cross-talk between A $\beta$  and tau pathological pathways [71]. These may clearly interact experimentally, with injections of fibrillar A $\beta$  inducing tau

hyperphosphorylation in primate brains [67] and injections of P-tau inducing A $\beta$  deposition in rat brains [72].

Frequent findings of plaque pathology in elderly without documented cognitive decline [73], only low correlation between plaque pathology and cognitive symptoms [46], and recent failures of anti-A $\beta$  drugs in clinical AD trials have provoked a debate about the validity of the amyloid cascade hypothesis [7, 49, 74] and raised some interest in A $\beta$ -independent disease mechanisms [75]. In particular, there is concern that the hypothesis may be less relevant for SAD than FAD [74]. In FAD, the situation is further complicated by  $\gamma$ -secretase having a large number of substrates besides APP [76], since some of these might be linked to development of neurodegeneration. Further, presenilin-1, which harbors the active site of  $\gamma$ -secretase, even has a non-proteolytic activity linked to lysosomal function [77]. Despite these objections, the amyloid cascade hypothesis still provides the most solid framework for understanding AD. A slight modification of the hypothesis might be that A $\beta$  works as a trigger for down-stream brain changes but is less important for progression after disease onset. This view is supported by the similar disease *durations* in patients with SAD, FAD and different *APOE* polymorphisms, despite different *ages-at-onset* [7].

### **Risk factors**

Ageing is the strongest risk factor for AD. The prevalence of AD dementia is about 1 % in people 60-64 years of age but exceeds 25 % in people 85 years or older [78]. Considering that brain pathology starts years or decades before clinical dementia, the true prevalence of AD is even higher [33, 79]. Other possible risk factors include head trauma, education, occupation, social and physical activity and mental ability during early life [3]. Risk factors for vascular disease, including smoking, hypertension and hyperlipidemia are also risk factors for AD dementia, but it is unclear if they are related to AD *per se*, or if vascular brain pathology lowers the threshold for symptoms in a patient with prodromal AD. It should be emphasized that the risk increase inflicted by these risk factors is very modest, and has not been possible to replicate in all studies.

### **Disease-causing genes**

FAD is caused by mutations in *PS1*, *PS2*, or *APP*. See the AD & FTD Mutation Database <http://www.molgen.ua.ac.be/ADMutations> for an updated list of mutations. Most FAD patients have mutations in *PS1* and there has been a debate as to whether these mutations lead to a gain- or loss of function in AD [80]. A loss of presenilin function in AD is supported by similarities

between the CSF A $\beta$  profiles in AD patients and the CSF profiles induced by  $\gamma$ -secretase inhibition [81, 82]. The existence of AD-causing mutations over the whole *PS1* gene also suggests that loss of function is involved in the disease mechanism. However, A $\beta$  pathology is not a necessary step for disease caused by altered presenilin function. In mice, loss of presenilin may lead to progressive neurodegeneration, synaptic loss and tau hyperphosphorylation without A $\beta$  pathology [83] (while APP-overproducing animals develop A $\beta$  deposits but not neurodegeneration [84]). In humans, some *PS1* mutations cause frontotemporal lobe dementia without A $\beta$  deposits [40].

Even if AD patients do have loss of presenilin function, this does not rule out A $\beta$  from the AD pathogenesis cascade. For example, APP/A $\beta$  might occupy the active site of  $\gamma$ -secretase and block an already reduced activity on non-APP substrates [85]. Also, a partial loss of presenilin function could increase the relative production of toxic A $\beta$  versus other A $\beta$  species [7].

*PS2* encodes presenilin-2 which is an alternative  $\gamma$ -secretase subunit. AD-causing mutations in *PS2* are very rare, which might be related to the minor role of presenilin-2 in APP degradation [86]. *APP* mutations affect APP degradation and/or A $\beta$  aggregation, and cause both cerebral vascular angiopathy and plaque pathology [40].

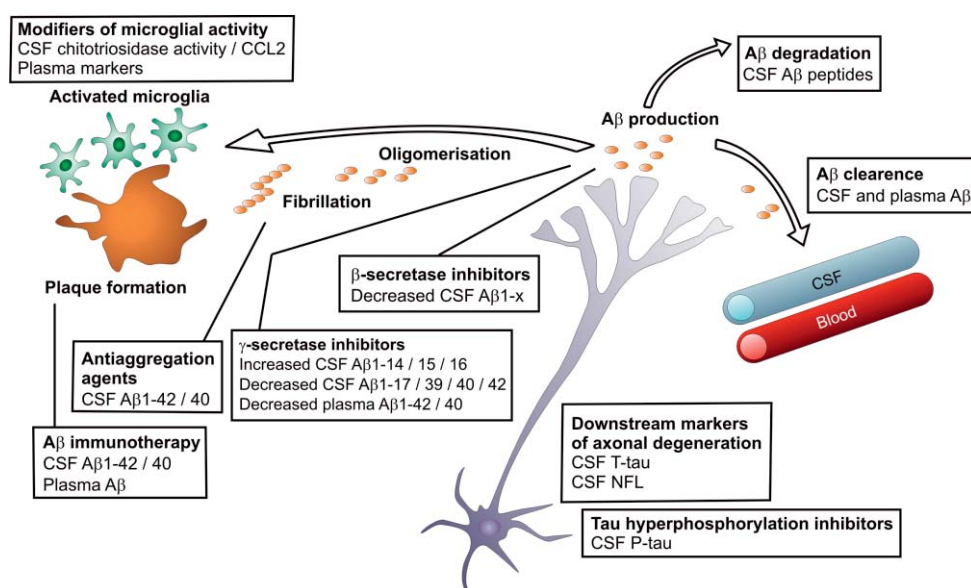
### Susceptibility genes

*APOE* is without comparison the most important risk gene for SAD [87]. *APOE* encodes the CNS cholesterol transporter apolipoprotein E, and the three common polymorphisms  $\epsilon 2$ ,  $\epsilon 3$ , and  $\epsilon 4$  profoundly alter AD risk, of which *APOE*  $\epsilon 4$  increases risk and lowers age-at-onset. The underlying molecular mechanisms are unclear, but might be linked to cholesterol redistribution and/or interactions with A $\beta$  accumulation.

www.AlzGene.org carries an updated database of AD risk genes [88] and currently (September 2011) highlights polymorphisms in 10 genes that affect AD risk. These genes fall into clusters with roles in vesicle and membrane trafficking (*BINI*, *PICALM*, *CD33*, and *CD2AP*), lipid metabolism (*APOE*, *CLU* and *ABCA7*), and immune function (*CRI*, *MS4A*, *CLU*, *ABCA7*, and *CD33*), pointing to these systems as important for disease mechanisms in SAD [89, 90]. However, compared with *APOE*  $\epsilon 4$ , which increases the risk 3-4 folds in heterozygotes and 8-10 folds in homozygotes, the risk increases for other susceptibility genes are very modest.

### Disease-modifying treatment

Symptomatic treatment for AD is available with acetylcholine esterase inhibitors and an NMDA-receptor antagonist, but these are not believed to modify the underlying disease progression [2]. Several putative disease-modifying drugs are under development, including modulators of A $\beta$  aggregation, inhibitors and modulators of APP processing enzymes and A $\beta$  immunotherapies (Figure 4).



**Figure 4.** Examples of putative disease-modifying AD treatments and corresponding possible CSF and plasma pharmacodynamic biomarkers. Adapted from [91] and [6].

At present, there is little evidence of beneficial effects of these novel treatment strategies in humans. Most large trials have either reported inefficiency at alleviating clinical symptoms or even harmful effects, despite some evidence of effects on A $\beta$  metabolism [92, 93]. Positive outcomes for anti-A $\beta$  drugs would support the amyloid cascade hypothesis, but negative results are more difficult to interpret. For example, they may be caused by failure of the drug to exert its desired effects within the CNS; collateral adverse effects rendering the net results negative; underpowered studies with erroneous inclusions of non-AD patients; inclusion of AD patients in disease stages too advanced for treatment; or errors in the very hypothesis underlying the treatment [49]. Current ongoing Phase III trials will provide further evidence whether this treatment principle will be effective. A possibility remains that drugs based on the amyloid cascade hypothesis may be more efficient in patients and carriers of FAD mutations than in SAD patients.

### **Niemann-Pick type C disease**

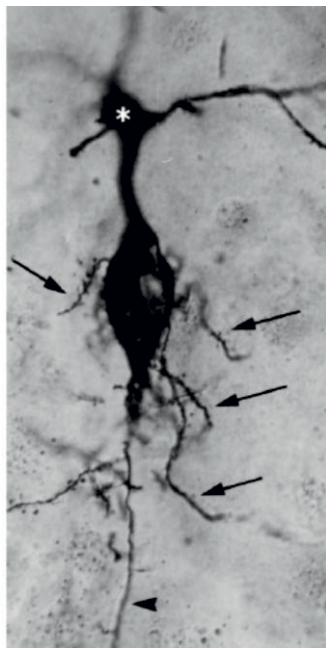
The diagnostic entity Niemann-Pick disease traces its origin to the work of the pediatrician Albert Niemann (1880-1921) and pathologist Ludwig Pick (1868-1944). Originally identified as a lipid storage disorder with hepatosplenomegaly and sometimes neurological engagement, it is now classified into Niemann-Pick disease types A and B, with sphingomyelinase deficiency, and NPC, which is a lipid trafficking disorder, with abnormal accumulation of unesterified cholesterol in late endosomes and lysosomes [94].

The incidence of NPC is around 1/120 000 live births, where 95% of the patients have mutations in *NPC1*, which encodes the trans-membranous protein NPC1 that is essential for normal cholesterol homeostasis [95-97]. About 5% of the patients have mutations in *NPC2* [98], which encodes the small soluble protein NPC2 that is believed to function together with NPC1 in the transport of cholesterol in late endosomes and lysosomes, and it is this system that malfunctions in the disease.

NPC may present at anytime in life, from the fetal period to the fifth decade and possibly later. Neurological symptoms dominate the clinical picture and include cerebellar ataxia, dysarthria, dysphagia and dementia. Infantile and juvenile patients usually die within a few years, but some juvenile patients may exceed 30 years of age. Patients diagnosed when adolescent or as adults have a more insidious onset, with progressive psychiatric problems, including delusions, hallucinations, depression, aggressiveness and dementia which may be misdiagnosed as AD [99].

### Neuropathology of NPC

Neuropathological findings in NPC include leukodystrophy, together with cerebellar and cortical atrophy. The neurons are filled with lipid storage material, primarily GM2 and GM3 gangliosides. The total brain concentration of cholesterol is not affected but the neuronal distribution of cholesterol is altered, with accumulation in cell bodies and reduced levels in distal axons. The neurons form meganeurites, axonal spheroids, ectopic dendrites and NFTs (Figure 5) [100]. There is a selective vulnerability among neuronal populations, with cell death primarily affecting Purkinje cells in the cerebellum. NPC brains show signs of abnormal APP/A $\beta$  brain metabolism, with increased levels of C99 in the cerebellum and A $\beta$ 42 in the hippocampus [10], but the patients generally lack A $\beta$  plaques, except for diffuse plaques in *APOE*  $\epsilon$ 4 homozygous patients [101]. CSF biomarkers of amyloid and axonal pathology have so far not been explored in NPC.



**Figure 5. NPC Neuropathology**

Golgi-impregnated cortical pyramidal neuron in a 3.5-year-old child with NPC. Spines and neuritic processes are sprouting from a meganeurite (arrows). The neuronal somata is indicated by the asterisk and the axon by the arrowhead. Image courtesy of Professor Steven U. Walkley, Albert Einstein College of Medicine, NY, USA.

### **Lyme neuroborreliosis**

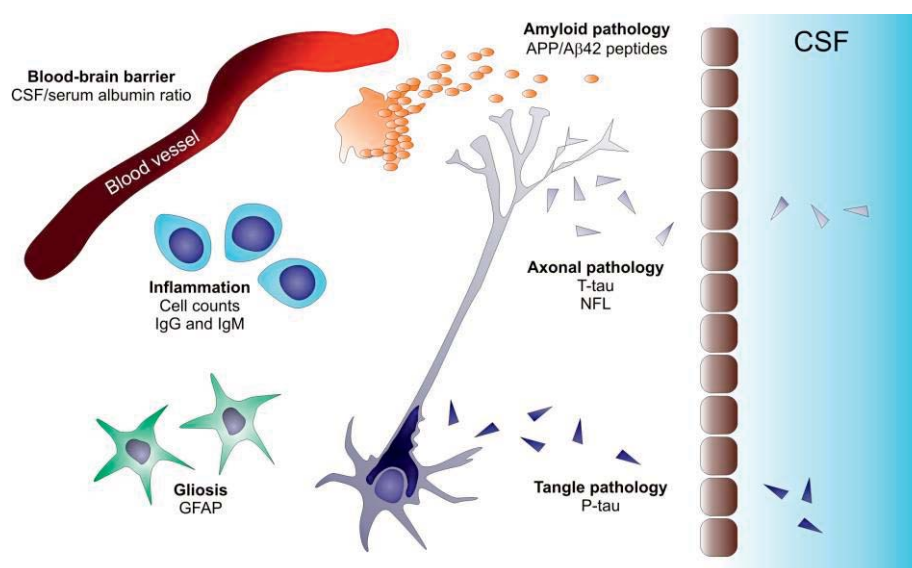
LNB is caused by a CNS infection by the tick-borne spirochete *Borrelia burgdorferi* sensu lato [102]. LNB is often manifested by cranial nerve engagement and common clinical findings are facial nerve palsy and radiculitic pain. The pathological spectrum of LNB is wide, ranging from peripheral axonal neuropathy, to mild encephalopathy and encephalomyelitis, with diffuse white matter lesions, and in rare cases even vasculitis with cerebral infarction. A chronic LNB may present with cognitive AD-like disturbances. Importantly, erroneous inclusion of LNB patients in AD drug trials may confuse interpretations of inflammatory reactions and adverse drug events [103]. LNB may be treated with intravenous ceftriaxone or oral doxycycline. Symptoms from an acute infection usually resolve within weeks after treatment, but chronic symptoms may improve more slowly.

### **Amyloid metabolism in neuroinflammation**

*Borrelia* infections have been suggested to be amyloidogenic [104], but CSF biomarkers of amyloid metabolism have so far not been studied in LNB. There is evidence of altered APP/A $\beta$  metabolism in other neuroinflammatory disease, with reduced CSF sAPP- $\alpha$  and sAPP- $\beta$  in multiple sclerosis, cerebral systemic lupus erythematosus [105], and HIV [106]. CSF A $\beta$ 42 has been described to be reduced in multiple sclerosis [105], bacterial meningitis [107] and HIV [106, 108].

## Cerebrospinal fluid biomarkers

CSF biomarkers have been used for over a century to identify and monitor disease processes within the CNS [109] and are routinely used in clinical diagnostics of neurological disorders [110]. The basis for sampling CSF for biomarkers is its proximity to the brain parenchyma, making it an optimal fluid for biochemical measurements of CNS abnormalities (Figure 6 and Table). The CSF fills the ventricles and surrounds the brain and the spinal cord. About two thirds of the CSF is produced by the choroid plexus in the ventricular system through passive filtration of capillary blood and active secretion [111]. The remaining part is released diffusely from the brain interstitium. There is a fast turnover of CSF, with a production of about 0.4 mL/min and a total volume of about 160 mL. CSF is accessible for sampling by lumbar puncture, which is a relatively simple and cost-effective procedure. Severe complications from diagnostic lumbar punctures are extremely rare, but headache occurs in 2–4% of elderly patients, and may be more frequent in younger individuals [112, 113]. Most knowledge concerning CSF biomarkers of amyloid and axonal pathology comes from studies in AD [114].



**Figure 6.** Summary of CSF biomarkers in relation to pathological processes.



**Table.** Selected CSF biomarker changes in different neurological diseases

<b>Biomarker</b>	<b>Change</b>	<b>Reflects</b>	<b>Diagnosis</b>
Albumin ratio (CSF/serum)	Increased	Blood-brain barrier damage	Infection, inflammation, vascular dementia, leukodystrophies, stroke
CSF white blood cell count	Increased	Intrathecal pleocytosis	Infection, inflammation, malignancy
CSF-specific oligoclonal IgG- or IgM-bands	Positive	Intrathecal immunoglobulin production	Infection, inflammation, malignancy
CSF A $\beta$ 42	Decreased	Amyloid plaque pathology	AD, dementia with Lewy bodies, LNB (none-mild decrease), Creutzfeld-Jakob's disease (none-marked decrease)
	Increased	?	NPC
CSF T-tau	Increased	Degeneration of cortical axons	AD, vascular dementia (none-mild increase), stroke, Creutzfeld-Jakob's disease, dementia with Lewy bodies, inborn errors of metabolism
CSF P-tau	Increased	Neurofibrillary tangles	AD
	Decreased	?	LNB
CSF NFL	Increased	Degeneration of myelinated axons	MS, vascular dementia, frontotemporal lobe dementia, leukodystrophies, amyotrophic lateral sclerosis, atypical Parkinsonian disorders
CSF GFAP	Increased	Astrocytosis, gliosis	MS, stroke, Alexander's disease, neuromyelitis optica

The aim of this table is to present a summary of CSF biomarkers used in neurological investigations, and not to be a complete review. Adapted from [115].

### **CSF biomarkers of amyloid pathology**

CSF APP metabolites may give clues to APP/A $\beta$  brain metabolism *in vivo* in humans. In 1995 it was discovered that AD patients have approximately 50% reduced concentrations of CSF A $\beta$ 42 compared with controls [116]. This is often explained by A $\beta$  deposition in plaques, and studies show that low CSF A $\beta$ 42 correlates to high numbers of plaques [117] and to brain retention of fibrillary A $\beta$ -binding positron emission tomography (PET) tracers [118-120]. Hypothetically, reduced A $\beta$  production due to neuronal loss or decreased synaptic activity [121] might also lower CSF A $\beta$ 42. CSF A $\beta$ 40 concentrations are largely unchanged in AD but some studies have found increased diagnostic performance for AD of the A $\beta$ 42/A $\beta$ 40 ratio compared to A $\beta$ 42 alone [122]. Several shorter A $\beta$  isoforms may also be monitored in CSF [123]. CSF levels of sAPP- $\alpha$  and sAPP- $\beta$  are unaltered [124] or mildly elevated in SAD [125] and reduced in neuroinflammation [105, 106].

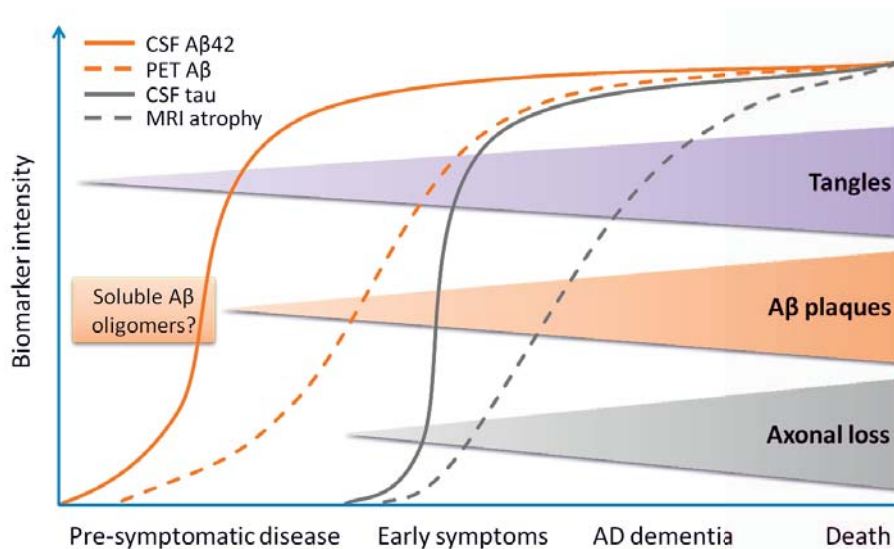
### **CSF biomarkers of axonal pathology**

CSF levels of the axonal markers neurofilament light protein (NFL) and tau can be used to monitor different types of axonal pathology. NFL is mainly found in large subcortical myelinated axons [126] and CSF NFL concentrations are elevated in response to damage of these structures in subcortical vascular dementia [127-129], multiple sclerosis [130], traumatic brain injury [131], spine trauma [132], amyotrophic lateral sclerosis [133], and LNB [9]. In contrast, tau is highly expressed in cortical axons [134]. CSF total-tau (T-tau) concentrations are elevated mainly in cortical diseases, such as AD [4], where mean levels increase about 300% compared to controls, and Creutzfeldt-Jakob disease [135, 136], where they often are even higher. CSF P-tau levels correlate with the number of NFTs in AD [137] and is not increased in other dementias, wherefore the ratio between CSF P-tau and T-tau is useful to differentiate AD from other neurodegenerative diseases with increased T-tau levels, such as frontotemporal lobe dementia [138], normal pressure hydrocephalus [139] and Creutzfeldt-Jakob disease [140]. CSF levels of axonal markers are believed to reflect the ongoing rate of axonal loss.

### **CSF biomarkers for diagnosis and prognosis**

Several studies support the use of CSF biomarkers to identify AD patients, with diagnostic sensitivities and specificities reaching 80-90% [4, 141, 142]. Changes in biomarkers are present already at the MCI stage, preceding clinical dementia [143-153], and may even be seen in cognitively normal individuals that will deteriorate several years later [152, 154-157] (Figure 7).

The slow disease progression creates a need for studies with long follow-up to verify clinical diagnoses in relation to baseline measurements. In a study of 137 MCI patients followed over 4-6 years, 57 were found to have MCI-AD, with CSF biomarkers at baseline achieving 95 % sensitivity and 83 % specificity [158]. Due to discrepancies between clinical AD diagnosis and autopsy confirmation [159, 160], higher diagnostic accuracies for biomarkers evaluated towards clinical diagnoses are difficult to achieve. In AD, high CSF tau levels at baseline predict a more malignant disease course [161], which is probably related to a higher rate of axonal loss.



**Figure 7.** Hypothetical model of possible relationships between biomarker intensities, neuropathological lesions and clinical disease development. Adapted and modified from [143]. Differences in slopes indicate possible differences in speed of development towards maximum biomarker intensities. Low CSF A $\beta$ 42 concentrations are generally stable over time in AD (suggesting that this is primarily a state marker) [162]. Low CSF A $\beta$ 42 may be an earlier biomarker than increased PET A $\beta$  signal [163] and the PET brain A $\beta$  signal may increase during disease development (stage marker) [164, 165]. CSF tau is stable or increases only slightly during symptomatic stages [162, 166], but brain atrophy as measured by MRI increases with disease progression. Large longitudinal studies with consecutive measurements may ultimately determine the precise time-points and slopes of development for these biomarkers.

### **CSF biomarkers of progression**

CSF A $\beta$ 42, T-tau and P-tau levels are stable over time for at least up to 2 years in AD [162, 167] which supports their role in early diagnosis, but also makes them unsuitable as markers of progression or accumulated neuronal loss. There is no clear consensus on how to use CSF biomarkers for this purpose, and studies are hampered by the need for serial samples taken during long-term follow-up.

### **CSF biomarkers: testing at what stage?**

If disease-modifying treatment becomes available, early diagnosis in conjunction with treatment would form a preventive strategy for AD. Disease prevention can be divided into primary prevention, which is the reduction of risk factors to prevent disease from occurring, and secondary and tertiary prevention, which is treatment to halt disease progression in the pre-symptomatic and symptomatic stages, respectively. Secondary prevention could incorporate biomarkers, but the low prevalence of pre-symptomatic AD in the general population makes this challenging [168]. Tertiary prevention equals diagnosis and treatment of MCI due to AD. Such patients may be diagnosed using CSF biomarkers, but it is still an open question if therapy at this disease stage will be efficient in a clinically meaningful way.

### **CSF biomarkers in clinical trials**

Biomarkers have different applications in clinical trials. They may be used to enrich study populations, in order to increase study power [141]. For AD treatment at the MCI stage this may be particularly useful, since about 50 % of unselected MCI participants are unlikely to be at risk for AD dementia. Biomarkers may also be used to stratify patients to different treatment arms, or in post-hoc analyses when interpreting outcome data. For example, an anti-A $\beta$  therapy likely has its strongest clinical effects on patients with biomarker evidence of A $\beta$  pathology. Biomarkers sampled before, during, and after therapy may be used to monitor drug effects. Finally, biomarker discovery in conjunction with drug development may also mean the opportunity for a company to launch an accompanying diagnostic tool.

### **CSF biomarkers as pharmacodynamic markers**

Several compounds that effectively reduce A $\beta$  pathology in AD animal models have failed to be clinically beneficial in human patients [3]. This reflects the difficulties in translating results from a short-lived genetically modified animal model to a complex human disease developing over decades. CSF pharmacodynamic markers may help to identify compounds with desirable CNS effects in small pilot studies, increase chances of success in large-scale trials, and minimize exposure of non-beneficial potentially harmful drugs to patients [114]. Pharmacodynamic markers may be primary or secondary. A primary pharmacodynamic marker is directly linked to the specific drug target, such as CSF A $\beta$  peptides, for a drug targeting A $\beta$  metabolism. A secondary pharmacodynamic biomarker measures a downstream effect of the intervention, such as a marker of axonal degeneration for an anti-A $\beta$  drug, since such treatment is supposed to have secondary effects on axonal loss. For example, a reduction of CSF T-tau after an intervention against A $\beta$  pathology may be interpreted as a reduced rate of axonal loss, which would be a very encouraging observation.

### **CSF biomarkers as surrogates?**

A surrogate marker is a regulatory term describing a validated substitute for a clinically meaningful endpoint. An effect on a surrogate predicts clinical effects (a drug should not only treat the biochemical measurement but actually affect the underlying disease in a way that is meaningful for the patient) [169]. Very few biomarkers fulfill these requirements [170]. The studies needed to establish surrogates are essentially the same that as those they are meant to overcome. Even if several studies on different drugs uniformly show similar results on a biomarker, for example reduced CSF T-tau in parallel with clear positive clinical outcomes, regulators might still ask whether reduced CSF T-tau for a novel drug predicts a positive effect or not. Qualified surrogate markers in neurology seem well in the future, but non-qualified surrogates may be used in early drug trials to select compounds likely to succeed in later stages [169]. The development of novel therapies goes hand in hand with the identification and use of such pharmacodynamic CSF biomarkers.

## **Aims and objectives**

The general aim of this dissertation was to study CSF biomarkers for amyloid and axonal pathology in different settings of AD and the related conditions NPC and LNB (Figure 8).

The specific aims of each paper were:

### **Paper I**

To study the diagnostic performance of CSF biomarkers in early-stage AD in a large multi-center setting

### **Paper II**

To study the diagnostic performance of CSF biomarkers in AD in a well controlled mono-center setting

### **Paper III**

To study the influence of age on CSF AD biomarkers

### **Paper IV**

To establish an external quality control (QC) program for CSF AD biomarkers.

To estimate the global variability between laboratories in CSF biomarker measurements

### **Paper V**

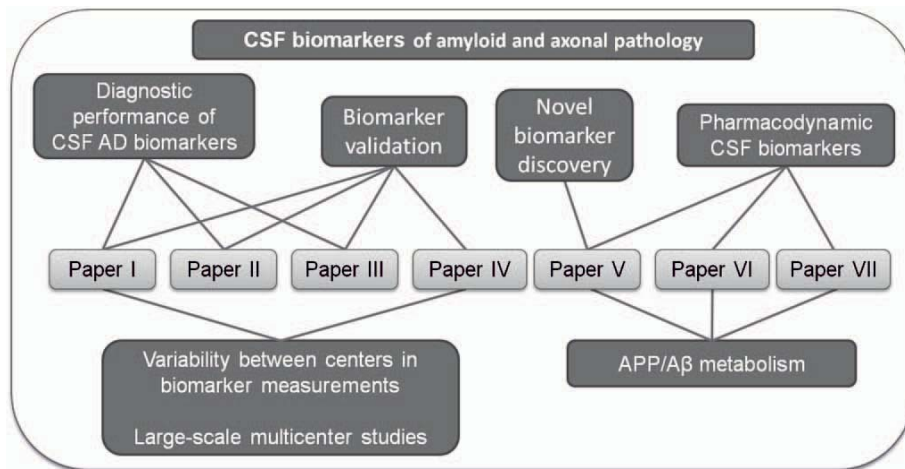
To study pharmacodynamic CSF biomarkers for BACE1 inhibitors

### **Paper VI**

To investigate CSF biomarkers of amyloid and axonal pathology in the lysosomal neurodegenerative disease NPC

### **Paper VII**

To investigate CSF biomarkers of amyloid and axonal pathology in the neuroinflammatory disease LNB



**Figure 8.** Major themes of the papers.

## **Methods**

Study participants are described in conjunction with each paper below.

### **CSF sampling and analyses**

All participants underwent lumbar puncture in the L3-4 or L4-5 interspaces. No serious adverse events were reported. If not stated otherwise, samples were stored in polypropylene tubes and frozen at  $-80^{\circ}\text{C}$  until analysis. All CSF samples were analyzed at the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, except for samples from Amsterdam, Kuopio and Munich (Papers I and III), and samples in the external QC program (Paper IV). Biochemical analyses were performed by experienced and certified laboratory technicians who were blinded to the clinical diagnoses and other clinical information.

### **Cell and animal experiments**

In the study on BACE1 inhibition, SH-SY5Y cells, 7PA2 cells, and HeLa cells were used. The SH-SY5Y cells (human neuroblastoma cells) expressed wild type APP695 or APP695 with the FAD-causing Swedish mutation. The 7PA2 cells (Chinese hamster ovary cells) expressed APP751 with the FAD-causing V717F mutation. HeLa cells (human liver cancer cells) expressed APP695 with the Swedish mutation. The cells were treated with the BACE1 inhibitors  $\beta$ -secretase inhibitor IV (Calbiochem, Merck), AZ-20 (AstraZeneca), or BACE1-siRNA, and cell media were analyzed for sAPP and A $\beta$  isoforms. The study also included dogs treated with the BACE1 inhibitors NB-B4 (Novartis), NB-C8 (Novartis) or inhibitor S (Janssen). Dog CSF samples were analyzed for A $\beta$  isoforms.

### **Analytical methods**

Biomarkers were analyzed by enzyme-linked immunosorbent assays (ELISAs), fluorescent bead-based assays on the Luminex xMAP platform (Luminex Corporation, Austin, TX, USA), electrochemiluminescent plate-based assays on the Meso Scale Discovery platform (MSD, Meso Scale Discovery, Gaithersburg, MD, USA) and mass spectrometry-based assay.



## **ELISAs**

ELISAs in this dissertation were sandwich assays, where the specific antigen is immobilized through binding onto an immobilized capture antibody, and then bound to a biotinylated detection antibody, which binds to a streptavidin-enzyme complex. The enzyme (such as horseradish peroxidase) reacts with a chromogen to develop color. The color intensity is a measure of the antigen concentration in the sample.

Commercial ELISAs were used for A $\beta$ 1-42 (INNOTEST  $\beta$ -amyloid1-42, Innogenetics, Ghent, Belgium) to measure A $\beta$  containing both the 1st and 42nd amino acids [171], T-tau (INNOTEST hTAU Ag, Innogenetics) to measure all tau isoforms [172] and P-tau (INNOTEST PHOSPHO-TAU(181P), Innogenetics) to specifically measure tau phosphorylated at the 181<sup>st</sup> amino acid [173]. These assays use the monoclonal capture/detection antibodies 21F12/3D6, AT120/HT7 and BT2, and HT7/AT270, for A $\beta$ 1-42, T-tau, and P-tau(181), respectively. A previously developed in-house sandwich ELISA was used for NFL [174].

## **Fluorescent bead-based assays**

Bead-based multiplex assays allow simultaneous quantification of several antigens and saves sample volume and analysis time. The xMAP platform uses antibody coated beads coded with unique fluorescent colors, where each color code corresponds to a specific antibody. Several sets of beads may be mixed with one sample for multiplex analysis. Biotinylated antibodies are used for detection and bind to streptavidin molecules conjugated to fluorescent phycoerythrin. The beads are then assayed in a flow cytometry system, where one laser is used for bead identification based on the bead color and another laser for quantification based on the detection antibody-phycoerythrin complex.

The xMAP assay INNO-BIA AlzBio3 (Innogenetics) was used for simultaneous quantifications of A $\beta$ 1-42, T-tau, and P-tau. This assay uses the monoclonal capture/detection antibodies 4D7A3/3D6, AT120/HT7, and AT270/HT7, for A $\beta$ 1-42, T-tau, and P-tau(181), respectively. Despite differences in absolute measurement values, the AlzBio3 kit and the individual INNOTEST kits for A $\beta$ 1-42, T-tau and P-tau produce highly correlating values, and have similar diagnostic performance [175-177].

The xMAP assay INNO-BIA A $\beta$  forms (Innogenetics) was used for simultaneous quantifications of A $\beta$ 1-40 and A $\beta$ 1-42 (format A) and A $\beta$ X-40 and A $\beta$ X-42 (format B). Both formats use the monoclonal antibodies 21F12 and 2G3, which specifically bind A $\beta$  peptides ending at Ala42 and Val40, respectively, as capture antibodies. In format A, 3D6, which selectively binds

A $\beta$  peptides starting at A $\beta$ 1, was used as detection antibody, providing specific quantifications of A $\beta$ 1-40/42 isoforms. In format B, 4G8 (epitope within A $\beta$ 18-22) was used as detection antibody, providing quantifications of A $\beta$ X-40/42 isoforms.

#### **Electrochemiluminescence assays**

MSD assays allow multiplex reactions with high sensitivity using electrochemiluminescence for detection.

The MSD Human/Rodent Abeta Triplex assay was used for quantifications of A $\beta$ X-38, A $\beta$ X-40 and A $\beta$ X-42. This assay employs C-terminal specific antibodies to specifically capture A $\beta$ X-38, A $\beta$ X-40, and A $\beta$ X-42. All isoforms are detected by SULFO-TAG-labeled 4G8 antibodies.

The MSD sAPP $\alpha$ /sAPP $\beta$  Multiplex Assay was used for quantifications of sAPP- $\alpha$  and sAPP- $\beta$ . This assay employs the 6E10 antibody to capture sAPP- $\alpha$  and a neopeptide-specific antibody to capture sAPP- $\beta$ . Both isoforms are detected by SULFO-TAG labeled anti-APP p2-1 antibodies.

#### **Immunoprecipitation and mass spectrometry**

A $\beta$  peptides were analyzed by immunoprecipitation and mass spectrometry (IP-MS) by a method previously developed at our laboratory [178]. Anti-A $\beta$  antibodies coupled to magnetic beads were used for IP. After elution, A $\beta$  isoforms were analyzed by mass spectrometry on an UltraFlex extreme matrix-assisted laser-desorption/ionization time-of-flight/time-of-flight (MALDI TOF/TOF) instrument or an AutoFlex MALDI TOF (Bruker Daltonics, Bremen, Germany). An in-house developed MATLAB (Mathworks Inc. Natick, MA, USA) program was used for relative quantifications of A $\beta$  isoforms in the spectra. For each peak the sum of the intensities for the three strongest isotopic signals were calculated and averaged followed by normalization against the sum for all the A $\beta$  peaks in the spectrum. This method primarily allows relative quantification of different A $\beta$  isoforms. The ratio between the different isoforms detected cannot be interpreted as a direct reflection of their absolute or relative abundance since the ionization efficiency might be different for different isoforms and since different isoforms are more hydrophobic and less soluble than others.

#### **Liquid chromatography and tandem mass spectrometry (LC-MS/MS)**

A $\beta$  isoform identities were confirmed by liquid chromatography (LC) combined with high resolution tandem mass spectrometry (MS-MS) [178].

LC-MS/MS analysis was performed on an Ettan MDLC nanoflow chromatographic system (GE Healthcare) using HotSep Kromasil C4 columns (G&T Septeck) coupled to a Thermo LTQ-FT Ultra electrospray ionization hybrid linear quadrupole ion trap/Fourier transform ion cyclotron resonance (ESI-LQIT/FTICR) mass spectrometer (Thermo Fisher Scientific). All spectra were acquired in FTICR mode and collision induced dissociation (CID) was used to obtain fragment ion data.

### **Statistical analyses**

As the distribution of quantitative measures were significantly skewed, non-parametric statistical methods were used for most assessments. For comparisons of quantitative data, the Kruskal-Wallis test was used across multiple groups, and the Mann-Whitney U-test between pairs of groups. For comparisons of dichotomized data, Chi-square statistics with Fisher's exact test was used. The Wilcoxon test was used for pair-wise comparisons between related samples. The Spearman correlation coefficient was used for correlation analyses, if not otherwise stated. The significance level threshold was set to  $P < 0.05$ , if not otherwise stated. These general statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA), SPSS v.15 and PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

Measurements of diagnostic performance included sensitivity, specificity, area under the receiver operating characteristic curve (AUROC), predictive values (positive and negative, PPV and NPV) and likelihood ratios (positive and negative, LR+ and LR-). These measurements were determined using MedCalc for Windows, version 11.4.4.0 (MedCalc Software, Mariakerke, Belgium).

Logistic regression models were constructed for diagnostic classifications using SPSS v.15 and PASW Statistics 18.

Multivariate discriminant analysis was performed using the orthogonal projections to latent structures (OPLS) algorithm using SIMCA P+ (v. 12, Umetrics, Umeå, Sweden). This is based on finding directions in the multivariate orthogonal space spanned by assayed parameters (for example biomarkers) that best separates defined groups (for example diagnostic groups). The generated vectors may be used in ROC statistics to calculate diagnostic accuracy.

Analysis of variance was performed using the mixed procedure of SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA).

**Ethics**

All subjects or their proxies gave informed and written consent. The studies were approved by the ethics committee at the University of Gothenburg and the home institutions of collaborators in the different studies. The animal studies in paper V were conducted in accordance with local animal regulations and ethical approvals.

## **Backgrounds, study settings and main results**

### **Paper I**

#### **Background**

Promising data support CSF biomarkers as diagnostic tools in early-stage AD, but previous studies have been small and mainly conducted at single centers, and there is a lack of large-scale multi-center studies. We hypothesized that CSF biomarkers would also be useful to identify MCI-AD patients also in a large heterogeneous in a multi-center setting.

#### **Subjects and study settings**

The study included patients with MCI or AD dementia, and healthy controls, recruited at memory clinics in Europe and USA. The study was designed in accordance with the Standards for Reporting Diagnostic Accuracy (STARD) criteria [179, 180]. Using STARD terminology, a clinical AD dementia diagnosis constituted the reference standard. Biomarker cut-offs were defined in a cross-sectional part of the study in AD dementia patients (N=529) and controls (N=304), and evaluated as index tests in MCI patients (N=750) in a longitudinal prospective part of the study. MCI patients were followed annually for at least 2 years (median 3 years, range 2-11) or until a dementia diagnosis.

Dementia was diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) criteria [181]. AD dementia patients met dementia criteria and the criteria of probable AD defined by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria [20]. MCI patients met the criteria established by Petersen et al. [25, 26]. Vascular dementia patients met the dementia criteria [181] and the requirements of the National Institute of Neurological Disorders and Stroke–Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS-AIREN) [182] or Erkinjuntti et al. [183]. The McKeith et al. criteria were applied for dementia with Lewy bodies [184] and the criteria of Neary et al. for frontotemporal lobe dementia [185]. The control population consisted of volunteers without objective cognitive symptoms (MMSE >25) and no active neurological or psychiatric disease. CSF samples were analyzed for A $\beta$ 42, T-tau and P-tau by ELISAs (xMAP at two centers, with values converted to ELISA values based on previous

conversion formulas [186]). Substantial differences were seen across centers in biomarker levels, wherefore data was normalized according to levels in controls. The diagnostic performance of biomarkers was tested at cut-offs with 85% sensitivity for AD dementia. Logistic regression was used to construct analytical expressions of combinations of biomarker measurements.

### **Main results**

During follow-up, 271 MCI patients were diagnosed with AD dementia and 59 with other dementias. MCI-AD patients had lower CSF A $\beta$ 42 and higher T-tau and P-tau at baseline than other MCI patients. The AUROCs were 0.76-0.79 for the individual biomarkers. For a combination of the biomarkers, a cut-offs with 85% sensitivity for AD dementia in the original cross-sectional cohort had 83% sensitivity for MCI-AD, 72% specificity for other MCI patients, 62% PPV and 88% NPV.

## **Paper II**

### **Background**

To assess the optimal performance of CSF A $\beta$ 42, T-tau and P-tau, we aimed to study them in a well defined homogeneous mono-center population with careful standardization of clinical and laboratory procedures. We also studied the potential gain from adding additional A $\beta$  related CSF biomarkers. We hypothesized that the diagnostic performance of CSF biomarkers would be strong in this setting.

### **Subjects and study settings**

Participants were consecutively recruited cognitively impaired patients at a memory clinic (N=60) and healthy controls (N=20). All subjects were examined by the same physician and care was taken to standardize all clinical and laboratory procedures. Patients were followed annually for a median of 3 years (range 1-7), and had stable MCI (N=13), AD dementia at primary evaluation or follow-up (N=32), or other dementias at primary evaluation or follow-up (N=15). Diagnostic criteria were as in Paper I. CSF samples were analyzed for A $\beta$ 42, T-tau, P-tau, A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, s-APP $\alpha$  and s-APP $\beta$ . Statistical comparisons between diagnostic groups to assess multiple biomarkers were carried out by multivariate discriminant analysis using the OPLS algorithm.

### **Main results**

The core CSF biomarkers A $\beta$ 42, T-tau, and P-tau clearly diagnosed AD versus controls and stable MCI with AUROC 0.97. The additional tested biomarkers had no major effect on the diagnostic performance.

## **Paper III**

### **Background**

CSF A $\beta$ 42, T-tau and P-tau levels reflect distinct disease processes in the AD brain. Thus, their diagnostic performance might be affected by age-dependent prevalence of AD-like brain pathology in the general population [73]. We hypothesized that the apparent diagnostic accuracy would decrease with age, due to increasing prevalence of AD-like brain pathology in the elderly.

### **Subjects and study settings**

We utilized the multi-center study population described in paper I, including AD dementia patients (median age 71, range 43-89 years), controls (67, 44-91 years), and longitudinally followed MCI patients (69, 43-89 years). The study population was divided into three age cohorts, for comparisons among subjects aged up to 64 years, 65-74 years, and 75 years or older.

### **Main results**

Biomarker distributions differed with age within the diagnostic groups, which primarily caused age-dependent decreased specificity for non-AD subjects. In contrast, the PPV for a combination of biomarkers remained essentially stable, while the NPV decreased slightly in old subjects, as an effect of the high AD prevalence in older ages.

## **Paper IV**

### **Background**

Previous studies have reported good diagnostic performance for CSF AD biomarkers but differences in absolute measurements, thus making it difficult to compare studies or introduce universal cut-offs. To support standardization and implementation of biomarkers, an international QC program for CSF AD

biomarkers was created. The program is supported by the Alzheimer's Association and administrated from the Clinical Neurochemistry Laboratory in Mölndal. It monitors variability of CSF measurements and aims to identify sources of variability for measurements of A $\beta$  and tau. This paper describes the construction of the program and the results of the first two rounds of samples. We hypothesized that there would be a large variability for CSF AD biomarkers among laboratories.

### **Study settings**

The study included 40 laboratories using kits for A $\beta$  or tau. Aliquots of pooled CSF were prepared and distributed from Mölndal. Two rounds with three samples per round were sent out to participants. The blinded samples had different biomarker profiles. Five experienced laboratories assessed within-laboratory precision by running each sample multiple times. Data was reported back to Mölndal for statistical interpretation. Mean levels and CVs were calculated.

### **Main results**

The total coefficients of variation between the laboratories were 13-36 % for different biomarkers and analytical platforms. Within-laboratory precisions differed considerably among biomarkers within individual laboratories, suggesting that kit performance also contributes to the total variability.

## **Paper V**

### **Background**

Drugs aiming to reduce the brain A $\beta$  load include inhibitors and modulators of APP degrading enzymes. BACE1 is a key enzyme for A $\beta$  production and an attractive therapeutic target in AD [55, 187-189]. We hypothesized that BACE1 inhibition would induce a specific neuronal release of A $\beta$  peptides that could be detected in CSF.

### **Study settings**

We used several different BACE1 inhibitors on cell models and two different cohorts of dogs. Cell media and CSF samples were analyzed by immunoassays and IP-MS to simultaneously study a large number of different A $\beta$  isoforms in response to treatment.



### **Main results**

BACE1 inhibition consistently increased the relative intensities of A $\beta$ 5-40 in cell and animal models. Dogs on active treatment had clearly increased ratios of CSF A $\beta$ 5-40/A $\beta$ 1-34. These results may be useful in future development of drugs directed against BACE1.

## **Paper VI**

### **Background**

NPC is a progressive neurodegenerative disease. Abnormal APP/A $\beta$  metabolism has been reported in NPC brains [10, 101] and in disease models [190], but CSF biomarkers of amyloid and axonal pathology have so far not been studied in NPC patients. We hypothesized that the abnormal lipid membrane composition, the altered vesicular trafficking and the lysosomal dysfunction in NPC would influence APP/A $\beta$  metabolism in a way that could be monitored in CSF, and that NPC neurodegeneration could be monitored by CSF biomarkers of axonal pathology.

### **Subjects and study settings**

Participants were NPC1 patients (N=38) enrolled in a longitudinal observational trial at the NIH, USA. NPC diagnoses were established by biochemical testing and mutation analysis. Patients undergoing CSF collection for other indications were enrolled as controls (N=14). CSF samples were analyzed for A $\beta$ 42, T-tau, P-tau, A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, sAPP- $\alpha$  and sAPP- $\beta$ . Eighteen NPC patients were being treated with the glucosylceramide synthase blocker miglustat at the study start (Zavesca, Actelion Pharmaceuticals Ltd, Allschwil, Switzerland).

### **Main results**

CSF A $\beta$  levels were markedly increased in NPC patients, with a shift toward the A $\beta$ 42 isoform. NPC patients also had increased T-tau. Patients on miglustat had lower A $\beta$ 42 and T-tau than untreated patients.

## **Paper VII**

### **Background**

Amyloid metabolism is altered in neuroinflammation. CSF markers of amyloid pathology have not been studied in LNB, and there is only limited data on axonal damage markers in this disease [9]. We hypothesized that LNB patients would have altered CSF levels of amyloid and axonal pathology and that these would respond to antibiotic treatment.

### **Subjects and study settings**

LNB patients with acute facial palsy (N=19), Bell's palsy patients (N=42) and controls (N=22) were investigated in a cross-sectional study. An independent cohort of LNB patients (N=26) were investigated with serial CSF samples in a longitudinal study to evaluate treatment effects. LNB was diagnosed by clinical symptoms, the presence of an inflammatory CSF profile and supportive findings. CSF was analyzed for A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, sAPP- $\alpha$ , sAPP- $\beta$ , T-tau, P-tau and NFL.

### **Main results**

LNB patients had lower CSF levels of sAPP- $\alpha$ , sAPP- $\beta$  and P-tau and higher levels of NFL than healthy controls and patients with Bell's palsy. In the prospective study, the low CSF sAPP- $\alpha$ , sAPP- $\beta$  and P-tau at baseline all increased towards normal at follow-up after treatment with antibiotics.

## Results and discussion

### CSF biomarkers for AD in multi-center studies

CSF biomarkers may achieve consensus recommendations of at least 85% sensitivity [191] even at an early clinical stage [4, 158]. However, mono-center studies may suffer from positive bias, and there is a lack of large-scale studies in heterogeneous settings. Paper I presents the largest study published so far on CSF biomarkers in early-stage AD. Of 750 MCI patients, 271 developed AD with dementia and a further 59 developed other dementing diseases during at least 2 years of follow-up. MCI-AD patients had lower CSF A $\beta$ 42 and higher P-tau and T-tau baseline values compared to other MCI patients. Following the STARD criteria, biomarker cut-offs were constructed in an independent cohort of AD dementia patients and controls with 85% sensitivity for AD dementia, and evaluated in the MCI cohort for MCI-AD, where they produced 79-86% sensitivity, 47-65% specificity, and 0.76-0.79 AUROC, confirming their high diagnostic accuracy for early-stage AD. A combination of A $\beta$ 42/P-tau ratio and T-tau with 85% sensitivity for AD dementia had 88% specificity for healthy controls, 83% sensitivity for MCI-AD, 72% specificity for other MCI patients, and 62% positive predictive value and 88% negative predictive value in the MCI cohort.

At the time of publication of Paper I, two other multi-center studies were also published. In the DESCRIPA study, with 193 MCI and subjective cognitive impairment patients [192], a previously defined CSF A $\beta$ 42 and T-tau AD biomarker profile had an odds ratio of 27 (95% CI 1.6–460) for MCI-AD in amnesic MCI, and 31% of controls (N=89) also had this biomarker profile [193]. In an Alzheimer's Disease Neuroimaging Initiative (ADNI) report including 196 MCI patients followed for 1 year, 87–89% of MCI-AD (N=37) and 88%–91% of mild AD dementia patients (N=100) had a CSF AD biomarker pattern (cut-offs from an independent set of autopsy confirmed cases, with accuracies 70-87%), and 34%–38% of controls (N=114) also had this pattern [194].

Together, these multi-center studies confirm that CSF biomarkers for AD have high diagnostic performance at an early clinical stage, especially regarding MCI-AD sensitivity, while specificities are generally lower. This is in accordance with findings of AD-type brain pathology in a large proportion of elderly with no documented cognitive decline [73], where it is unclear how many that will eventually deteriorate.

### **CSF biomarkers for AD in mono-center studies**

There are several possible reasons for the somewhat lower diagnostic performances in multi-center than in certain mono-center studies, including inter-center or inter-laboratory variability, and short follow-up time that prohibits verification whether all stable MCI cases really would have had a benign course also with longer follow-up. In the mono-center study described in Paper II, a homogeneous patient population was included and all clinical and laboratory procedures were carefully standardized. The diagnostic performance of the biomarkers for AD versus controls (including stable MCI) was among the highest ever reported, with a combination of CSF A $\beta$ 42, T-tau, and P-tau achieving an AUROC level of 0.97. Adding additional biomarkers related to APP/A $\beta$  metabolism (A $\beta$ X-38, A $\beta$ X-40, A $\beta$ X-42, sAPP- $\alpha$ , sAPP- $\beta$ ) had no major impact on the differentiation of AD versus controls/stable MCI, demonstrating the excellent potency of the core CSF biomarkers to identify AD pathological processes when a stringent protocol for clinical management and analyses is used. However, although AD patients did not differ from other dementia patients in individual CSF A $\beta$  peptide measurements, they had elevated CSF A $\beta$ X-38/A $\beta$ X-42 and A $\beta$ X-40/A $\beta$ X-42 ratios, suggesting that an extended CSF A $\beta$  pattern analysis still may be clinically useful to increase the diagnostic precision towards other dementias. AD patients with additional vascular pathology (N=7) did not differ in CSF biomarkers from remaining AD patients, arguing against a large influence from vascular pathology on the biomarkers' diagnostic accuracy in AD. The available data on CSF biomarkers is promising for clinical application in AD, but detailed studies including several diagnostic modalities are needed to elucidate how to optimally use combinations of biomarkers, and in which subgroups of MCI patients that they add most information to the clinical examination [195].

### **The influence of age on biomarker potential**

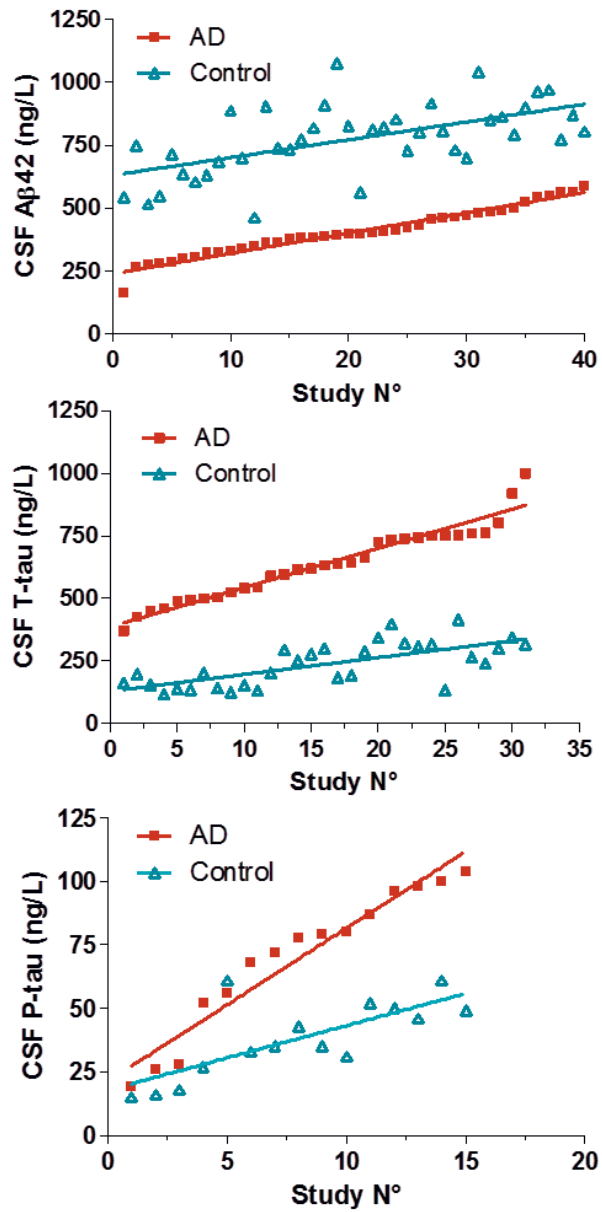
An in-depth analysis of CSF biomarker distributions revealed increasing overlap between AD patients and non-AD patients with age (Paper III). AUROCs for the biomarkers decreased with age, primarily due to age-dependent higher prevalence of AD-like biomarker patterns in non-AD persons. This probably reflects age-dependency in brain pathology and illustrates that late-onset AD forms a continuum towards normal aging, similar to other age-related diseases, such as atherosclerosis and certain cancer forms. AD-like brain pathology in elderly with no documented cognitive decline may suggest very-early-stage AD. In any event, they indicate that AD-like brain pathology *per se* is insufficient for clinical

disease, and that other factors such as disease duration, cognitive reserve capacity or cerebral co-morbidities modulate symptom onset. Only long term follow-up studies in cognitively stable subjects may fully resolve this issue.

Young AD patients had more extremely shifted CSF A $\beta$ 42 levels than older AD patients. Such a difference is in agreement with previous findings of differences between early- and late-onset AD, including differences in neuropathology [196] and earlier reports of CSF biomarker profiles [171]. This may point to differences in disease-mechanisms between different forms of AD. Generally, late-onset SAD is thought of as a heterogenic and polygenic condition, which is clinically influenced by brain co-morbidities, especially vascular disease. In contrast, some early-onset SAD cases may have autosomal recessive inheritance [197] and be less influenced by age-associated vascular pathology. Also, some SAD patients have very rapid disease progression, suggesting distinct or aggravated disease mechanisms [19]. It is still not fully resolved how autosomal dominant early age-at-onset FAD cases relates to SAD, but it is noteworthy that the FAD-causing genes *APP*, *PS1* and *PS2* are not major susceptibility genes for SAD [198], and that even the development pattern of neuropathological changes may be different in some FAD cases [40].

### **Variability of CSF biomarkers**

Studies have reported varying absolute biomarker levels in AD patients and controls, but with robust relative differences between diagnostic groups (Figure 9). One of the findings in Paper I was an inter-site variability in CSF biomarkers that required normalizations. Such variability across (highly specialized) centers highlights a need for standardization of both analytical techniques and clinical procedures. A first step towards this is the establishment of a program to systematically monitor the variability. In the QC program study (Paper IV and Figure 10), 26 participating laboratories used INNOTEST ELISAs, 14 used Luminex xMAP with the INNO-BIA AlzBio3 kit, and 5 used Meso Scale Discovery with the A $\beta$  triplex or T-tau kits to measure biomarkers for AD. The total inter-laboratory CVs were 13-36 %. Five laboratories analyzed the samples 6 times on different occasions. For these, within-laboratory CVs (2-19%) differed considerably between biomarkers within individual laboratories, suggesting influence by assay-related factors.



**Figure 9.** Variability among studies. Average levels of biomarkers in AD patients and controls in 40 studies using INNOTEST ELISAs. Adapted from supplementary figure 1, Paper IV.



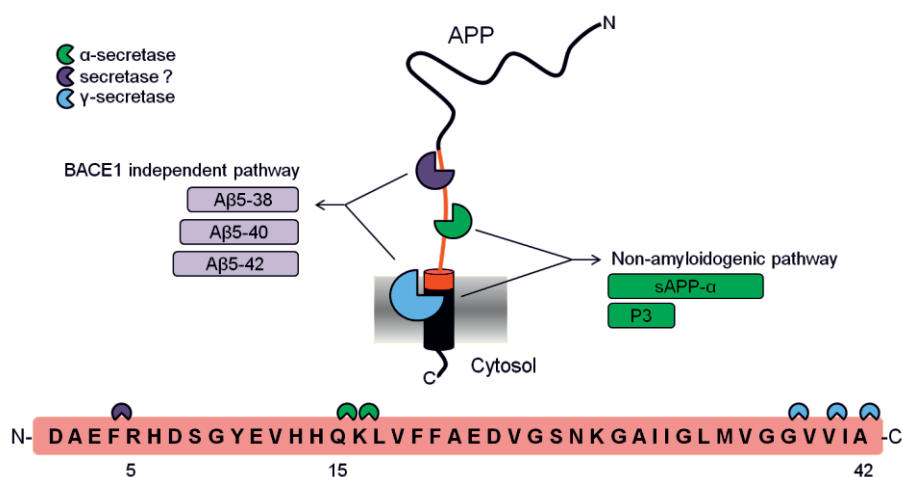
**Figure 10.** Participants in the Alzheimer's Association external QC program, in the latest completed round of the program (round 6, N=70).

The results in paper IV were in agreement with previous smaller inter-laboratory surveys. Lewczuk et al. found inter-laboratory CVs 21-38% in a survey of 14 laboratories, but intra-assay CVs were usually below 5%, suggesting that differences between laboratories and/or analytical kits were responsible for most of the total variability [199]. Verwey et al. found inter-laboratory CVs 31-37 % for A $\beta$ 42 ELISAs in a survey of 20 laboratories, while CVs for T-tau and P-tau were 13-21 %, and intra-laboratory CVs over time were 7-25 % [200]. Shaw et al found inter-laboratory CVs 13-18 % and intra-laboratory CVs 5-11 % for A $\beta$ 42, T-tau and P-tau at 7 labs using the xMAP AlzBio3 kit [201]. Possible causes of the variability among CSF biomarker studies and within QC programs are pre-analytical factors (including patient selection or differences in sample handling and storage), laboratory procedures and factors related directly to the assays (including differences between individual immunoassays and batches of kits) [5, 202]. The Alzheimer's Association QC program now includes check-lists to pinpoint differences in analytical procedures between laboratories, which will be analyzed in an upcoming study. Other ongoing initiatives aim at standardizing pre-analytical procedures. Major efforts are also being made by kit manufacturers to provide more robust analytical kits with minimized variability. Clinically established CSF parameters, including albumin and immunoglobulin levels, often reach inter-laboratory CVs below 10-15%. Such low variability would increase the usefulness of CSF biomarkers both in research and in clinical settings. To achieve this, the biomarker community may need to go even further and construct certified reference materials and methods for CSF biomarkers, which has been important in the corresponding standardization of clinical chemistry tests for serum biomarkers [203, 204].

## CSF pharmacodynamic biomarkers

Clinical trials for disease-modifying treatments in neurodegenerative diseases are hampered by the very nature of these diseases. Beneficial drug effects are difficult to detect due to slow progressions and long lag phases between pathological changes and clinical signs, as well as difficulties in staging and scoring of disease severity. It might therefore be useful, not having to rely only upon clinical endpoints of treatment effects, but to balance these with biomarkers that reflect a beneficial intervention [205]. In paper V, BACE1 inhibition had a specific effect on the CSF A $\beta$  peptide pattern, with reduced A $\beta$ 1-34 and increased A $\beta$ 5-40 levels, which were more sensitive measurements than effects on A $\beta$ 1-40 and A $\beta$ 1-42. For comparison,  $\gamma$ -secretase inhibitors increase CSF A $\beta$ 1-14, A $\beta$ 1-15 and A $\beta$ 1-16 [82], while A $\beta$ 1-40 and A $\beta$ 1-42 levels are less affected [206]. CSF A $\beta$ 1-40 and A $\beta$ 1-42 may be less suitable as sensitive pharmacodynamic markers also for BACE1 inhibition. A $\beta$ 5-40 and A $\beta$ 1-34 may therefore be useful in future drug trials as sensitive markers of drug effects in patients.

The findings also points to *in vivo* activity of an alternative pathway of APP processing (Figure 11). The shift towards A $\beta$ 5-40 is consistent with previous reports on A $\beta$ 5-40 being resistant to BACE1 inhibition [57, 207] and the sharp decrease in A $\beta$ 1-34 is consistent with findings that both cuts, at position 1 and position 34, depend on BACE1 [208, 209]. The enzyme responsible for the cut at position 5 is still unidentified, but might be linked to the  $\alpha$ -secretase pathway [207].



**Figure 11.** BACE1 independent APP degradation, releasing A $\beta$ 5-X peptides, which are measurable in CSF.



### **CSF biomarkers in NPC**

NPC shares intriguing features with AD, including exacerbation by *APOE ε4* [101], lysosomal dysfunction [210, 211], lipid dysregulation [212, 213] and tangle pathology [41, 214]. In its adult form, NPC may be a clinical differential diagnosis to AD. The NPC1 protein is upregulated in brains of AD patients and transgenic AD mice, which might be related to altered cholesterol metabolism [215], and some reports suggest increased A $\beta$  production in NPC [10, 101]. The study in paper VI is the first on CSF A $\beta$  and tau in NPC patients. The increased CSF A $\beta$  levels in combination with unaltered CSF sAPP- $\beta$  are consistent with increased  $\gamma$ -secretase-dependent A $\beta$  release. Furthermore, the increase in A $\beta$ 42 relative to A $\beta$ 40 and A $\beta$ 38 suggest a shift towards A $\beta$ 42 release in NPC patients. Patients with high disease severity scores had lower CSF A $\beta$  and sAPP- $\beta$  levels (closer to control values), which might have been due to a reduced number of neurons, neuronal dysfunction or brain sequestration of A $\beta$  in advanced disease. The elevated CSF A $\beta$  level is a unique finding since it has not been found in other disorders. Other lysosomal diseases need to be investigated to determine if this abnormality is specific to NPC or present in additional lysosomal diseases.

### **Amyloid metabolism in NPC**

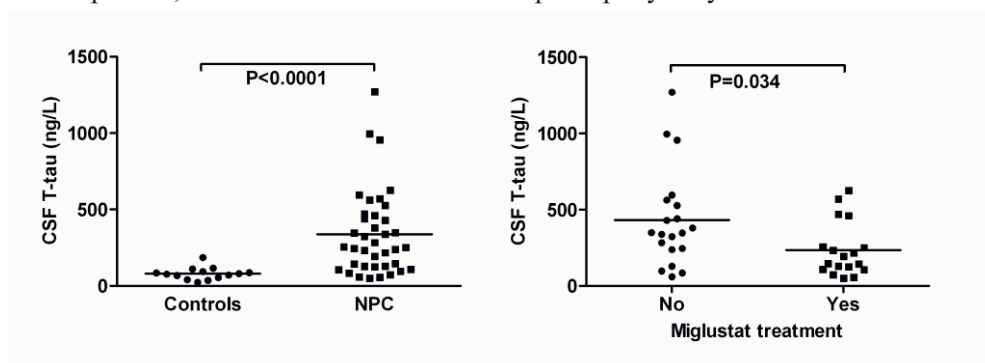
The results in paper VI add to previous (sometimes contradictory) evidence of abnormal APP/A $\beta$  metabolism in NPC. It has been reported that NPC mice neurons have increased  $\gamma$ -secretase activity, and accumulate A $\beta$ 40, A $\beta$ 42 and C99 (but have normal mRNA and protein levels of APP, BACE1 and presenilin-1) [10, 190, 216-218]. NPC1-null CHO cells accumulate CTFs in lipid rafts and have increased release of sAPP- $\beta$  [219, 220]. U18666A (3- $\beta$ -[2-(diethylamino)-ethoxy]androst-5-en-17-one) treatment is used in cell models to mimic NPC cholesterol accumulation (it inhibits NPC1 function and blocks cholesterol transport from late endosomes/lysosomes to the endoplasmic reticulum) [221]. U18666A has been described to reduce release of A $\beta$ 40 and A $\beta$ 42 from rat cortical neurons, mouse cortical neurons and SH-SY5Y cells [218, 222, 223] (but not from CHO cells [190]); increase intracellular accumulation of A $\beta$ 40, A $\beta$ 42 and  $\beta$ -CTF [10, 190, 223]; increase APP levels at the cell surface, increase  $\alpha$ -secretase cleavage and reduce  $\beta$ -secretase cleavage [222]; cause presenilin-1 to accumulate in vesicular organelles involved in cholesterol sorting [218]; and increase expression of both presenilin-1 and presenilin-2 [224]. Most of these effects are possible down-stream results from inhibited membrane trafficking. We recently found that SH-SY5Y cells treated with U18666A release increased relative levels of

the peptides A $\beta$ 5-38, A $\beta$ 5-40 and A $\beta$ 5-42 (manuscript in preparation), corresponding to the BACE1 independent peptides described in paper V. It is not known if the altered APP/A $\beta$  metabolism found in patients and different model systems contribute to pathology in NPC.

### CSF tau as a marker of axonal pathology in NPC

The increased levels of CSF T-tau in NPC patients support the usability of CSF T-tau as an axonal degeneration marker across neurodegenerative diseases [225]. Patients on miglustat had lower CSF T-tau, suggesting that this treatment had interfered with axonal degeneration (Figure 12). This is consistent with clinical effects on neurological symptoms from this drug (although the present study was not a randomized trial) [226, 227]. Also, in a follow-up study where serial samplings were carried out on a subset of the patients in paper VI, treatment start reduced CSF T-tau levels [228].

CSF P-tau levels were normal in NPC patients. This might come as a surprise since NPC patients often present NFTs, but CSF P-tau is usually normal in tauopathies, and increased levels are seen principally only in AD.



**Figure 12.** NPC patients had increased CSF T-tau levels. Patients on miglustat treatment had lower levels than untreated patients.

### Links between AD and NPC: APP/A $\beta$ and the fat connection

NPC is characterized by pathological accumulation and distribution of different lipid species. Lipid dysregulation is closely linked to A $\beta$  pathology, as suggested by the frequent brain A $\beta$  deposits in non-demented patients with ischemic heart disease [229], the risk for AD cause by hypercholesterolemia in epidemiological studies [230], the AD susceptibility genes related to lipid metabolism, the possible effects of cholesterol-lowering statin treatment on APP/A $\beta$  metabolism [231-233] and correlations between dyslipidemia and

plaque pathology (but not NFTs) [234]. The key APP-processing enzymes  $\gamma$ -secretase and BACE1 are affected by the lipid composition of cellular membranes, in particular the cholesterol content [212], but clinical trials with cholesterol-lowering treatments have produced ambiguous results [232, 233, 235, 236]. Future studies combining CSF biomarkers of lipid dysregulation and A $\beta$  metabolism may untangle these molecular relations *in vivo* in patients.

### **Links between AD and LNB: APP/A $\beta$ and neuroinflammation**

Neuroinflammation is a prominent feature of AD, where activated or dysfunctional microglia are associated with neuritic plaques [237]. Studies of the relations between A $\beta$  metabolism and inflammation suggest that there is an extensive cross-talk between A $\beta$  production and inflammatory regulation [238], but the precise role of inflammation in AD in human patients is still unclear [239]. In paper VII, LNB patients had reduced levels of CSF sAPP- $\alpha$  and sAPP- $\beta$  compared with healthy controls and Bell's palsy patients, and these measurements increased towards normal after antibiotics treatment. Together, studies on different neuroinflammatory diseases point to general effects of neuroinflammation on APP metabolism, with reduced CSF sAPP- $\alpha$  and sAPP- $\beta$  levels [105-108]. One possible explanation for this is increased degradation due to inflammatory proteolysis or phagocytosis. The physiological functions of different sAPP species in the brain are unclear but sAPP- $\alpha$  has been attributed with neuroprotective properties [240]. A neurotoxic N-terminal APP-fragment located within the sAPP-sequence may induce axonal degeneration through interaction with axonal death receptor 6 (DR6) receptors [241]. Further studies could explore if sAPP- $\alpha$  or sAPP- $\beta$  are metabolized to DR6-activating peptides in neuroinflammation. Regarding markers of axonal pathology, the increased CSF NFL in LNB described in paper VII is consistent with earlier reports of white matter damage in this disease [102]. Hypothetically, the reduced CSF P-tau levels may have been due to disease-linked effects on neuronal kinases or phosphatases, and it is noteworthy that P-tau levels were shifted towards normal after treatment.

## **Concluding remarks and outlook**

The findings presented in these studies show that CSF biomarkers of amyloid and axonal pathology have high diagnostic performance for AD, even in early stages. In particular, the diagnostic accuracy is high when careful standardization is done for clinical and laboratory procedures. CSF biomarker distributions vary with age, probably reflecting age-dependent differences in AD-like brain pathology. An external QC program has been launched to monitor and, hopefully, help to reduce variability and facilitate global implementation of CSF biomarkers in AD diagnostics. CSF biomarkers may detect CNS drug effects *in vivo*, and be used to detect both disease-specific and disease-converging amyloid and axonal pathology in different medical conditions.

### **Early-stage testing for AD: ethical considerations**

Until disease-modifying therapy for AD is available, there are few strong arguments for biomarker testing in MCI. As in other medical practices, any testing should be preceded by a thorough discussion with the patient on possible consequences and interpretations of the test. A result indicating AD may cause feelings of hopelessness, agony and despair. There might even be an increased risk of suicide in dementia, although it is unclear if this is linked to the stigma of the diagnosis or caused by mood disorders secondary to the disease itself [242]. On the other hand, a correct early diagnosis may be clarifying and appreciated by patients, even without the existence of disease-modifying treatment. A correct diagnosis allows informed planning for the future, as in other diseases without efficient treatment, such as incurable cancers. An early diagnosis may also support early start of symptomatic treatment. Ultimately, each patient deserves a personal ethical analysis before disclosing a diagnosis, and it is crucial to involve the patient in this process [243].

### **The future of AD treatment: a role for CSF biomarkers?**

There is still no disease-modifying AD therapy, but there is room for optimism. For example, positive reports have come from studies on the A $\beta$  immunotherapy bapineuzumab, with beneficial effects on clinical symptoms and A $\beta$  pathology measured by Pittsburgh compound B (PiB) PET, and a trend towards reduced CSF P-tau levels [7, 244, 245]. In the long run, drugs are most likely to achieve a clinically meaningful outcome in early-stage AD. The importance of early intervention against A $\beta$  pathology is supported by

animal studies [246, 247], but for a clinical response, the precise importance of early intervention will depend on the exact role of A $\beta$  in the disease cascade. If an initial A $\beta$  dysregulation triggers a pathological cascade that continues independently of brain A $\beta$  load, early intervention is crucial [7]. Biomarkers could help to identify windows of reversible pathology. One group to consider for future interventions might be patients with low CSF A $\beta$ 42 but no amyloid PET binding, if these are shown to have early-stage AD in follow-up studies, since such a biomarker pattern might indicate very early alterations in APP/A $\beta$  metabolism [163]. Wide-spread clinical diagnosis and treatment of pre-symptomatic AD, pose an enormous challenge, since applied screening tools must have extraordinary predictive power and treatments must be safe and relatively inexpensive. Any preclinical screening method requiring amyloid PET scans presently fails at broad-scale implementation due to insufficient availability of scanning equipment for the vast number of eligible possible AD patients.

### **The outcome of treatment**

One may conceive of at least two outcomes of an effective disease-modifying AD treatment. The best possible scenario is full prevention of neurodegeneration and total rescue of cognitive functions. A poorer, but more realistic scenario is delayed disease progression, with unclear benefits in individual patients, and uncertain prognosis of life prolongation and degree of disability. The latter scenario is comparable to other age-related disorders, such as atherosclerosis, where a large number of patients are treated with anti-hypertensive or anti-coagulant drugs to prevent a vascular insult in one single patient. Biomarkers identifying likely treatment responders would be valuable, especially considering potential side-effects and high cost of disease-modifying AD therapies.

Several AD drugs are now in clinical trials. One danger with the large number of ongoing trials is the risk for random positive results, which scientists must bear in mind when communicating with patients, relatives and lay media. Ultimately, the nature of the trials needed to prove beneficial effects in AD may require a review of drug development procedures (perhaps including patent durations) and support strong collaborations between industry, academia and regulators. As explained in this work, CSF biomarkers can play several roles in this complicated effort.

### **Future directions**

CSF biomarkers are being continuously developed for different purposes. Efforts are devoted to validation, standardization and implementation of established biomarkers, and identification of novel ones. Targeted and general proteomics and peptidomic approaches hold great promise for future biomarker discovery. For clinical usefulness in AD, such ventures should probably aim at very specific applications and not only plain diagnostics, since the core AD biomarkers already achieve high diagnostic performance if utilized correctly. However, there is a lack of biomarkers for disease progression, markers identifying specific disease stages, and markers predicting responsiveness to therapy, to name but a few applications that could be specifically pursued. Biomarker discovery may also be achieved in other body fluids, primarily blood. Novel ultra sensitive techniques may allow quantification of neuronal components in blood with such precision as to have clinical impact, and alternative molecules may be investigated, such as autoantibodies in blood [248]. Aiming beyond immediate clinical usefulness, biomarkers will continue to provide unique opportunities to increase our understanding of brain mechanisms in health and disease.

## Acknowledgments

This dissertation is the work of many people. I am especially grateful to:

My main supervisor: **Professor Henrik Zetterberg**. Your scientific curiosity and your energy, enthusiasm and generosity have been enormously inspiring. Thank you for the opportunity to work with you and your group. I guess Louis Pasteur was thinking about you when he said that “To be astonished at anything is the first movement of the mind toward discovery” (although probably in French). You have been a brilliant supervisor.

My co-supervisors: **Professor Kaj Blennow** for excellent scientific leadership, enlightening discussions and guidance in both research and clinical work. I am also grateful for your trust in me to represent the laboratory at many occasions. **Johan Gobom** for never-ending enthusiasm, patience when I was devoted to clinical issues and faster-than-light sense of humor, which saved me from boredom more often than you know.

All co-authors and present and previous co-workers at the Clinical Neurochemistry laboratory and the rest of the Department of Psychiatry and Neurochemistry: **Erik Portelius**, my friend and mentor, your input was absolutely essential, **Ulf Andreasson** for journeys into multidimensional space, **Gunnar Brinkmalm** for equally brilliant comments on mass spectrometry and Resistance strategies and **Jan-Eric Månsson** for sharing your deep knowledge of biology and chemistry, you made me a believer in the lysosome! **Dzemila Secic, Maria Olsson, Mikael Gustavsson, Monica Christiansson, Sara Hullberg and Åsa Källen** for outstanding experimental work. **Staffan Persson** for being a superb coordinator and for sometimes sneaking up behind me and staring like a madman. **Shirley Fridlund** for excellent experimental work and help with language review. **Ulla Rüetschi, Inger Fagerberg Blixter, and Susanne Li** for supervising my first stumbling steps in the laboratory. **Rita Persson** for being a wonderful roommate. All other co-workers for creating a scientifically exciting atmosphere, especially: **Alexandra Abramsson, Ann Brinkmalm, Annika Lekman, Annika Öhrfelt, Annika Sjölander, Annika Thorsell, Bob Olsson, Bozena Zayer, Gösta Karlsson, Joel Jakobsson, Josef Pannee, Kerstin Andersson, Lena Olvén-Andersson, Magdalena Nutu, Malin von Otter, Maria Bjerke, Mariann Wall, Mikko Hölttä, Petronella Kettunen, Rolf Ekman, and Ronald Lautner**. All at the section for neurometabolic diseases, including **Maria Blomqvist, Eva Johansson, Karin Jansson, Kazemieh Magedian, Elmira Fatigh, Linda Duphorn, Irene Rasmusson,**

**Britt-Marie Rynmark and Eva Lena Ackers. Ann-Charlotte Hansson, Monica Olsson, Ulla Ohlson, Mari Klaesson, and Inger Almgren** for practical and administrative help.

All other co-authors, especially: **Anders Wallin** and the staff at the Sahlgrenska University Hospital Memory Clinic for a good collaboration, and for welcoming me during clinical rotations. **Lennart Minthon, Oskar Hansson** and co-workers for welcoming me in Kirseberg in 2007 and a good collaboration since. **Per Johansson and Johan Svensson** for the rewarding GDHEM collaboration and further contacts. **Lars Hagberg and Daniel Bremell** for the exciting work on infectious diseases. **Mony de Leon** for a warm welcoming in NYC in 2008. **Leslie Shaw** for many good discussions. **Maria Carrillo and the rest of Alzheimer's Association** for your kind and enthusiastic support. **Forbes D Porter** for the unique opportunity to study NPC biomarkers with you and your team, and **Chris Hempel** for the support of the NPC studies. All the collaborators in the BACE1 project, especially: **Lawrence Rajendran, Johan Lundkvist, Fredrik Jeppsson, Laura Jacobson, Ulf Neumann and Marc Mercken.**

**Steven Walkley and Nenad Bogdanovic,** for kindly sharing images included in this dissertation.

Indirect contributors are people that I have worked with in previous or present projects not included in the dissertation, especially: **Oluf Andersen,** thank you for introducing me to the realm of neurology in 2005, this work would not have happened without your support. **Lars Rosengren, Radu Constantinescu, Björn Holmberg, Claes Malmström, Markus Axelsson, Sara Haghigi, Albert Hietala, Niklas Darin and Peter Berglund** for productive team works in research, clinical issues and teaching. My previous scientific tutors **Martin Garwicz** for introducing me to motor system development, **Einar Björnsson** for guidance through the mysteries of the gut, and **Rogan Tinsley** for training in immunohistochemistry and excitotoxicity. **Katarina Kågedal, Caroline Hastings, Silva Hecimovic, Edyta Merisson, Michael Schöll, Mats Stridsberg Karin Sävman, Charles Vite and Gustaf Österlundh** for other scientific collaborations.

I have learnt much from the enthusiastic students that I have supervised during the last years, especially: **Kristin Augutis, Karin Nagy, Pashtun Shahim, Shahrzad Tabatabaei, and Christoffer Rosén.** I am also grateful to all the other students that I have met during my teaching at the University



of Gothenburg and the Sahlgrenska University Hospital, for many challenging questions and discussions.

In parallel with conducting this doctorate, I was a resident doctor in clinical chemistry/clinical pathology at the Sahlgrenska University Hospital. I would like to acknowledge all my clinical colleagues, especially **Professor Anders Lindahl** for your supportive attitude towards research.

Friends may become co-workers. **David Brax**, thank you for helping me penetrate the ethical dilemmas of dementia, **Erik Rosén** for clarifying both logistic regression and kip-ups, **Ina Skagervik** for the collaboration on intracranial bleeding diagnostics and your support in the neurochemistry course 2011, **Lisa Mouwitz** for digging up ancient scrolls of lumbar punctures, and **Andreas Pikwer** for uniting A $\beta$  metabolism, sedatives and semi-abstract coloration of my home.

Min familj och alla vänner utanför hjärnsjukdomarnas värld, som har gett mig den energi som har behövts för dessa djupdykningar i likvor! Ni vet vilka ni är, men särskilda tack för all uppmuntran och stöd till **mina föräldrar, Britta, Dag, Liv, Per, Henrik, Simon, Ella, David S, Ellis, Erik E, Erik F, Jakob E, Johanna D, Johanna E, Johannes, Kerstin och Jakob, Linnea, Lovisa, Maria B, Martin, Mitra, Niklas F, Petter, Sam, Tette, Gösta, Tobias, Ulla, SEG och Viktor!** Ni är fantastiska!

**Lisa**, du har det största hjärtat i världen och har stöttat mig något otroligt i det här arbetet. Tack för all hjälp och all kärlek. <3 <3 <3

Finally, I want to express my deepest gratitude to **all the patients and other participants** in these studies. This work is dedicated to you.

During the preparation of this dissertation, I received financial support from Sahlgrenska University Hospital, Sahlgrenska Academy, the Lundbeck Foundation, Stiftelsen Psykiatriska Forskningsfonden, Stiftelsen Gamla Tjänarinnor, Uppsala Universitets Medicinska Fakultet stiftelse för psykiatrisk och neurologisk forskning, Swedish Brain Power, the Swedish Brain Fund, Göteborgs läkaresällskap, Svenska läkaresällskapet, Neurologiskt Handikappades Riksförbund, Svenska kemistsamfundet, Anna-Lisa och Bror Björnssons stiftelse, Thuréus stiftelse, Unilab travel grant, Pfannenstills stiftelse, Lars Hiertas minne, Demensfonden, Sweden and Alzheimers's Association.

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