The Postsynaptic Protein Neurogranin:

A New Item in the Alzheimer's Disease Biomarker Toolbox

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia affecting more than 50 million people worldwide today and is characterised by progressive cognitive decline. One of the earliest events in AD, which is also closely related to neuronal loss and the degree of dementia, is synaptic degeneration. The degree of dementia has been found to correlate better with synaptic loss compared to other neuropathological changes, such as plaques and tangles. Synaptic proteins are therefore highly suitable as biomarkers for AD, possibly also for diagnosis, even at early stages.

The aim of this thesis was to characterise the postsynaptic protein neurogranin (Ng) in cerebrospinal fluid (CSF), plasma, and brain tissue, develop methods for quantification of Ng as well as to test the hypothesis that Ng in CSF and plasma is a possible biomarker for AD. Using hybrid-immunoaffinity mass spectrometry (HI-MS) 15 endogenous Ng peptides in CSF, 16 in plasma and 39 in brain tissue were identified. Based on the peptide profiles, it seems that there are most likely two separate pools of Ng; one derived from the central nervous system (CNS) and one from the periphery. In particular, Ng peptides ending at amino acid 76 were specifically detected in the CNS but not in the periphery, and the levels of the specific peptide Ng48-76 was increased in CSF from sporadic AD (sAD) patients in two separate cohorts. While plasma Ng was not significantly altered in sAD compared to controls, CSF Ng quantified by immunoassays was increased in sAD in multiple independent cohorts. In addition, CSF Ng was also increased in patients with mild cognitive impairment that progressed to AD, thus showing that Ng can be used to detect AD even at early stages. Furthermore, when comparing CSF Ng across eight different neurodegenerative diseases, including Parkinson's disease, frontotemporal dementia, and sAD, CSF Ng was only increased in patients having AD pathology. Increased CSF Ng in sAD was also demonstrated in autopsy-confirmed cases. Finally, Ng in both CSF and brain tissue was found to correlate very well with the degree of neuropathological changes, thus showing that there is a close relationship between Ng and disease-specific changes in AD.

Examination of Ng in brain tissue revealed that the concentrations of several Ng peptides were increased in relationship to full-length Ng, in both sAD and familial AD compared to controls and individuals that are cognitively intact but have developed AD pathology. These data indicated a shift from full-length Ng to Ng peptides in AD, demonstrating that the formation of Ng peptides in brain tissue might be connected to AD-related synaptic degeneration leading to cognitive decline. The increase of peptides in brain tissue is most likely what causes the mirrored increase of CSF Ng as well.

In conclusion, the work included in this thesis has shown that the postsynaptic protein Ng is a CSF biomarker for AD, even at early stages, and that it also is specific for sAD compared to other major neurodegenerative diseases. The increase of Ng in sAD CSF is most likely caused by elevated levels of Ng peptides being produced in the brain as a result of synaptic degeneration. Thus, Ng is indeed a new, and useful, item in the AD biomarker toolbox.

Keywords: Alzheimer's disease, biomarker, neurogranin, CSF, brain tissue

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SAMMANFATTNING PÅ SVENSKA

Alzheimers sjukdom (AD) är den vanligaste typen av demens och har blivit en av vår tids största folksjukdomar med över 100 000 drabbade enbart i Sverige. Då de kliniska symptom och minnestester som idag används för att ställa diagnos delvis är för ”trubbiga” för att identifiera patienter med lindrig minnesstörning, vilka ännu inte har fullt utvecklad AD, eller individer med mycket tidig AD, är behovet av mer exakta metoder stort.

Ett av de tidigaste fynden i AD är skador på och förlust av nervceller och synapser, vilket leder till minnesstörningar. Synapser är kontaktpunkterna mellan nervceller, där minnen skapas, överförs och lagras, och dessa känsliga kopplingar är mycket sårbara för de förändringar som sker vid AD. Synapserna är till stor del uppbyggda av unika proteiner som bara finns just där och många av dem går att mäta i ryggvätska (även kallad cerebrospinalvätska eller likvor), vilken är en färglös vätska som omger hjärnan och bl.a. skyddar den från stötar samt transporterar bort restprodukter. Eftersom ryggvätska är i direktkontakt med hjärnan lämpar den sig mycket väl för att identifiera samt mäta relevanta sjukdomsmarkörer. Störd synapsfunktion är som tidigare nämnts en av de tidigaste förändringarna vid utvecklingen av AD och tidigare studier har visat att neurogranin, ett protein som uttrycks specifikt i synapserna, är ett nyckelprotein för synapsernas funktion och har en mycket viktig roll i bildningen samt lagringen av minnen. Fram tills nu har det dock inte funnits några sätt att mäta synapshälsa i den åldrande hjärnan.

I avhandlingen har fokus varit dels att karakterisera neurogranin i blod, ryggvätska samt hjärnvävnad, för att få en större förståelse för hur neurogranin påverkas av sjukdomsförloppet i AD, och dels att utveckla robusta metoder för att mäta neurogranin. Genom att analysera neurogranin i ryggvätska från individer med AD, tidig AD, andra former av demens samt friska individer med flera olika metoder har vi kunnat visa att ökade koncentrationer av neurogranin i ryggvätska fungerar som en sjukdomsmarkör för synapshälsa samt att den är specifik för just AD, vilket är viktigt för att kunna utesluta andra neurodegenerativa sjukdomar. Vidare har vi också visat att den är användbar även vid identifiering av individer med tidig minnesstörning som senare utvecklar AD. Koncentrationen av neurogranin i både ryggvätska och hjärnvävnad visade ett starkt samband med mängden patologiska förändringar i hjärnan vilket indikerar att neurogranin med största sannolikhet speglar sjukdomsförloppet väl.

Sammanfattningsvis visar resultaten att neurogranin i ryggvätska är en ny och mycket användbar sjukdomsmarkör för AD, men större studier och även uppföljningsprover över en längre tid behövs för att utvärdera neurogranin i större skala.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Kvartsberg H**, Duits FH, Ingelsson M, Andreasen N, Öhrfelt A, Andersson K, Brinkmalm G, Lannfelt L, Minthon L, Hansson O, Andreasson U, Teunissen CE, Scheltens P, Van der Flier WM, Zetterberg H, Portelius E, Blennow K. *Cerebrospinal fluid levels of the synaptic protein neurogranin correlates with cognitive decline in prodromal Alzheimer's disease.* Alzheimer's & Dementia 2015, 11(10):1180-90.

II. **Kvartsberg H**, Portelius E, Andreasson U, Brinkmalm G, Hellwig K, Lelental N, Kornhuber J, Hansson O, Minthon L, Spitzer P, Maler JM, Zetterberg H, Blennow K, Lewczuk P. *Characterization of the postsynaptic protein neurogranin in paired cerebrospinal fluid and plasma samples from Alzheimer's disease patients and healthy controls*. Alzheimer's Research & Therapy 2015, 7(1):40.

III. Portelius E, Olsson B, Höglund K, Cullen NC, **Kvartsberg H**, Andreasson U, Zetterberg H, Sandelius Å, Shaw LM, Lee VMY, Irwin DJ, Grossman M, Weintraub D, Chen-Plotkin A, Wolk DA, McCluskey L, Elman L, McBride J, Toledo JB, Trojanowski JQ, Blennow K. *Cerebrospinal fluid neurogranin concentration in neurodegeneration: relation to clinical phenotypes and neuropathology*. Acta Neuropathologica 2018, 136(3):363-376.

IV. **Kvartsberg H,** Lashley T, Murray CE, Brinkmalm G, Cullen NC, Höglund K, Zetterberg H, Blennow K, Portelius E. *The intact postsynaptic protein neurogranin is reduced in brain tissue from patients with familial and sporadic Alzheimer's disease.* Acta Neuropathologica 2019, 137(1):89-102.

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CONTENT

ABBREVIATIONS

aa	Amino acid
ACN	Acetonitrile
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	β-amyloid
BBB	Blood-brain barrier
BCSFB	Blood-CSF barrier
BS^3	Bis[sulfosuccinimidyl] suberate
bvFTD	Behavioural variant FTD
CaMKII	Calcium–calmodulin-dependent protein kinase II
CBD	Corticobasal degeneration
CBS	Corticobasal syndrome
CERAD	Consortium to Establish a Registry for AD
CID	Collision-induced dissociation
CNS	Central nervous system
CSF	Cerebrospinal fluid
CU-AP	Cognitively-unaffected amyloid positive
DC	Direct current
DLB	Dementia with Lewy bodies

DMP	Dimethyl pimelimidate
DSM-5	5th edition of Diagnostic and Statistical Manual of Mental Disorders
ECL	Electrochemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
EWS	Ewing's sarcoma
FA	Formic acid
fAD	Familial AD
FBS	Frontal behavioral-spatial syndrome
FDG	Fluoro-2-deoxy-D-glucose
FDG-PET	Positron emission topography with fluoro-2-deoxy-D-glucose
FTD	Frontotemporal dementia
FTLD	Frontotemporal lobe degeneration
FTLD-FET	Frontotemporal lobe degeneration-Fused in sarcoma/ Ewing's sarcoma/TATA-binding protein-associated factor 15
FUS	Fused in sarcoma
GAP-43	Growth-associated protein 43
GSH	Glutathione
HCD	Higher-energy collisional dissociation
HI-MS	Hybrid-immunoaffinity mass spectrometry

HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IP	Immunoprecipitation
IP-MS	Immunoprecipitation mass spectrometry
IWG	International Working Group
IWG-2	International Working Group 2
LC	Liquid chromatography
LC-MS	Liquid chromatography combined with mass spectrometry
LP	Lumbar puncture
LTD	Long-term depression
LTP	Long-term potentiation
lvPPA	Logopenic variant PPA
MALDI	Matrix-assisted laser desorption/ionisation
MALDI-TOF	Matrix-assisted laser desorption/ionisation-time-of-flight
MCI	Mild cognitive impairment
MCI-AD	MCI progressing to dementia due to AD
MMSE	Mini-Mental Status Examination
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MS/MS	Mass selection/mass separation
MSD	Meso Scale Discovery
m/z	Mass/charge

naPPA	Non-fluent/agrammatic variant of primary progressive aphasia
NfL	Neurofilament light
NFT	Neurofibrillary tangle
nfvPPA	Non-fluent variant PPA
Ng	Neurogranin
NIA-AA	the National Institute on Aging-Alzheimer's Association
NINCDS-ADRDA	the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association
NINDS	the National Institute of Neurological Disorders and Stroke
NINDS-SPSP	the National Institute of Neurological Disorders and Stroke and Society for PSP
NMDA	N-methyl-D-aspartate
PAGE	Polyacrylamide gel electrophoresis
PCA	Posterior cortical atrophy
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PD MCI	Parkinson's disease with mild cognitive impairment
PET	Positron emission topography
PiB	Pittsburgh compound B
PiB-PET	Positron emission topography using Pittsburgh compound B
PKC	Protein kinase C
PPA	Primary progressive aphasia

PSP	Progressive supranuclear palsy
PSPS	Progressive supranuclear palsy syndrome
PTM	Post-translational modification
p-tau	Phosphorylated tau
REM	Rapid eye movement
RF	Radio frequency
[Ru (bpy)3]2^{+}	Tris(bipyridine)ruthenium
sAD	Sporadic AD
SDS	Sodium dodecyl sulphate
Simoa	Single Molecule Array
sMCI	Stable MCI
SNAP-25	Synaptosomal nerve-associated protein 25
SPECT	Single-photon emission computed tomography
SV2A	Synaptic vesicle protein 2A
svPPA	Semantic variant PPA
TAF15	TATA-binding protein-associated factor 15
TDP-43	TAR DNA binding-protein 43
TOF	Time-of-flight
TOF/TOF	Time-of-flight tandem mass spectrometry
t-tau	Total tau
WB	Western blot

1 INTRODUCTION

Dementia is defined as a significant decline in one or several aspects of cognitive performance which has a negative impact on daily life and function[1]. 50 million people worldwide were living with dementia in 2018, and the number is expected to have tripled by 2050[2]. Dementia is diagnosed based on the history of the illness and which cognitive domains that are affected. In addition, structural imaging of the brain, as well as blood tests, are often used in order to rule out non-degenerative causes such as stroke or tumours. As of today, there is no cure, *i.e.* a way to stop the neurodegenerative process and onset of neuropathological changes, and the medications that are available only treat or modify the symptoms[3].

1.1 CEREBROSPINAL FLUID

Cerebrospinal fluid (CSF) is produced by specialised cells in the choroid plexus of the ventricles of the brain and circulates within the brain and spinal cord. As CSF is in direct contact with the central nervous system (CNS) it provides a valuable diagnostic window into the brain. At any given moment, the total volume of CSF in an adult is around 150 mL, but it is continuously reabsorbed and reproduced[4]. CSF has several functions, and apart from providing hydromechanical protection of the CNS, it also allows circulation of substances[5] and removal of waste products[6]. The CNS is separated from the periphery, *i.e.* blood, by the blood-brain barrier (BBB) and the blood-CSF barrier (BCSFB), both of which prevent free diffusion of soluble molecules across them by tight junctions between the endothelial cells of CNS microvessels (BBB) or choroid plexus epithelial cells (BCSFB). Instead, transport of nutrients, ions, etc. is enabled by specialised transport proteins[7]. It has been shown that soluble extracellular forms of β-amyloid (Aβ) can be cleared from the brain by various routes[8], including across the BBB[9]. Thus, there are multiple ways through which proteins from the CNS can end up in the periphery[8]. It should be noted that BBB function can be altered by various causes, such as traumatic brain injury[10], inflammation[11], and neurodegenerative diseases[12, 13].

CSF can be sampled through a lumbar puncture (LP) between the L3/L4 or L4/L5 vertebrae, and there are standardised procedures to follow with regard to certain aspects of the procedure[14]. The most common complication after an LP is post-lumbar puncture headache, which, although harmless, might last for several hours up to a week[15-17].

1.2 BIOMARKERS

What is a biomarker? According to the definition of the International Programme on Chemical Safety, led by the World Health Organization and in coordination with the United Nations and the International Labor Organization, a biomarker is defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease"[18]. In the human body, biomarkers can be measured in tissue or bodily fluids such as blood, saliva, or CSF. In the setting of neurodegenerative diseases, biomarkers could potentially have multiple uses. They can be diagnostic, *i.e.* used in order to make a diagnosis; they can be prognostic, *i.e.* used to determine if a person will remain at the same cognitive level or deteriorate; and finally they can also be predictive, *i.e.* used to determine who will get the disease or not. In addition, biomarkers may also be used for discriminating between different neurodegenerative diseases, as well as staging or determining the extent of the disease and monitoring response to a drug either in the clinic or during pharmaceutical trials[19].

1.3 NEURODEGENERATIVE DISEASES

Neurodegenerative disease is an umbrella term used to describe diseases that result from progressive neurodegeneration, *i.e.* loss of neurons, and include both dementia and movement disorders. The presentation of the disease or syndrome varies depending on the kind of neurodegenerative process, what type of cell(s), and what area(s) of the brain that is affected. To further complicate matters, different combinations of affected areas and types of neuropathological changes might give a heterogeneous presentation of symptoms between patients. Neurodegenerative diseases are often categorised and classified either based on the type and location of neuropathological change, presentation of clinical symptoms, or a combination of both[20].

Something that many neurodegenerative diseases have in common is protein aggregation which most likely is the end stage of a series of molecular processes. These aggregates may be toxic to neurons thus ultimately causing cell death[21]. Sometimes, mutations in specific genes make proteins more prone to aggregate, and such mutations can result in hereditary variants which are often called familial versions of the disease in question[22].

There are many challenging aspects to neurodegenerative diseases, for instance, that most of them have a fairly long presymptomatic phase, during which neuropathological changes accumulate but do not yet cause any clinical symptoms, meaning that when clinical symptoms do arise, the disease is most likely already fairly advanced[20, 23]. Moreover, making a clinical diagnosis can be quite difficult as it is

relatively common with atypical presentations of symptoms or that symptoms correlate poorly with the degree of neuropathological changes. In addition, even though post-mortem neuropathological examination is the gold standard for many diseases[24-26], meaning it is the only way to make a definitive diagnosis, there is a high incidence of multiple pathologies, so-called comorbidities[27-29].

1.3.1 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) was first described in 1906 by the German psychiatrist and pathologist Alois Alzheimer and was later named after him. He described a single case, characterised by memory disturbances and neuropathological findings of extracellular miliary bodies and dense bundles of neurofibrils. Today, these are all recognised to be hallmarks of AD[30]. Representing approximately 60-80% of all dementia cases, AD is the most common cause of dementia[31] with a prevalence of around 5% in the population of 60 years and above[32]. The incidence increases with age and roughly doubles every five years after the age of 65 years[33]. AD is characterised by an insidious onset and progressive decline of cognitive functions. The most common clinical presentation is impaired episodic memory, but other common symptoms include changes in personality, judgement, and behaviour, as well as aphasia, apraxia, and agnosia[30]. There are also cases which have an atypical presentation, with language, visual or executive problems that are more pronounced and earlier than memory deficits. These are discussed under posterior cortical atrophy (PCA). AD is characterised by a long pre-clinical phase, and neurodegeneration is estimated to start 20-30 years before the onset of clinical symptoms[34], during which pathological changes accumulate. As a result, by the time the symptoms are pronounced enough to make the patient seek medical attention, and a diagnosis is made, the neurodegenerative processes will have caused considerable and irreversible damage manifesting as cognitive decline. As treatments are most likely to have maximum impact and efficiency at earlier stages when the brain is relatively intact, early diagnosis is of utmost importance[16, 35]. As of today, there is no cure or a way to slow neurodegeneration in AD, and the only approved medications are aimed at treating or modifying the symptoms. For instance, acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) agonists such as mementin are used to treat cognitive symptoms but do not affect the underlying disease process[36].

1.3.1.1 Pathology

The neuropathological hallmarks of AD are neuronal loss in certain areas of the brain, with early affected areas comprising the medial temporal lobe (hippocampus and entorhinal cortex), in addition to intraneuronal neurofibrillary tangles (NFTs) and extracellular neuritic plaques. The NFTs consists of aggregated, and sometimes truncated, hyperphosphorylated tau protein while the plaques are composed of Aβ peptides[30], mainly ending at amino acid (aa) 42, *i.e.* $A\beta_{X-42}$[37].

Plaques follow a general path of spreading from cortical to subcortical regions[38], while NFTs spread in an opposite pattern thus starting in subcortical and ending in the cortical regions[39, 40]. In the more advanced stages of AD, neuroanatomical distribution of NFTs correlates both with the cognitive domains that are affected as well as areas of neuronal death[41]. However, it is rare that only one type of neuropathological change is present in an elderly individual. Studies show that irrespective of clinical symptoms and diagnosis, up to around 70% of cases display multiple pathologies, such as AD pathology, Lewy body pathology, vascular pathologies, TAR DNA binding-protein 43 (TDP-43) proteinopathy, and hippocampal sclerosis, and comorbidity of pathological changes has been shown to increase with age[29]. Almost 40% of dementia cases that are not clinically diagnosed as AD display enough AD neuropathology to fulfil the criteria for an AD diagnosis[42]. It is also worth to note that AD pathology is quite common in non-demented older individuals[39], with studies estimating 30-40% of cognitively healthy elderly individuals classified as positive for AD pathological changes upon autopsy[43-45]. From here on, such individuals are referred to as cognitively-unaffected amyloid positive (CU-AP). However, even though CU-AP individuals have plaques, the composition of these might differ compared to plaques in AD brains[46], indicating that the exact mechanisms leading to neuropathological changes might differ.

There are scoring systems for determining the extent, *i.e.* presence, distribution, and frequency, of plaques and NFTs. Thal phases (0-5) are used to determine the spread of the amyloid plaques throughout the brain[38], while Braak stages (0-VI) determine the distribution of tau pathology[47]. In addition, there is the Consortium to Establish a Registry for AD (CERAD) neuritic plaque score which is used to classify AD neuropathology into four groups (no or negligible – high level)[48]. The Braak, Thal, and CERAD scoring can also be combined into an ABC score, which thus incorporates all of the described neuropathological aspects[48].

1.3.1.2 Heritability and Risk Factors

Most AD cases have no known cause and are called sporadic (sAD) but autosomal dominant mutations in genes related to the metabolism of amyloid precursor protein (APP), *i.e. PSEN1* and *PSEN2*, are the major underlying cause of the hereditary form called familial AD (fAD) which accounts for around 1%, or less, of all cases. APP is the precursor of Aβ peptides and is cleaved by presenilin 1 and 2, encoded by *PSEN1* and *PSEN2*, which are both included in the γ-secretase complex[49, 50]. fAD can also be caused by mutations in *APP* which affect how the secretases, *e.g.,* γ-secretase, process APP[49] and today a total of more than 300 mutations in *PSEN1, PSEN2* and *APP* have been identified[51]. While sAD usually presents after the age of 65, fAD generally has a much earlier disease onset and a more rapid progression. The pathogenic mutations in *APP* are so called missense mutations, *i.e.* leading to the coding of a different aa, and the mutations are most often located in the Aβ-encoding gene sequence, often near

protease cleavage sites which alter the proteolytic processing of APP. The effects of *APP* mutations include increased overall production of Aβ peptides, an increase of the $A\beta_{1-42}/A\beta_{1-40}$ ratio, increased secretion of $A\beta_{1-42}$ and $A\beta_{1-40}$, and increased oligomerisation, to mention a few. A detailed list can be found at https://www.alzforum.org/mutations/app[52]. The vast majority of *PSEN* mutations are also missense mutations which among other things results in an increase in the $A\beta_{1-42}/A\beta_{1-40}$ ratio[50]. There are also examples of protective mutations[53].

Even though no single genetic cause of sAD has been identified there are genetic risk factors associated with sAD, the most prominent one being *APOE*, which encodes for apolipoprotein E (apoE). *APOE* has three alleles, *ε2*, *ε3*, and *ε4*, where *ε2* appears to be protective, *ε3* neutral, and *ε4* harmful. The most common allele is *ε3*, followed by *ε4*, and *ε2*[54]. *ε2* exerts its protective effect by decreasing the overall risk of developing sAD as well as delaying onset[55]. In contrast, *ε4* lowers age of onset and increases risk in a dose-dependent manner. Individuals that are heterozygous or homozygous for *ε4* have an increased risk, three and 15 times more likely, respectively, to develop sAD compared to an individual without a copy of the *ε4* allele[50]. The mechanism by which *APOE* modulates risk might be connected to clearance of Aβ peptides from the brain, which appears to differ between the isoforms[56]. It has also been shown that *ε4* carriers exhibit lowered cerebral glucose metabolism and have greater atrophy of certain areas of the brain, including the hippocampus[57].

1.3.1.3 The Amyloid Cascade Hypothesis

The underlying cause of AD is still not known, but the most widely accepted suggested pathological pathway is called the "amyloid cascade hypothesis". The amyloid cascade hypothesis was introduced in 1992, and identifies increased production and aggregation of $A\beta_{1-42}$ as the primary driving mechanism of the disease, leading to the formation of plaques and NFTs and ultimately neuronal death and dementia[58]. The Aβ peptides are generated by proteolytic processing of APP by β- and γ-secretase[59] and there are many Aβ peptides present in both human CSF[60] and brain tissue[37]. Evidence supporting the amyloid cascade hypothesis include for instance that mutations in genes related to APP metabolism cause fAD[49-51] and that an extra copy of *APP*, which occurs in Down's syndrome, leads to the formation of plaques even in adolescents with AD pathology increasing with age, presumably due to overproduction of Aβ[61]. In many ways, due to the popularity of the amyloid cascade hypothesis, principally all disease-modifying drugs in clinical trials have been aimed at altering the concentrations of Aβ in the brain in different ways. However, so far all have failed, and no new drugs for AD have been approved since 2003[62] leading to that the amyloid cascade hypothesis has been extensively criticized[63, 64], mainly because of the failures to modify the course of AD by targeting Aβ.

While most of the AD field acknowledge that Aβ is central to the disease, not all agree that Aβ peptides are the driving force behind the disease. There are a few alternative hypotheses for the underlying cause of AD, two of which focus on inflammation[65] and oxidative stress[66]. It has been known for a long time that there is a neuroinflammatory component to AD, but the inflammation has most often been assumed to be a consequence of, or response to, the pathophysiological changes[67]. The inflammatory processes within the brain are driven by microglia, and the degree of inflammation increases with disease progression[65]. Moreover, strong associations between AD and mutations in several genes related to the immune system have been identified in addition to reports of increases in inflammatory cytokines, chemokines, and other molecules related to inflammation[67]. The other major alternative hypothesis, oxidative stress, also has a lot of supporting evidence, including increased DNA and protein oxidation in AD as well as studies showing that Aβ peptides are capable of generating free radicals[66]. Oxidative damage occurs early in the disease process, preceding a high plaque load and has been liked to abnormal phosphorylation of tau and mitochondrial dysfunction[68].

1.3.1.4 Diagnosis

The first diagnostic criteria for AD were presented in 1984 by the Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). However, since no biomarkers were available at the time, these criteria were for probable, possible, or definite dementia due to AD based on clinical symptoms and exclusion of other dementias[69]. Probable AD was defined as dementia with progressive impairment of memory and cognitive function in the absence of other disease causing the symptoms, while definitive AD could only be diagnosed by neuropathology upon autopsy[69]. The specificity and sensitivity for these criteria have been reported to 80% and 70% respectively[70]. The American Psychiatric Association released the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) in 1994 (revised in 2000), in which the AD criteria were comparable to the NINCDS-ADRDA[71, 72]. According to DSM-IV, a dementia diagnosis requires the loss of two or more of the following cognitive domains: memory, language, calculation, orientation, or judgement. As both fluid and imaging biomarkers emerged and the reported changes were shown to be consistent in AD, the International Working Group (IWG) for New Research Criteria for the Diagnosis of AD proposed new criteria which were presented in 2007. The IWG criteria were based on identifying the core symptoms for progressive memory impairment in combination with one or more positive biomarkers; either imaging (hippocampal volumetric magnetic resonance imaging (MRI), or positron emission topography (PET)), or fluid (CSF $A\beta_{1-42}$, total tau (t-tau), and phosphorylated tau (p-tau)). In addition, these criteria provided tools for the diagnosis not only of AD dementia but also for the different stages of the disease, including prodromal AD, also

called mild cognitive impairment (MCI)[73]. Revised criteria based on the 1984 NINCDS-ADRDA were presented in 2011 by the National Institute on Aging-Alzheimer's Association (NIA-AA) in which biomarkers, as well as MCI, was introduced into the NIA-AA framework[74].

MCI is a clinical syndrome characterised by an evident decline in memory or other cognitive abilities, that does not impair day-to-day functioning, and does not meet criteria for dementia defined by DSM-IV[75]. In addition, MCI subjects have prominent amnestic symptoms, above what is considered to be normal for age, that indicate a pre-stage of AD[75]. As with AD, the pathological changes in MCI might not exclusively be AD-related but rather a mixture of different pathologies[76, 77]. One study reported 88% of MCI subjects to have AD neuropathological hallmarks[78], thus indicating that MCI indeed is a pre-stage of AD in a majority of cases. Other commonly underlying causes are vascular pathology and depression[79]. The prevalence of MCI differs between studies, but is somewhere between 3-19% after the age of 65, and while some remain cognitively stable over time, around half progress to dementia within 5 years[80]. As mentioned, MCI due to AD is generally characterised by amnesia[75], but there is also non-amnestic MCI which is mostly caused by other neurodegenerative diseases such as frontotemporal dementia (FTD), dementia with Lewy bodies (DLB) or vascular dementia[80].

IWG-2 is a recent update of the IWG criteria, and include only the core CSF AD biomarkers and amyloid PET, but not hippocampal volumetric MRI, as supporting evidence of AD pathology. In the IWG-2 criteria, biomarkers are now included as research criteria for the diagnosis of AD, meaning that the disease can be diagnosed using these supportive biomarkers. In short, one of the three following biomarkers need to be positive: low CSF $A\beta_{1-42}$ and high t-tau or p-tau; PET scan indicating the presence of plaques; an autosomal dominant mutation[81]. In 2018, NIA-AA presented updated guidelines for researchers in order to incorporate the current understanding of the disease as defined by biology rather than clinical symptoms. According to the 2018 criteria, an AD diagnosis is defined by the pathological appearance of plaques and tangles and that these can be documented *in vivo* using biomarkers[26]. This biological approach is in stark contrast to the other criteria which put a lot of weight on symptoms and signs, such as cognitive decline, that rather are clinical manifestations of the neurodegeneration and pathological changes.

There are a number of different tests aimed at assessing various aspects of cognitive performance which can be used when evaluating suspected dementia in a patient[33]. The most widely used screening test for evaluating cognition is called Mini-Mental Status Examination (MMSE) and was first described by Paul R. McHugh and colleagues in 1975, but it is still extensively used. The MMSE test is comprised of 30 points distributed on 19 items measuring orientation, memory, concentration, language, and

praxis[82]. Generally accepted cut off scores are cognitively intact (27-30), mild AD (21-26) and moderate AD (12-20)[83]. However, it is not possible, or at the very least very difficult, to discriminate between AD and other dementias solely based on clinical history coupled with cognitive and neuropsychiatric tests, which is why biomarkers are essential.

1.3.1.5 Biomarkers

In AD, there are currently three core biomarkers that are measured in CSF and used for diagnosis as well as inclusion criteria in clinical trials: $A\beta_{1-42}$, p-tau, and t-tau[16, 84] (Figure 1). AD and MCI due to AD are characterised by lowered CSF concentrations of $A\beta_{1-42}$, as this peptide is retained in the plaques, combined with increased concentrations of both t-tau and p-tau, which reflect axonal damage and tangle formation, respectively[16, 23, 35, 84, 85]. The core AD biomarkers show high sensitivity and specificity for AD, around 85-90%[85]. However, recent studies have highlighted the fact that there is much to gain from utilising the $A\beta_{1-42}/A\beta_{1-40}$ ratio compared to $A\beta_{1-42}$ alone in order to increase diagnostic accuracy [86-88]. By adding the $A\beta_{1-42}/A\beta_{1-40}$ or $A\beta_{1-42}/A\beta_{1-38}$ ratio, there is very little overlap between AD and healthy controls[89]. The hypothesis is that using the ratio normalises for high- and low-producers of Aβ[90].

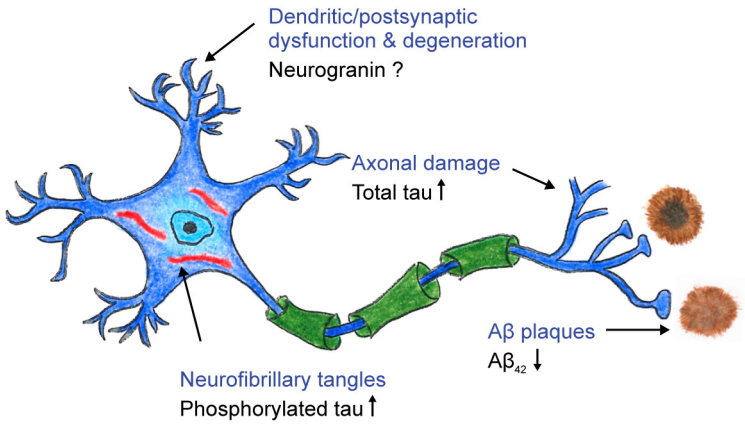

Figure 1. Core cerebrospinal fluid biomarkers in Alzheimer's disease. *A neuron containing NFTs with adjacent plaques. In AD, CSF $A\beta_{1-42}$, which reflects plaques, is decreased while CSF t-tau and p-tau, reflecting axonal damage and NFTs respectively, are increased. The postsynaptic protein neurogranin might be used in order to evaluate dendritic and/or postsynaptic dysfunction and degeneration.*

Apart from fluid biomarkers in CSF, considerable efforts are being put into identifying plasma biomarkers, which would be very useful since plasma is much more easily accessible. One of the most widely pursued is plasma Aβ, and recent mass spectrometry (MS)-based assays have shown that the plasma $A\beta_{1-42}/A\beta_{1-40}$ ratio has a

good diagnostic ability[91, 92]. However, more studies need to be performed before it can be implemented as an accurate biomarker.

In addition to the fluid biomarkers, there are also imaging techniques which allow monitoring of pathophysiological changes in the brain of living patients. Structural MRI can be used in order to determine the degree of atrophy. Typically, the entorhinal cortex is the earliest area affected by atrophy, followed shortly by the hippocampus and amygdala. PET scans are based on assessing the retention of radioactive ligands in the brain. The metabolism of the brain can be assessed using the radioligand 2-[^{18}F]-fluoro-2-deoxy-D-glucose (^{18}F-FDG), *i.e.* FDG-PET, and AD is characterised by hypometabolism of certain areas including the hippocampus. The metabolic deficiencies gradually worsen with disease progression[93]. There are also PET tracers that bind to Aβ plaques, the most widely evaluated being Pittsburgh compound B (PiB)[94], *i.e.* PiB-PET, but there are now several amyloid tracers available[95]. A high inverse correlation has been shown between PiB-PET and the CSF $A\beta_{1-42}/A\beta_{1-40}$ ratio thus indicating that the two methods both reflect the amyloid burden of the brain[96]. Recently, tau tracers capable of visualising NFTs have also been developed and are reported to associate well with both brain atrophy and cognitive decline[97] in addition to being able to discriminate between AD and other neurodegenerative diseases[98].

Dysfunction and loss of synapses are directly linked to cognitive symptoms even at early stages of the disease and are thought to precede neuronal loss[99]. It has also been shown that the severity of dementia is more closely associated with the extent of synaptic loss than plaques and NFTs[100-103]. Thus, synaptic proteins could potentially fill a gap in the diagnostic panel that is used today, not only as early biomarkers for AD, but they might also be used to monitor disease progression and evaluating prospective disease-modifying therapies. Recently, several novel synaptic markers have been investigated including synaptosomal nerve-associated protein 25 (SNAP-25)[104], synaptotagmin[105], synaptic vesicle protein 2A (SV2A)[106], growth-associated protein 43 (GAP-43)[107], and neurogranin (Ng)[108], the latter of which will be discussed in detail in this thesis.

1.3.2 AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a motor neuron disease in which patients display both upper and lower neuron signs[109]. ALS is one of the major neurodegenerative diseases along with AD and Parkinson's disease (PD), and descriptions date back at least until the early 19th century, but the connection between symptoms and underlying neurological problems was first described in 1874 by Jean-Martin Charcot[110]. The incidence of ALS is around 2 per 100,000 persons/year with males having a slightly higher incidence than females [109, 111], at least in the sporadic variant. There is no cure for the disease[112] and cause of death is most commonly

respiratory failure, which usually occurs 2-5 years after the onset of first symptoms, but survival time have been known to vary between a few months up to several decades[113, 114]. However, there is one disease-modifying drug that has shown moderate promising results in terms of extending survival time mainly by delaying the need for artificial respiration[115].

1.3.2.1 Pathology

Neuropathological changes associated with ALS include loss of myelinated axons in the lateral and anterior columns of the spinal cord, small round or oval shaped eosinophilic inclusions in motor neurons of the spinal cord and brain stem, called Bunina bodies, and ubiquitinated inclusions containing TDP-43 in the cytoplasm of anterior horn cells[116].

1.3.2.2 Heritability and Risk Factors

There are two variants of ALS, one sporadic and one familial, of which the latter accounts for approximately 5-10% of the cases. Familial ALS can be caused by mutations in *SOD1* (20 %), *TARDBP* (2-5%)[117], *FUS,* and the most recently discovered hexanucleotide repeats (GGGGCC) in *C9ORF72*, which encodes for superoxide dismutase, TDP-43, fused in sarcoma (FUS), and chromosome 9 open reading frame 72 respectively, to mention a few[118]. *C9ORF72* has been shown to be the most common mutation in both the sporadic (6%) and familial form (40%) of ALS[119]. In addition, *C9ORF72* has also been linked to autosomal-dominant FTD[120], and 20-50% of all ALS patients fulfil consensus criteria for probable or definite FTD[121]. Age of onset is around 60 years for sporadic ALS while familial ALS manifests earlier with a mean onset of around 50 years of age[122]. Most cases of familial ALS show an autosomal dominant pattern of inheritance, but some cases of autosomal recessive inheritance have been reported as well[123, 124].

1.3.2.3 Diagnosis

The El Escorial criteria for diagnosis of ALS were established in 1994[125], with the purpose of standardising the diagnosis, and have since then been revised twice[126, 127]. The El Escorial criteria stage ALS into probable, possible, and definitive diagnosis, although in the latest revision it was stated that the former categories of probable and definite ALS should be replaced by a new and validated staging system as a diagnosis can be made simply if the criteria for possible ALS are fulfilled[127]. According to the El Escorial criteria, the diagnosis of ALS requires the presence of signs of both upper and lower motor neuron degeneration as well as progressive spread of symptoms within a region or into other regions. Such signs must also be in the absence of electrophysiological evidence of other signs of disease that might explain the degeneration of upper and lower motor neurons and neuroimaging evidence of disease

processes that might explain clinical manifestations and electrophysiological results[125]. Neuronal loss in the lower motor system results in denervation of muscles and symptoms such as cramps and muscle weakness while degeneration of upper motor neurons presents as spasticity and fasciculations[128]. There is also a second set of criteria called the "Awaji-Shima" criteria in which electromyography, used to evaluate electrical activity produced by skeletal muscles, is added to the El Escorial criteria[129].

1.3.2.4 Biomarkers

There are several potential CSF and blood biomarkers for ALS, including neurofilaments, TDP-43[130], and cytokines[131], with CSF neurofilament light (NfL) showing especially high promise[132].

1.3.3 CORTICOBASAL DEGENERATION

Corticobasal degeneration (CBD) is a rare, progressive neurodegenerative disorder which can only be diagnosed upon autopsy[133]. The disease is called corticobasal syndrome (CBS) when diagnosed by clinical criteria. It was first described in 1968 as corticodentatonigral degeneration with neuronal achromasia[134], and the term CBD was introduced in 1989[135]. The symptoms of the first described cases emphasized progressive movement abnormalities including slowing of voluntary movements as well as presence of involuntary movements. Neuropathological findings included frontoparietal atrophy with neuronal loss, gliosis, and pigment loss in the substantia nigra[134]. However, clinicopathologic studies have revealed that CBD can be considered to be an atypical parkinsonian disorder as it mainly manifests as a movement disorder (or at least many case series have had data biased towards movement disorders as a lot of cases have been identified from movement disorder clinics) with symptoms such as tremor and bradykinesia[136], but it may also present as a cognitive disorder with neuropsychological impairments[137]. Mean age of onset is around 64 years[24] and mean disease duration is less than 7 years[133]. There is no cure, but the individual symptoms can be treated[136].

1.3.3.1 Pathology

Neuropathological findings in CBD are frontoparietal atrophy, accumulation of NFTs consisting of the insoluble four-repeat isoform of tau in neurons, astrocytes, and oligodendroglial cells, ballooned neurons, substantia nigra and focal cortical neuronal loss[136, 138, 139]. CBD pathology is very closely related to progressive supranuclear palsy (PSP), which also is classified as an atypical parkinsonian disorder, and the most reliable way to discriminate between them is by looking at what type of astrocytic tau-containing inclusion that is present; plaques in CBD vs. tufted in PSP[140]. In addition, the ballooned neurons which are characteristic for CBD are not present in PSP[139]. To make matters more complicated, CBD is very heterogenic as the underlying pathology

can be AD, FTD, Pick disease, PSP, or PCA, to mention a few[138, 141, 142]. Since pathological findings associated with CBD also have been found in patients with FTD[143] and the FTD subgroup primary progressive aphasia (PPA)[143, 144] it has been proposed to call the clinical presentation CBS in order to distinguish it from the neuropathological entity of CBD[143], which can only be definitely diagnosed upon autopsy[24].

1.3.3.2 Heritability and Risk Factors

Although most cases are sporadic, there have been reported cases of familial CBD as well. However these are quite rare and such cases seems to be associated with mutations in *GRN*, encoding for progranulin, mutations and frontotemporal lobe degeneration (FTLD) with TDP-43 immunoreactive inclusions (FTLD-TDP) rather than tau[24].

1.3.3.3 Diagnosis

CBD is a complex neurodegenerative disorder and diagnosis is often complicated. Based on clinical phenotypes of autopsy-confirmed CBD cases, diagnostic criteria for CBD have identified four phenotypes; CBS (possible and probable), frontal behavioural-spatial syndrome (FBS), non-fluent/agrammatic variant of primary progressive aphasia (naPPA), and progressive supranuclear palsy syndrome (PSPS). Probable CBS have an asymmetrical presentation of at least two motor symptoms including limb rigidity or akinesia, limb dystonia or alien limb phenomena[24].

1.3.3.4 Biomarkers

Imaging to evaluate patterns of atrophy may be promising and could also be used in order to exclude other conditions with similar clinical presentations. CSF biomarkers of CBD have not yet been sufficiently studied[24].

1.3.4 DEMENTIA WITH LEWY BODIES

DLB is the second most common type of dementia in elderly people, accounting for 10-20% of all cases[145]. Lewy bodies were first described by Friedrich Heinrich Lewy in 1912[146] and received their name in 1917[147], but it was not until much later that the association with dementia was recognised and the term DLB was introduced[148, 149]. The prevalence is around 0.4% in people aged 65 or above, and symptom onset usually occurs between 50 and 70 years of age[150]. There is no cure, but most symptoms can be improved upon treatment[151].

1.3.4.1 Pathology

In DLB, the primary pathological finding is intraneuronal Lewy bodies, comprised of inclusions consisting of aggregated α-synuclein[152] and ubiquitin[153], in many areas of

the brain including the neocortex, forebrain, and brainstem. There are also Lewy neurites, which correspond to normal neurites that contain filaments similar to those found in Lewy bodies[154], and thus also contain α-synuclein[155]. However, Lewy bodies and Lewy neurites are not exclusive to DLB but are also the main pathological component of multiple system atrophy [156, 157] and PD[158, 159]. As all of these diseases have aggregated α-synuclein in Lewy bodies as a common pathology, they are sometimes referred to as α-synucleinopathies[157]. The presence of plaques and tangles, *i.e.* AD pathology, in DLB can modify the clinical symptoms, for instance resulting in a lowered rate of both parkinsonism and visual hallucinations. Thus, comorbidity of AD pathology in DLB makes it more challenging to distinguish AD and DLB clinically[154].

1.3.4.2 Heritability and Risk Factors

Most cases of DLB are sporadic, but there have also been reports of occurrences in families[160]. Also, several loci[161, 162], including *APOE ε4*[163], have been associated with increased risk of developing DLB.

1.3.4.3 Diagnosis

The newly revised criteria for clinical diagnosis of possible and probable DLB state that "dementia, defined as a progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational functions, or with usual daily activities, is an essential requirement for DLB diagnosis". Core clinical features are defined as fluctuation; visual hallucinations; parkinsonism; rapid eye movement (REM) sleep behaviour disorder. There are many supportive clinical features such as severe sensitivity to antipsychotic agents, postural instability, transient episodes of unresponsiveness, and hypersomnia. A diagnosis of possible DLB can be made if one core clinical feature is present, with or without indicative biomarkers (see below), or if at least one indicative biomarker is present in the absence of core clinical features. Probable DLB can be diagnosed when at least two core clinical features are present with or without the presence of any indicative biomarkers, or only one core clinical feature but with at least one indicative biomarker[151].

1.3.4.4 Biomarkers

Biomarkers are not yet required for the diagnosis of DLB, but, as mentioned above, they are included as an indication of the disease. Indicative biomarkers include reduced dopamine transporter uptake in basal ganglia, demonstrated by single-photon emission computed tomography (SPECT) or PET, and polysomnographic confirmation of REM sleep without atonia, but there are also several supportive biomarkers. Certain specific electroencephalography (EEG) patterns is an example of a supportive biomarker[151]. Imaging, such as MRI, to determine grade and localisation of atrophy[164], and CSF α-

synuclein might be useful in order to differentiate DLB from AD[165]. Two other promising biomarkers are plasma concentrations of epidermal growth factor and apolipoprotein A1, but these need to be confirmed in larger studies[164].

1.3.5 FRONTOTEMPORAL DEMENTIA

FTD is the clinical neurodegenerative syndrome of the neuropathological entity FTLD. FTLD displays a complex pathological pattern of neurodegeneration[166] and molecular neuropathology[167] resulting in progressive deficits in language, behaviour, and executive functions[168]. Arnold Pick published the first description of FTD in 1892 of a patient that had aphasia, lobar atrophy, and presenile dementia[169]. FTD is the most common form of early-onset dementia and overall the third most common type of dementia, after AD and DLB[168]. Age of onset is typically around 65 years of age, and the prevalence is 15-22 per 100,000 individuals[170]. Based on clinical presentation, FTD can be further divided into either a behavioural variant FTD (bvFTD), which is the most common variant, or language disorders of primary progressive aphasia (PPA). In addition, PPA has three clinically distinct language disorder subgroups of logopenic variant PPA (lvPPA), non-fluent variant PPA (nfvPPA), and semantic variant PPA (svPPA)[171]. Current medication strategies for treatment of FTD symptoms are mostly based on increasing or replacing neurotransmitters and modifying behavioural symptoms[172].

1.3.5.1 Pathology

Neuropathological changes in FTLD are in most cases characterised by relatively selective atrophy of the frontal and temporal lobes. There are several distinct molecular pathologies included in FTLD, which are classified according to the major constituents of the intracellular protein inclusions into FTLD-tau, FTLD-TDP, and FTLD-FUS. The most common form is FTLD-tau, where hyperphosphorylated tau forms inclusions, which is found in 40-50% of all cases. Apart from FTLD, Pick disease, CBD, and PSP are also characterised by the same inclusions as in FTLD-tau. FTLD-TDP is found in roughly half of FTLD and can be further divided into the three major subtypes of FTLD-TDP: type A, B, and C, which all have distinct patterns of cytoplasmic or intranuclear pathology and cortical association. Finally, FTLD-FUS accounts for about 10% of all FTLD cases[168]. It is worth to note that FTLD-FUS is sometimes grouped with pathological inclusions of FUS, Ewing's sarcoma (EWS), and TATA-binding protein-associated factor 15 (TAF15), and in these cases this pathologically distinct group is called FTLD with FUS/EWS/TAF15 (FTLD-FET)[167].

1.3.5.2 Heritability and Risk Factors

The heritability of the disease differ between FTD variants, and up to 50% of the cases report a positive family history, but 10-27% of all FTD cases have been found to

display an autosomal dominant mode of inheritance[173]. Mutations in *VCP*, encoding for valosin-containing protein, *CHMP2B*, which encodes for charged multivesicular body protein 2B, *TARDBP*, and *FUS* are found in less than 5% of familial FTD cases[173]. It was long known that familial FTD was linked to chromosome 9 and in 2011 researchers managed to identify a hexanucleotide (GGGGCC) repeat in *C9ORF72* causing autosomal dominant FTD[120]. Mutations in *C9ORF72*, *MAPT*, encoding for tau, and *GRN* together account for around 60% of the familial cases[174].

1.3.5.3 Diagnosis

The first diagnostic criteria for bvFTD were presented in 1994 by the Lund and Manchester groups[175]. The criteria were updated in 1998 and included recognition of semantic dementia and progressive non-fluent aphasia (now called nfvPPA) as well as introducing both core and supportive diagnostic features[176]. Autopsy studies have shown that the 1998 diagnostic criteria correctly classify 80-90% of bvFTD cases, although they lacked sensitivity in early phases of the disease[173]. An additional set of revised criteria for bvFTD were published in 2007 where neuroimaging and genetics were added, and the role of supportive behavioural features was given more importance[177]. In the most recent bvFTD diagnosis criteria possible bvFTD require three of the following symptoms: disinhibition; apathy; hyperorality and/or executive deficits; compulsive behaviour or loss of empathy. For probable bvFTD at least three of the described symptoms need to be displayed, in addition to significant functional decline and that imaging results must be consistent with bvFTD, *e.g.* MRI showing frontal and/or temporal lobe atrophy. bvFTD with definitive FTLD pathology can be diagnosed either by histopathological evidence, *i.e.* upon autopsy, or by the presence of a known pathogenic mutation[178]. There are also criteria for the three variants of PPA, in which a patient first needs to meet basic PPA criteria which include insidious onset and gradual progression of impairment in language such as object naming or syntax. Everyday life should not be impaired except for activities related to language. Classification into one of the PPA variants may occur on a clinical, imaging or definitive pathological level. For a diagnosis supported by imaging, the localisation of neuroimaging changes should be consistent with those previously associated with each variant of PPA. To summarise the clinical core criteria, lvPPA is characterised by impairments in word retrieval such as names and repetition of phrases, nfvPPA displays agrammatism and/or apraxia of speech, while svPPA is identified by impaired word comprehension and/or confrontation naming[171]. Guidelines for clinical staging and disease progression in FTD and its variants have also been proposed[179].

1.3.5.4 Biomarkers

Currently, there are no fluid biomarkers used in FTD diagnosis but the core AD biomarkers, *i.e.* CSF $A\beta_{1\text{-}42}$, p-tau, and t-tau, can be used to differentiate FTD patients from AD patients. However, AD-like CSF profiles are also found in lvPPA[178] patients,

which often have underlying AD pathology[180]. NfL is one of the most promising biomarkers for FTD and increased concentrations compared to control individuals have been reported in both CSF and blood[181]. Both volumetric MRI, which measures grey matter atrophy, and FDG-PET, used to visualise and quantify alterations in brain metabolism, are very useful in FTD[181]. In FTD, volumetric MRI shows regional grey matter atrophy in certain areas including the frontal and temporal lobes and FDG-PET reveals hypometabolism[181]. Neuroimaging is included in the diagnostic criteria for probable bvFTD[178] as well as classification of PPA variant[171].

1.3.6 PARKINSON'S DISEASE

PD is the second most common neurodegenerative disease and is characterised by loss of dopamine-secreting, *i.e.* dopaminergic, neurons in the substantia nigra which cause dopamine deficiency. The prevalence is approximately 1% in individuals of 60 years of age or above[182]. PD was first described by James Parkinson in 1817 who referred to it as shaking palsy or *paralysis agitans*, and observed symptoms included tremors and a forward bent posture[183]. Treatments for motor symptoms consist mainly of drugs that enhance the concentrations of intracerebral dopamine or stimulate dopamine receptors and include levodopa and dopamine agonists. Another option to treat moderate-to-severe PD is deep brain stimulation, where a neurostimulator, which sends electrical impulses, is implanted to stimulate either the subthalamic nucleus or globus pallidus internus[184]. Studies have shown that deep brain stimulation can have positive effects on both motor and non-motor features of PD[185]. A potential problem when it comes to both treatment and research is that by the time a patient starts to display symptoms, a loss of 70-80% dopaminergic neurons may already have occurred[186].

1.3.6.1 Pathology

The main pathological finding in PD is intraneuronal Lewy bodies, containing aggregated α-synuclein, which appear in six suggested stages spreading from the medulla oblongata, through the midbrain and mesocortex into the neocortex[158].

1.3.6.2 Heritability and Risk Factors

As with many other neurodegenerative diseases, there are both sporadic and familial forms of PD, with familial PD being estimated to account for less than 10% of all cases. At least five genes have been shown to have a clear link to PD including *SNCA*, also called *PARK1* and *PRKN*, also called *PARK2*, which encodes α-synuclein and parkin respectively, as well as *LRRK2*, encoding for leucine-rich repeat serine/threonine-protein kinase 2. Mutations in *SNCA* and *LRRK2* have been identified in families showing autosomal dominant inheritance while *PRKN* mutations have been liked to autosomal recessive PD. *LRRK2* mutations are the most common cause of both sporadic and autosomal dominant PD[187].

1.3.6.3 Diagnosis

PD diagnostic criteria developed by the National Institute of Neurological Disorders and Stroke (NINDS) includes staging into possible, probable, and definite PD. Criteria for possible PD include at least two of four PD motor characteristics, *i.e.* resting tremor, rigidity, asymmetric onset, or bradykinesia, of which one must be rest tremor or bradykinesia. Also, no symptoms suggestive of alternative diagnosis should be present, and the patient should have a substantial response to a dopamine receptor agonist or levodopa, which is a precursor to dopamine that is converted to dopamine once it has entered the CNS. In order to meet the criteria for probable PD at least three PD characteristics should be present for at least three years with no features suggestive of an alternative diagnosis and a substantial response to levodopa or dopamine receptor agonist. Definite PD can only be diagnosed upon autopsy as all criteria for probable PD should be met with the addition of histopathological confirmation of diagnosis, meaning neuronal loss and Lewy bodies within the substantia nigra[188]. Non-motor symptoms include for instance cognitive impairment and olfactory dysfunction[189], and can be present at prodromal stages[190].

1.3.6.4 Biomarkers

There are currently no biomarkers used in the diagnosis of PD. However, biomarkers capable of diagnosing PD in the prodromal stage would be of high value both for the development of treatments and accurate identification of patient groups[191]. Potential future biomarkers include CSF α-synuclein[192, 193] and CSF protein deglycase DJ-1[192], which is a chaperone that inhibits α-synuclein aggregation[194].

1.3.7 POSTERIOR CORTICAL ATROPHY

PCA, which describes a progressive neurodegenerative syndrome, was first introduced by Benson *et al.* in 1988 after observation of five patients that had progressive dementia preceded by disorders of higher visual functions as well as predominant parieto-occipital atrophy[195]. However, others disagreed and argued that it was merely an unusual presentation of AD[196]. In later years it has been shown that the majority of autopsied brains from patients with PCA have AD pathology in the form of plaques and tangles[197]. In light of these findings, PCA is also commonly referred to as atypical AD[198, 199]. What sets PCA apart from AD is that age of onset tends to be much earlier than AD, with symptom onset reported from mid-50s to early 60s[200, 201], and some clinical features that are not typical of AD[202], such as fewer memory difficulties and greater insight of illness[200]. The effectiveness of pharmacological treatments for AD is not known in PCA[203], but most PCA patients require treatments with antidepressants[200].

1.3.7.1 Pathology

Pathological studies have shown that the underlying neuropathological cause of PCA is AD, but some cases are attributable to other causes such as DLB or CBD, meaning the presence of either Lewy bodies or astroglial inclusions of tau, respectively[203]. Using neuroimaging to localise and quantify patterns of atrophy it has been shown that patients with PCA have significant reductions of grey matter in several areas, especially in the occipital and parietal lobes, compared to controls[204, 205]. Also, greater right parietal and less left medial temporal and hippocampal atrophy was found in PCA compared to AD. Several studies have also reported asymmetric atrophy patterns in PCA with atrophy more pronounced in the right hemisphere[203]. Compared to a typical case of AD, most neuropathological examinations of PCA have revealed increased numbers of plaques and tangles in the occipital areas as well as fewer lesions in the prefrontal cortex[206, 207]. Nevertheless, considerable overlap between AD and PCA have also been reported in several regions, including inferior parietal lobe and precuneus[208] and CSF biomarkers seem to be very similar between the two diseases[209-212].

1.3.7.2 Heritability and Risk Factors

PCA has so far not been connected with any autosomal dominant mutations[203]. As in AD, *APOE* seems to be associated with PCA, but the associated risk may be smaller compared to AD. Other genes suggested to be connected to PCA are *CR1* and *ABCA7*, which encodes for complement receptor type 1 and ATP-binding cassette sub-family A member 7, respectively[213].

1.3.7.3 Diagnosis

Clinical manifestations of PCA include insidious onset and gradual progression of symptoms, prominent visuoperceptual and visuospatial impairments without significant impairment of actual vision, indication of complex visual disorders, such as visual field defects, but relatively intact memory and insight. However, once neurodegeneration spreads to anterior cortical regions patients may also experience symptoms similar to AD, such as memory loss and deficits in cognition and language [203, 214, 215]. In PCA, working memory and anterograde memory tends to be impaired while episodic memory is still intact, which is also in contrast to AD, as AD patients typically have reduced episodic memory[203, 216].

Two sets of diagnostic criteria of PCA have been proposed with suggested core features of: gradual progression and insidious onset; presentation of visual deficits without any ocular disease; relatively unaffected episodic memory, verbal fluency, and personal insight; presence of symptoms including dyspraxia and apraxia; absence of stroke or tumour. Supportive features include onset before the age of 65 and neuroimaging indicating PCA[200, 217]. The consensus classification proposes several

classification levels in order to define the disease and an extensive definition of suggested core criteria as well as staging into prodromal/suspected/possible PCA, PCA, and advanced PCA. In prodromal/suspected/possible PCA, the patient displays subtle deficits in posterior cortical functions, but symptoms are too mild or few to fulfil the core criteria. PCA is defined as fulfilment of the core criteria, and advanced PCA also fulfils the core criteria with the addition that the progression of neurodegeneration has led to impairment of other cognitive functions such as episodic memory, language, and executive functions[27].

1.3.7.4 Biomarkers

The core AD biomarkers show very similar results in AD and PCA[209-212], which, together with the fact that most PCA cases have underlying AD pathology[197], is why PCA is considered to be an atypical variant of AD[203].

1.3.8 PROGRESSIVE SUPRANUCLEAR PALSY

PSP, also called Richardson's syndrome, was first described in 1964 by John Steele and collegues[218] and is the most common form of atypical parkinsonism[219]. The prevalence is 5 per 100,000 individuals[220] and age of onset is around 63 years of age[221]. PSP is a clinical syndrome characterised by supranuclear palsy, postural instability resulting in frequent falls, and mild dementia. Since the symptomatic picture often is complex, the treatment strategy is diverse but often include levodopa, although the benefits are limited[222].

1.3.8.1 Pathology

Neuropathological changes include accumulation of tau in NFTs and neuropil threads mainly in pallidum, subthalamic nucleus, red nucleus, substantia nigra, pontine tegmentum, striatum, oculomotor nucleus, medulla, and dentate nucleus. However, these pathological changes can also be found in other diseases. The most specific pathological features of PSP are star-shaped astrocytic tufts and NFTs that can be seen with light microscopy and strongly immunostain against tau[223].

1.3.8.2 Heritability and Risk Factors

PSP is considered to be a sporadic disease. Although rare variants caused by mutations in *MAPT* have been identified, these do not share identical aetiology with the sporadic cases. Mutations in *LRRK2* and *PRKN* have been observed in autopsy-confirmed cases, but it is not clear how these mutations may influence PSP[224].

1.3.8.3 Diagnosis

The most widely used criteria for ante-mortem diagnosis of PSP are the National Institute of Neurological Disorders and Stroke and Society for PSP (NINDS-SPSP)

clinical diagnosis criteria published in 1996[225]. The 1996 NINDS-SPSP diagnostic criteria have very good specificity for both possible and probable PSP, with around 80-93% and 95-100% respectively, as confirmed by autopsy[224]. However, these criteria failed to diagnose cases that were not typical or that were early in the disease course. Therefore, revised guidelines aimed to optimise early, sensitive, and specific clinical diagnosis were recently presented. The revised criteria for possible or probable PSP contain mandatory inclusion criteria of: sporadic occurrence; age 40 or more at onset of first PSP symptoms; gradual progression of PSP symptoms. There are also mandatory exclusion criteria as well as context-dependent exclusion criteria. Symptoms are divided into four functional domains: ocular motor dysfunction; postural inability; akinesia; cognitive dysfunction. Each functional domain also has three levels of certainty. In addition, there are also supportive features such as levodopa-resistance and photophobia[224].

1.3.8.4 Biomarkers

CSF NfL might be useful for diagnosis as it is increased in PSP compared to PD and DLB[226]. In a clinical trial, where several biomarkers were monitored longitudinally, CSF NfL was the only biomarker that changed over time, and it also correlated with changes in several clinical symptoms, indicating that it could be used to monitor progression of the disease[227]. NfL can also be detected in plasma, and PSP plasma NfL is increased compared to controls and PD. In addition, baseline concentrations of plasma NfL can be used to predict disease progression[226].

1.4 SYNAPSES

Synapses are the functional units of the brain, communicating signals between neurons. A human brain is estimated to have approximately 100 billion neurons with an estimated average of 7000 synapses each, and the adult human brain is thought to have 100 to 500 trillion synapses[228]. Synapses are composed of a presynaptic (axonal) terminal that is connected with a postsynaptic dendritic domain called the dendritic spine. Neurotransmitters are released by the transmitting synapse in response to an influx of Ca^{2+} caused by an action potential. When the neurotransmitters are received by receptors on the dendrite, it results either in a changed transmembrane potential, which is either inhibitory or excitatory, or a modulated production of messenger molecules which can amplify both excitatory and inhibitory signals. Excitatory transmembrane potentials trigger an influx of positive ions, *i.e.* K^+, Na^+ and Ca^{2+}, resulting in a slightly positive overall charge of the cell, while inhibitory potentials result in a slightly negative net charge[229].

Research has shown that synapses are not static, but in fact plastic. Synaptic plasticity is the basis for most models of memory and learning. Long-term potentiation (LTP), and the converse process long-term depression (LTD), is carefully controlled activity-dependent modification of synapses resulting in a long-lasting increase or decrease of synaptic efficiency[230]. LTP causes an influx of Ca^{2+} which subsequently results in the activation of calcium-calmodulin-dependent protein kinase II (CaMKII) by calmodulin. Ca^{2+} alters the affinity of calmodulin for its target proteins thereby increasing the probability of it to activate CaMKII. However, the availability of calmodulin is regulated by Ng. Upon activation, CaMKII interacts with neurotransmitter receptors at the synapse. CaMKII also have a role in enlargement and strengthening of synapses in later stages of LTP[231]. It has been shown that both Aβ dimers isolated from AD brain tissue[232] and naturally secreted Aβ oligomers[233] are capable of inhibiting LTP as well as enhancing LTD[234]. It has been proposed that the association between AD and LTP is mediated by Aβ peptides that reduce LTP and/or promote LTD, which leads to delays in basal synaptic transmission ultimately resulting in synaptic loss and neurodegeneration[235]. As already mentioned, synaptic loss is a central event in AD[236], but it also plays a part in many other neurodegenerative diseases such as PD and multiple sclerosis[237].

1.5 NEUROGRANIN

Ng is a 78 aa long postsynaptic protein that was first identified in bovine brain tissue in the late 1980s[238]. Although Ng is considered to be neuron-specific, it is also expressed in low levels in lung tissue, bone marrow, and the spleen. Also, platelets have been shown to express high levels of Ng[239]. In the brain, Ng is present in dendritic spines of neurons within associative cortical areas, including the hippocampus, amygdala, and cerebral cortex[239, 240]. Studies on the human brain using *in situ* hybridisation have shown that there is a selective translocation of Ng mRNA to dendrites, and that such translocation is impaired in the cortex of sAD patients[241]. Ng is an important unit for LTP function through its interaction with calmodulin. During resting state, Ng binds to calmodulin via an IQ motif (aa 33–46) which is well conserved among other calmodulin-binding proteins. Upon Ca^{2+} entry in response to neurotransmitters, Ng is phosphorylated at S36 by protein kinase C (PKC) and is thereby inhibited from binding calmodulin[242] (Figure 2). However, the phosphorylation is transient[243], and Ng is in this manner able to regulate the concentration of calmodulin by responding to intracellular concentrations of Ca^{2+} levels following neuronal excitation. Phosphorylation of Ng seems to be needed for efficient LTP, as mutated Ng that could not be phosphorylated resulted in significantly reduced LTP, most likely through sequestering of calmodulin, thus stopping it from activating its targets, such as CaMKII[244]. In mice, concentrations of both Ng protein and mRNA decrease with age and these changes are related to CNS dysfunction[245].

Furthermore, reduced cognition and LTP is displayed in knockdown mouse models[246]. In contrast, upregulation of Ng expression results in improved cognition and LTP[247], thus signifying the importance of Ng in these processes. Staining of human cortex for Ng shows a synaptic-like pattern[248].

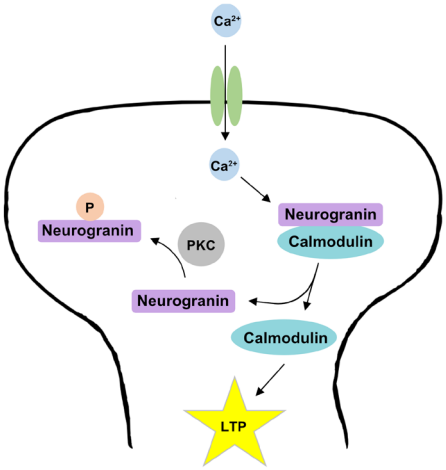

Figure 2. The role of neurogranin in long-term potentiation*. In response to the influx of Ca^{2+}, Ng dissociates from calmodulin and is phosphorylated at S36 by PKC, which prohibits Ng from binding calmodulin. Once calmodulin no longer is bound by Ng, it will interact with downstream targets, such as CaMKII which results in increased LTP.*

When studying Ng in human brain tissue using semi-quantitative western blot (WB) it was shown that Ng levels in both the frontal cortex and hippocampus of AD were decreased compared to controls[249]. In 1999 it was shown that Ng, as well as other synaptic proteins, are present in human CSF[250], and in 2010, a first study showed that, in contrast to brain tissue, the relative CSF levels of Ng are increased in AD compared to controls[108]. During the last few years, the interest of Ng as a possible biomarker for AD has increased. Several independent studies have shown that CSF Ng, measured by different immunoassays, is increased in sAD compared to controls[251-255] and it is now a well-replicated biomarker for sAD (http://www.alzforum.org/alzbiomarker).

2 AIM

2.1 GENERAL AIM

The overall aim of the work included in this thesis is to characterise Ng in CSF, plasma, and brain tissue of controls and AD patients, develop methods for quantification of Ng and to test the hypothesis that Ng is a novel biomarker for AD.

2.2 SPECIFIC AIMS OF EACH PAPER

Paper I: Evaluate in-house developed monoclonal Ng antibodies and use them to characterise Ng in CSF and brain tissue. Furthermore, the purpose is to quantify Ng in CSF using both HI-MS and ELISA in order to evaluate CSF Ng as a novel biomarker in AD.

Paper II: Characterise Ng in plasma and to evaluate if plasma Ng can be used as a novel biomarker for AD. In addition, the aim is to test the stability of CSF Ng during storage and investigate if the detected peptides are continuously generated in CSF and plasma.

Paper III: Investigate if increased CSF Ng is specific for AD compared to other neurodegenerative diseases and evaluate how CSF Ng, as measured by ELISA, correlates with pathology in AD.

Paper IV: Characterise endogenous Ng peptides as well as full-length Ng, including possible post-translational modifications (PTMs), in brain tissue in addition to quantifying both peptide and full-length Ng in brain tissue from two regions of sAD, fAD, controls and CU-AP individuals.

3 MATERIALS AND METHODS

3.1 SUBJECTS AND SAMPLE COLLECTION

All subjects gave their informed consent, and recruitment, as well as sample collection, was performed in accordance with ethical permissions approved by regional ethical committees. Detailed demographics of all participants, as well as diagnosis criteria, can be found in the respective papers.

De-identified CSF samples from the Clinical Neurochemistry Laboratory at the Sahlgrenska University Hospital, Mölndal, Sweden, was used for method development.

3.1.1 CEREBROSPINAL FLUID

In Papers I-III, CSF was collected according to standardised procedures. Briefly, 12 mL of CSF was acquired via LP through the L3/L4 or L4/L5 interspace and collected in polypropylene tubes. Insoluble material was subsequently removed by centrifugation at 2000 × g at +4 °C for 10 min before the supernatant was transferred and aliquoted into new tubes. The CSF was finally stored at −80 °C.

3.1.2 PLASMA

Plasma, which was used in Paper II, was obtained by centrifugation of whole blood that had been collected in tubes coated with the anticoagulant ethylenediaminetetraacetic acid (EDTA) at 2500 × g at +4 °C for 10 min. The plasma was aliquoted in polypropylene tubes and stored at −80 °C.

3.1.3 BRAIN TISSUE

Brain tissue was used in Papers I and IV. One hemisphere was fixed in formalin solution for several weeks, followed by dissection and both histological and immunohistological staining. Neuropathological diagnosis and scoring were made by neuropathologists. The other hemisphere was flash frozen and stored at −80 °C.

3.2 IMMUNOPRECIPITATION

In CSF, the three most abundant proteins together constitute around 80% of the total protein content, making sample clean-up and/or enrichment helpful for some analyses as well as necessary before MS analysis[256]. Immunoprecipitation (IP) is a highly efficient way to enrich peptides and/or proteins from complex samples such as CSF, plasma, or tissue homogenates. IP combined with MS (IP-MS) (further on referred to as hybrid-immunoaffinity mass spectrometry (HI-MS)) can be used in many ways such

as determining PTMs, identifying protein complexes[257] or characterising endogenous peptides from a protein[60]. There are several ways to perform an IP; in this thesis, the method was based on magnetic beads to which in-house generated anti-Ng antibodies were bound. Briefly, the sample is incubated with the bead-antibody complex on a mixer to allow binding of the protein of interest. The incubation is followed by several washing steps and finally elution[60] (see Figure 3 for an overview). In order to minimise binding of unspecific proteins to the beads as well as prohibit large amounts of the Ng antibodies from being eluted with the sample, which can disturb MS analysis, in particular, MALDI analysis of full-length Ng, crosslinking can be utilised. By crosslinking, the antibodies are covalently bound to the beads often using either dimethyl pimelimidate (DMP) or bis[sulfosuccinimidyl] suberate (BS^3), but the crosslink reaction can also decrease the efficacy of antigen binding[258]. Quantification is possible by the addition of a known concentration of one or more stable heavy isotope-labelled peptide(s), using for instance ^{15}N or ^{13}C, preferably corresponding to the peptide(s) one is interested in quantifying. In Paper I, IP followed by WB was used to show that the in-house generated monoclonal anti-Ng antibodies were able to detect endogenous Ng in brain tissue homogenates. HI-MS was used in Papers I, II, and IV in order to characterise and relatively quantify full-length and peptide Ng in CSF, plasma, and brain tissue.

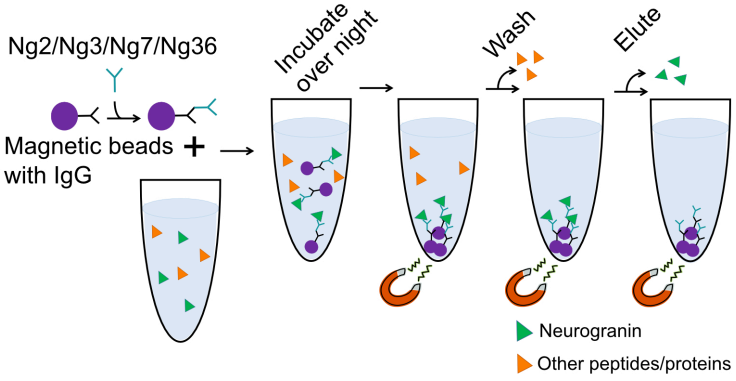

Figure 3. Schematic picture of immunoprecipitation of neurogranin used in this thesis.

3.3 IMMUNOASSAYS

There are many different types of immunoassays, but what they all have in common is that antibodies are used to detect and quantify a certain analyte. It should also be noted that immunoassays can be used to determine the presence of antibodies in a sample as well, and in those instances, an antigen is used for detection instead. Typically, an immunoassay involves binding of the analyte, several washing steps, binding of the detection antibody that is coupled to some type of detectable label followed by a

chemical or enzymatic reaction that allows detection and quantification. There are many types of labels, including radioactive isotopes, fluorogenic reporters and electrochemiluminescent (ECL) tags, but the most commonly used is enzymes. Immunoassays that are based on enzymatic reactions are called enzyme-linked immunosorbent assays (ELISAs). In a sandwich ELISA, two antibodies against the same antigen are used; one for coating the microtiter plate that is used for capturing of the antigen, and another for detection. A common set up in ELISA is to have the detection antibody conjugated to biotin and add streptavidin-conjugated horseradish peroxidase (HRP), followed by the addition of chromogenic substrate. It is the colour change following the HRP enzymatic reaction that is detected and quantified using a spectrophotometer, *i.e.* ELISA reader. In ECL, light is emitted from electrochemically generated intermediates that undergo a highly exergonic reaction. Luminophores, which are the functional groups responsible for luminescence of a compound, generally ruthenium complexes such as tris(bipyridine)ruthenium ([Ru (bpy)3]2^{+}), emits light after excitation following electron-transfer reactions[259]. Some results indicate that ECL assays might provide increased sensitivity and broader dynamic range compared to conventional ELISA, and some systems also offer the possibility to multiplex analytes in the same assay[260].

In Papers, I, II, and III immunoassays were used to quantify $A\beta_{1\text{-}42}$, p-tau, and t-tau using commercially available kits in order to biochemically classify patients as AD or control. In addition, in Papers I, III, and IV, Ng was quantified in CSF and brain tissue using in-house developed ELISAs based on in-house generated monoclonal anti-Ng antibodies. In Paper II, an in-house developed assay using in-house generated monoclonal anti-Ng antibodies and Meso Scale Discovery (MSD) Technology, which is based on ECL, was used to quantify Ng in CSF and plasma samples.

3.4 WESTERN BLOT

WB is a technique used for detection of specific proteins in for instance tissue samples or bodily fluids such as CSF. The sample can also be purified or enriched before WB by for instance IP. The basis for WB is gel electrophoresis which separates the proteins in a sample according to size. Gel electrophoresis can be performed in denaturing or non-denaturing, so-called native, conditions. Native gel electrophoresis can be useful since the activity of enzymes is kept intact. The difference between native and denaturing gel electrophoresis is that in native gels the proteins are separated based on both their molecular weight and tertiary structure, while separation during denaturing conditions is based only on molecular weight as the tertiary structure is destroyed. In WB, proteins in the sample are denatured and separated according to size during gel electrophoresis after which they are transferred to a membrane and detected using an antibody that is specific to the protein of interest. Lastly, a secondary antibody, that

recognises the first antibody is added and detected using for instance chemiluminescence based on streptavidin, biotin, and HRP, similar to the previously described ELISA procedure, but with a luminescent substrate instead of a chromogenic one. A very common set up is polyacrylamide gel electrophoresis (PAGE) combined with sodium dodecyl sulphate (SDS). In SDS-PAGE, the proteins are negatively charged by SDS and are then forced to migrate through the polyacrylamide network by applying a current[261]. Larger proteins will migrate more slowly compared to smaller ones, and different percentages or concentration gradients of polyacrylamide can be used depending on the size of the protein(s) of interest. Using commercially available software, such as ImageJ (Rasband, WS, ImageJ; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij), the detected bands can be relatively quantified. In Paper I WB was used to evaluate the specificity of in-house generated monoclonal anti-Ng antibodies and in Paper IV to relatively quantify full-length Ng in brain tissue.

3.5 STATISTICS

Statistical analyses were performed using GraphPad Prism for Windows software (GraphPad Software, La Jolla, CA, USA), SPSS (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp.) and R programming language (R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/). Because biomarker values were skewed, non-parametric tests were used; Mann-Whitney U-test was used when comparing two groups or Kruskal-Wallis test when comparing three or more groups with post-hoc pairwise comparisons when appropriate. Associations between biomarker values were determined using Spearman's rank correlation. All tests were two-sided with a significance level set to $p \leq 0.05$. p values for each family of comparisons were adjusted for multiple comparisons using Holm's method to control the family-wise error rate. Graphs were created using GraphPad Prism.

3.6 LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY

Proteomics is a research field often associated with the identification of new biomarkers. In short, proteomics is the study of the proteome, which refers to the entire set of proteins that is expressed by a cell, a tissue or an entire organism[262]. There are many methods that can be applied in proteomics, and they are generally based on a sample preparation step, in order to reduce the complexity of the sample prior to MS analysis. MS is extremely useful as it allows for the identification of proteins and peptides in a biological sample. However, biological samples such as CSF are very complex, containing a multitude of proteins, salts, and cell debris, sometimes referred

to as interfering matrix components, which can interfere with sample analysis thus making identification difficult[263]. For example, the difference in concentration between high and low abundant proteins in plasma is up to ten orders of magnitude[264]. Sample clean-up and preparation is therefore important and sometimes even necessary. However, each pre-analytical step might also cause losses of the protein of interest, in addition to the risk of introducing contaminants, which may compromise the MS analysis. Many sample clean-ups and preparations utilise separation of the analytes based on size or biochemical properties in order to decrease the complexity and total protein concentration of the sample. One of the most used methods in today's proteomics is liquid chromatography (LC) combined with MS (LC-MS) as it takes advantage of the separation abilities of LC together with the very sensitive mass analysis capabilities of MS. The principle of MS is that molecules are transferred from a solid or liquid state into gas phase, ionised, and separated based on their mass/charge (m/z). There are many different ionisation techniques, two of the most common ones are matrix-assisted laser desorption/ionisation (MALDI) and electrospray ionisation (ESI), both of which are soft ionisation techniques meaning that there will be relatively little degradation or fragmentation of the analyte. MS can be used to determine the aa sequence which is accomplished by tandem MS, often called MS/MS (mass selection/mass separation). MS/MS can also be employed to identify PTMs. PTMs are chemical alterations to proteins occurring after protein synthesis is finished and more than 300 kinds of PTMs have been discovered[265]. PTMs can alter protein function, and some PTMs can also be reversible, a common example being phosphorylation in signal transduction pathways[266]. Identification of PTMs and understanding their impact on protein function is therefore very important. Although MS is a very useful tool for identification of PTMs on proteins, analysis and correct identification can be very complicated[265]. In Paper IV, PTMs that had not previously been described on human Ng were characterised and identified using MS. Depending on the aim and methodology, MS can be used for both identification and quantification[267]. In Papers I, II, and IV, HI-MS using both nano-LC-MS and matrix-assisted laser desorption/ionisation-time-of-flight (MALDI-TOF) was used.

3.6.1 LIQUID CHROMATOGRAPHY

A chromatographic system consists of a stationary phase through which the sample is carried by a mobile phase. LC separates molecular species according to their physicochemical properties, *e.g.* hydrophobicity, charge, isoelectric point etc., using liquid as the mobile phase and a stationary phase. The stationary phase is a column packed with either monolithic material or silica beads onto which carbon chains of different lengths (C4, C8, C18 etc.) can be attached. Sample molecules that interact strongly with the stationary phase will have a longer retention time from the LC system while those that do not interact very strongly will elute faster. The system used in this work was mainly reversed-phase chromatography utilising a non-polar stationary

phase and a polar liquid phase. Here, analytes were separated based on their hydrophobicity through interactions with carbon chains (C18) inside the column and were eluted from the stationary phase by an increasing gradient of organic solvent, in this case, acetonitrile (ACN), in the mobile phase[268]. In order to obtain higher resolution and faster analyses, high-performance (previously high-pressure) liquid chromatography (HPLC) was utilised. In HPLC, the column packing material is smaller and more uniform in size combined with increased flow pressure compared to conventional LC at low pressure. LC can be performed either online, *i.e.* with the LC system connected directly to the mass spectrometer, or offline[269].

3.6.2 IONISATION TECHNIQUES

3.6.2.1 Matrix-Assisted Laser Desorption/Ionisation

In MALDI, the analyte is directly embedded in a matrix in crystallized form consisting of a light absorbing low molecular mass molecule[269] on a target plate[270]. Important features of matrices are the capability to absorb the appropriate wavelength, rapid transition into gas phase, and the ability to ionise embedded analyte molecules without too much heating[269]. One of the most common matrices for analysis of smaller proteins and peptides is α-Cyano-4-hydroxycinnamic acid[271]. The molecules of the matrix/analyte mixture are evaporated into gas phase by a laser pulse from which the matrix absorbs light energy, resulting in excitation and ionisation of a fraction of the analyte molecules. One advantage of using MALDI is that it is quite tolerant to sample contamination. However, a complex sample such as CSF needs to go through some type of sample clean-up or enrichment, as analysis otherwise most likely only will detect the most abundant proteins or suffer from too high salt content. MALDI is most commonly combined with a TOF mass analyser[272].

3.6.2.2 Electrospray Ionisation

In ESI, the sample is dissolved in a polar, volatile solvent and passed through a needle which is placed at high electric potential relative to the inlet of the mass spectrometer. The high difference in potential causes the fluid to form a so-called Taylor cone, causing a spray of charged droplets. As the sample droplets approach the heated inlet, they will evaporate and enter the spectrometer as gaseous ions. However, it is not entirely known how ions are formed during this process[273-275]. In ESI, the sample needs to be dissolved in a mixture of water and organic solvent. ESI can be run in negative or positive ionisation mode, and to aid in the formation of positive ions formic acid (FA) can be added while ammonia is often added to help form negatively charged ions. ESI is less tolerant to contaminants than MALDI and often requires separation of the proteins/peptides in the sample. However, ESI is readily combined with on-line LC in order to separate the analytes based on their chemical properties, thus reducing the complexity at a given acquisition time point, which in the end increases the overall

sensitivity and specificity of the MS analysis. ESI ions are especially favourable for fragmentation using collision-induced dissociation (CID) in which molecular ions are fragmented by collision with inert gas atoms or molecules for MS/MS analysis. Higher flow rates between 1 and 1000 μL / min are used in conventional ESI-MS, but lower flow rates between 20 and 300 nL / min can be used in nano-ESI. The advantages of nano-ESI compared to conventional ESI are lower sample consumption, significantly higher sensitivity as well as higher tolerance to salt[272].

3.6.3 MASS ANALYSERS

3.6.3.1 Time of Flight

In TOF, the molecular m/z's of the analytes are calculated from their time of flight. Ions are accelerated in an electrical field, and all ions with the same charge will obtain the same kinetic energy, but their velocity is mass-dependent, meaning that heavier ions will have lower velocity compared to lighter ions. A double-charged (2+) ion will have a greater velocity compared to the corresponding single-charged (1+) ion. The analytes will travel from the target plate through a drift region where they will be separated based on their velocity towards a detector, and the difference in time between the starting pulse and when the ions hit the detector is the time of flight (t_{TOF}). TOF requires a well-defined starting signal which makes MALDI and TOF a very good match since the MALDI laser pulse can be used as a starting point for the time measurement. TOF can be used in two modes; linear and reflector mode. When using linear mode, ions with identical m/z will, due to a small initial velocity spread, arrive at the detector at slightly different time points which will result in broader peaks in the mass spectrum subsequently limiting the resolution and thereby mass accuracy. In order to reduce the spread, a reflector is used as an energy focusing device through de- and re-acceleration of the ions in a strong electrical field which will give differentiated flight paths thus compensating for the initial spread in velocity[272]. TOF can also be used for identification of proteins and peptides by TOF tandem mass spectrometry (TOF/TOF), for details see Westman-Brinkmalm and Brinkmalm[276]. In Papers I, II, and IV MALDI-TOF/TOF was employed to identify multiple endogenous Ng peptides in CSF, plasma, and brain tissue. MALDI-TOF was used to detect and relatively quantify several Ng peptides in several cohorts included in Papers I, II, and IV.

3.6.3.2 Quadrupole

The quadrupole consists of four parallel rods in a symmetrical configuration. The rods have superimposed direct current (DC) and radio frequency (RF) potentials. Opposing rods are electrically connected pairwise with the potential of the other pair having the opposite sign. The potentials can be applied so that only ions within a desired m/z range will have a stable trajectory allowing them to travel through the entire quadrupole. The quadrupole allows for very high sensitivity when operated as a mass

filter, *i.e.* monitoring a fixed m/z. When analysing a broader m/z range, it has to be operated in scanning mode which will significantly decrease sensitivity[272]. Quadrupoles are commonly used in hybrid instruments where it often functions as a mass filter to select precursor ions[272].

3.6.3.3 Orbitrap

The most recently developed mass analyser is the orbitrap. It utilises an electric field in order to trap ions in an orbital motion. The orbitrap consists of two electrodes; a symmetrical, barrel-shaped outer electrode which surrounds the inner electrode. The injected ions are trapped in a circular motion around the inner electrode. At the same time, they are also trapped in an oscillatory motion along the inner electrode axis in a manner that is dependent on their m/z. Contrary to the case in, for example, TOF-MS, the detection in the orbitrap is achieved by means of image current detection of the oscillating ions[272]. Hybrid instruments combining the orbitrap with a linear ion trap or a quadrupole have become popular today as they provide both high mass accuracy, high resolution, and high sensitivity[272]. In Papers I, II, and IV the hybrid instrument Q Exactive from Thermo Fisher Scientific was used as a high-resolution instrument in order to maximise the identification of Ng peptides as well as identifying PTMs on full-length Ng in Paper IV. The Q Exactive consists of a quadrupole mass filter, the orbitrap, and a higher-energy collisional dissociation (HCD, a variant of CID) cell, where precursor ions are fragmented by collision with N_2 gas molecules for MS/MS[277].

3.6.4 DATABASE SEARCHES

MS allows for determining the aa sequence of proteins and peptides present in a sample, and that information can, in turn, be used to identify the protein. However, as the human proteome consists of roughly 20,000 proteins, it is not feasible to identify the protein or peptide manually. The sequence, *i.e.* primary structure, is known for all human proteins, thus allowing for searching against a database containing all sequences. The most common method that database searches are based on today is MS/MS spectra derived from individual peptides from an enzymatically digested protein. It is also possible to use databases to search for endogenous peptides, *i.e.* peptides that are not created from protein digestion during sample preparation but that are naturally present in the biological sample. There are a number of software available which are based on different algorithms[278]. Most software also allows for identification of PTMs as well. In Papers I, II, and IV all database searches were performed using MS/MS data from endogenous Ng species.

4 RESULTS AND DISCUSSION

4.1 PAPER I

As synaptic pathology is an early event in AD and also correlates better with cognitive decline compared to AD neuropathology[100, 102, 103], synaptic proteins are candidates for new biomarkers of AD. The postsynaptic protein Ng has in mouse models been shown to be important in the formation and storage of long-term memories through LTP[246, 247]. In addition, by using semi-quantitative WB, it was previously shown that the CSF levels of Ng are increased in AD compared to controls[108].

In order to study Ng in detail, three monoclonal Ng antibodies were developed; Ng2, Ng3, and Ng7. These, along with the commercially available polyclonal antibody ab23570 (Upstate), were shown to detect both recombinant and endogenous full-length Ng using WB, HI-MS, and direct ELISA. Ng2, Ng3 and Ng7 were epitope mapped, using a series of short overlapping peptides in ELISA and HI-MS. All three antibodies were found to bind the C-terminal region of Ng with Ng2 recognising aa 52-63, Ng3 recognising aa 54-65, and Ng7 recognising aa 52-65 (Figure 4). In the HI-MS experiments, a combination of Ng2 and Ng3 was used as this combination yielded the best signal-to-noise using MALDI-TOF. In order to relatively quantify Ng peptides in clinical samples, a known concentration of an internal standard, consisting of a heavy isotope-labelled version of Ng43-75, was added to all samples. A sandwich ELISA using Ng7 for capture and Upstate for detection was also developed.

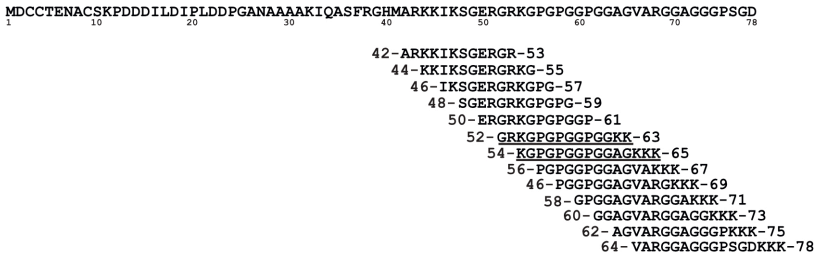

Figure 4. Epitope mapping of in-house generated anti-neurogranin antibodies. *The antibody Ng2 reacted with the peptide Ng52-63, Ng3 reacted with Ng54-65, and Ng7 reacted with both of these peptides.*

Using HI-MS, we showed that Ng is present as several endogenous peptides in both CSF (Figure 5) and brain tissue, and that full-length Ng is readily detectable in brain tissue, but not CSF, and that it is most likely subjected to PTMs. In total, 15 endogenous Ng peptides were identified in CSF and nine in brain tissue, of which several were present in both. The major peaks corresponded to Ng43-75 and Ng44-75 in CSF (Figure 5), and Ng48-76 in brain tissue. Most of the identified peptides ended at aa 75, 76 or 78 in both CSF (Figure 5) and brain tissue.

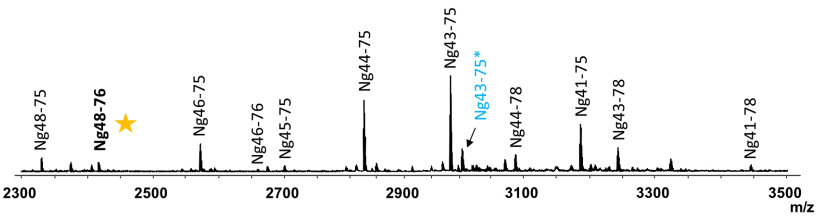

Figure 5. MALDI-TOF spectrum displaying several C-terminal neurogranin peptides in cerebrospinal fluid. *Ng48-76, which is marked by a star, was significantly increased in sAD compared to controls in two separate cohorts in this paper. Ng43-75* represents the heavy isotope-labelled peptide that was used as an internal standard during quantification.*

In order to evaluate CSF Ng as a potential novel biomarker for AD, Ng was quantified using both ELISA and HI-MS in a pilot study of CSF from 16 sAD patients and 10 healthy controls. HI-MS analysis of C-terminal peptides showed that Ng48-76 was significantly increased in sAD CSF compared to controls ($p = 0.002$) and that several other peptides had a trend towards increased concentrations in sAD but failed to reach statistical significance. ELISA showed similar results with increased CSF Ng concentrations in sAD compared to controls, $p < 0.0001$ (Figure 6A). A second study on CSF from 44 sAD patients and 30 controls was analysed, and increased CSF Ng in sAD compared to controls was confirmed by both ELISA ($p < 0.001$) (Figure 6B) and HI-MS (Ng48-76, $p < 0.05$). The ELISA and HI-MS results showed a significant correlation in both study 1 ($r_s = 0.88$, $p < 0.0001$) and study 2 ($r_s = 0.70$, $p < 0.0001$). A third independent study included 40 individuals each of sAD, MCI, and healthy controls. This cohort was aimed to verify the results of the two pilot studies as well as testing the performance of CSF Ng in the early stages of sAD and was analysed only by ELISA due to the large volume needed for HI-MS analysis (1 mL). The MCI patients were followed between 2 and 4 years. The analysis showed that CSF Ng was increased in sAD compared to controls ($p < 0.001$) also in this cohort. Furthermore, when the MCI group was divided into stable MCI (sMCI) and MCI progressing to dementia due to sAD (MCI-AD), MCI-AD had significantly increased CSF Ng compared to both controls ($p < 0.001$) and sMCI ($p < 0.001$) while sMCI had lower CSF Ng compared to sAD ($p < 0.05$). There was no difference between controls and sMCI, or MCI-AD and sAD (Figure 6C).

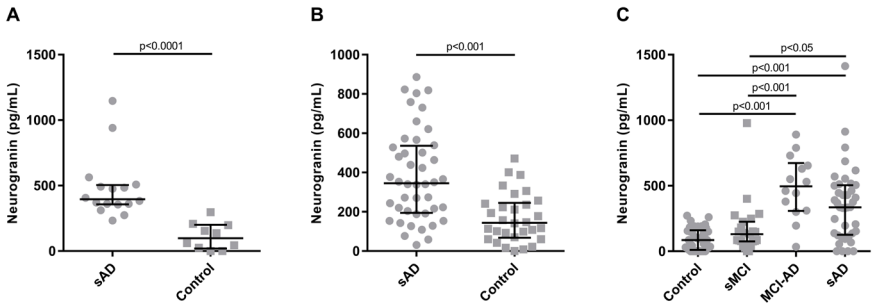

Figure 6. Scatter plots displaying cerebrospinal fluid neurogranin concentrations quantified by ELISA *from* ***(A)*** *Study 1* ***(B)*** *Study 2* ***(C)*** *Study 3. Data presented are median and interquartile ranges.*

The concordance between biomarker positive for $A\beta_{1\text{-}42}$ and Ng, defined as <634 pg/mL and >237 pg/mL respectively, was 75%. Cox proportional hazard models were used to assess the capability of CSF Ng to predict conversion from MCI due to sAD and dichotomised CSF Ng concentrations of 152 pg/mL predicted conversion with a hazard ratio of 12.8, thus indicating an increased risk of conversion to AD. Although CSF Ng was not associated with MMSE at baseline, there was a high association with annual decline in MMSE ($p = 0.001$). Both t-tau ($r_s = 0.70$, $p < 0.0001$) and p-tau ($r_s = 0.61$, $p < 0.0001$), but not $A\beta_{1\text{-}42}$, were strongly correlated with CSF Ng, especially in sAD and MCI but also in controls.

In this paper, we showed for the first time that Ng is present as multiple endogenous peptides in both CSF and brain tissue, some of which were found in both. Furthermore, the data also show that full-length Ng in brain tissue most likely has several PTMs present but that concentrations of full-length Ng in CSF probably are very low compared to brain tissue. We found that CSF Ng is markedly increased in both sAD and MCI compared to controls and that CSF Ng, as measured by ELISA, was associated with CSF tau as well as decline in MMSE. These results suggest that CSF Ng reflects cortical synaptic degeneration as defined by CSF increase of tau and that CSF Ng is a prognostic biomarker for cognitive decline. As the antibodies used for HI-MS both had epitopes in the C-terminal region, no N-terminal peptides were found. Future studies using antibodies with epitopes near the N-terminal or mid-region are needed to elucidate if N-terminal fragments are also present in brain tissue and CSF.

Just as the specific peptide $A\beta_{1\text{-}42}$ is a biomarker for AD, Ng48-76 was increased in sAD in two separate cohorts. Such an increase could indicate that it may be useful to monitor specific Ng peptides rather than, or perhaps as a complement to, ELISA which most likely detects many different peptide species, even though the two methods

correlated well. Since Ng48-76 was the dominating peptide in brain tissue as well as significantly increased in sAD CSF, our data indicate that this specific peptide is closely related to AD pathology.

The most important findings in Paper I were that CSF Ng is increased in sAD and MCI due to AD, and that the specific peptide Ng48-76 is increased in sAD CSF.

4.2 PAPER II

Although Ng is considered to be a neuron-specific protein, it is also expressed in the periphery by platelets and a couple of other cell types and tissues[239]. Since blood is much easier to access compared to CSF, blood-based biomarkers are highly sought after. Therefore, the aim was to characterise Ng in plasma and investigate if plasma Ng could be used as a biomarker for AD, as well as to compare peripheral Ng with Ng originating from the CNS, *i.e.* CSF and brain tissue.

In this paper, an MSD immunoassay, based on Ng7 for capture and Upstate for detection, and HI-MS with Ng2 and Ng3 were used for analysis.

Using HI-MS, plasma Ng was characterised and 16 endogenous Ng peptides were identified, most ending at aa 75 and 78, of which seven had not been detected in CSF or brain tissue previously. The most abundant peptides were Ng43-75 and Ng44-75 (Figure 7). Full-length Ng was also present in plasma and was most likely altered by PTMs.

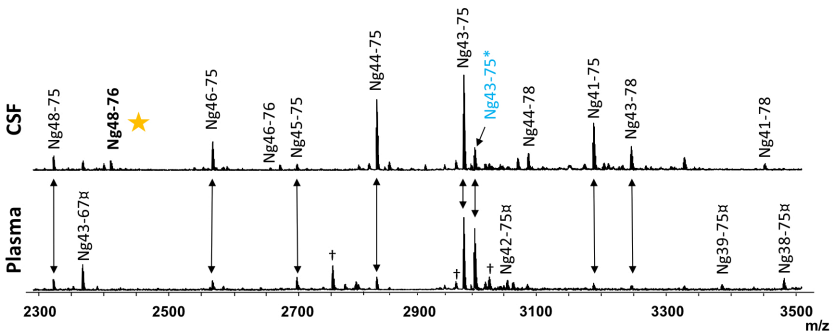

Figure 7. MALDI-TOF spectra displaying several C-terminal neurogranin peptides in cerebrospinal fluid and plasma. *In plasma, none of the detected peptides differed significantly between sAD and controls. Ng43-75* represents the heavy isotope-labelled peptide that was used as an internal standard during quantification. Ng48-76, which is marked by a star, was found to be increased in sAD CSF in Paper I. ¤ signifies peptides that were only found in plasma. † denotes peaks caused by non-specific binding of other plasma proteins to the magnetic beads used in HI-MS.*

A cohort of paired plasma and CSF samples from 25 sAD individuals and 20 healthy controls was analysed by both HI-MS and MSD (plasma samples) or only MSD (CSF samples). In the HI-MS analysis, several Ng peptides were relatively quantified in plasma, but none were found to be significantly different between the groups. MSD analysis showed similar results. A second validation study of plasma samples analysed by HI-MS also failed to identify any significant difference between the groups for any of the detected peptides. However, MSD analysis of the paired CSF samples showed a marked increase of Ng in sAD compared to controls ($p < 0.0001$). There was no correlation between plasma and CSF Ng. In the paired plasma and CSF cohort the controls were significantly younger than the sAD group, but there was no association between Ng and age.

The stability of CSF Ng during storage was evaluated by MSD analysis of CSF kept at room temperature, +4 °C, or −20 °C for between 1 and 7 days. At both +4 °C and −20 °C Ng concentrations were not significantly altered during 7 days. When stored at room temperature for 2 days, the CSF Ng concentrations did not change. However, after day two there was a 30% drop in concentration. To address the question of whether any degradation of full-length Ng is taking place in CSF and plasma, multiple plasma and CSF samples were depleted of all detectable native Ng by three consecutive IPs before recombinant full-length Ng was added. After a 24h incubation at room temperature, the samples were analysed by HI-MS in order to screen for degradation products in the form of newly formed peptides. In CSF, there was no *de novo* formation of Ng peptides, while in plasma, 14 Ng peptides were detected after the incubation.

In this paper, we showed for the first time that Ng is present in plasma as both modified full-length protein and many endogenous peptides. Seven of the identified peptides have only been detected in plasma and not in CSF or brain tissue (see Paper I), and thus seems to be specific for the plasma Ng profile. We also found that recombinant full-length Ng was degraded into 14 peptides in plasma. These proteolytic peptides are most likely formed by some of the proteases present in plasma. Some of the *de novo* peptides corresponded to naturally occurring Ng peptides in plasma and CSF, while four did not have any previously identified native counterparts.

It is of particular worth to note that Ng48-76, which was identified as increased in sAD CSF in Paper I, was not naturally present nor produced upon incubation of recombinant full-length Ng in plasma. It is therefore unlikely that the enzyme(s) responsible for these specific cleavages (both at the N- and C-terminus) are present or active in plasma, at least under the conditions of the experiments. Since there are several peptides ending at aa 76 present in both brain tissue and CSF, but not plasma, and this particular cleavage does not seem to occur in the periphery, the data points towards two separate pools of Ng with very little to no exchange between them; a CNS pool consisting of

the species found in CSF and brain tissue and a peripheral pool, *i.e*. plasma and perhaps other tissues as well. This scenario is further supported by the fact that the cluster of peaks representing full-length Ng with PTMs looked distinctly different in plasma compared to brain tissue.

The key findings in Paper II were that full-length Ng is not degraded into peptides in CSF and that there are no native or *de novo* formed Ng peptides in plasma that ends at aa 76. This is of great importance because it suggests that CSF Ng, and in particular Ng48-76, reflects the active neurodegenerative processes of the brain in AD. In conclusion, CSF Ng might be a novel clinically accessible biomarker for synaptic function in sAD. However, this hypothesis needs to be thoroughly investigated in future studies.

4.3 PAPER III

Others have previously shown that increased CSF Ng seems to be specific for AD[279-282]. However, these studies have been fairly small and did not include a definitive neuropathological diagnosis. In this paper, the aim was to investigate if elevated CSF Ng is specific to AD by quantifying Ng in CSF from seven other neurodegenerative disorders, including cases with definitive neuropathological diagnosis, in order to test the hypothesis that increased CSF Ng is specific for AD.

We developed and optimised an ELISA based on the in-house generated monoclonal antibodies Ng2 and Ng22 (epitope aa 63-75). The study included CSF from a total of 915 subjects including 75 healthy controls, 114 MCI, 397 sAD, 6 PCA, 96 FTD (of which 46 bvFTD, 12 lvPPA, 20 nfvPPA, 18 svPPA), 68 ALS, 37 PD without dementia, 19 PD with MCI (PD MCI), 29 PD with dementia (PDD), 33 DLB, 21 CBS, and 20 PSP patients. 116 of all subjects had a definitive diagnosis by neuropathological examination and included 75 sAD, 16 DLB, 12 FTD, 7 ALS, and 6 PSP subjects.

ELISA analysis of sAD, MCI, and controls showed that CSF Ng was significantly increased in sAD compared to controls ($p = 0.0001$) as well as MCI ($p < 0.0001$). Moreover, when each group was split into AD biomarker-positive or negative, biomarker-positive defined as t-tau > 93 pg/mL and $A\beta_{1-42}$ < 192 pg/mL and biomarker-negative as the opposite, biomarker-positive individuals had significantly higher CSF Ng concentrations compared to biomarker-negative for both controls ($p = 0.03$), sAD ($p < 0.0001$), and MCI ($p < 0.0001$). Also AD biomarker-positive DLB subjects had increased CSF Ng compared to biomarker-negative DLB ($p = 0.0005$) and sAD ($p = 0.002$). Biomarker-negative subjects from the sAD (n=83) and MCI (n=74) groups as well as biomarker-positive controls (n=9) were excluded from further analysis.

When comparing CSF Ng concentrations of sAD with the other neurodegenerative diseases, sAD had significantly increased concentrations compared to PD ($p < 0.0001$), PD MCI ($p = 0.005$), PDD ($p < 0.0001$), DLB ($p = 0.002$), CBS ($p = 0.03$), PSP ($p = 0.004$), FTD ($p < 0.0001$), and ALS ($p < 0.0001$), but not PCA (Figure 8). There were no differences in CSF Ng concentrations between any of the other groups. When FTD was split into clinical subgroups, sAD had increased concentrations of CSF Ng compared to nfvPPA ($p = 0.0004$), svPPA ($p = 0.01$), and bvFTD ($p < 0.0001$), but not lvPPA. In contrast, lvPPA concentrations of CSF Ng were similar to those of sAD and were in fact higher compared to bvFTD ($p = 0.02$).

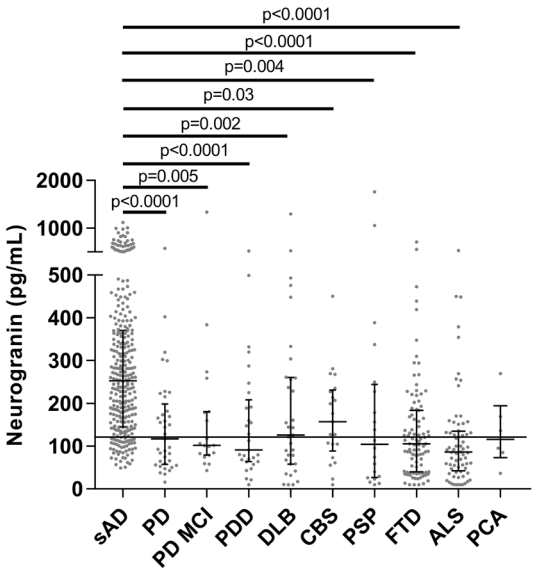

Figure 8. Scatterplot displaying cerebrospinal fluid concentrations in biomarker-positive Alzheimer's disease compared to seven other neurodegenerative diseases. *The data presented are median with interquartile ranges. The line represents the median CSF Ng concentration of the healthy control group.*

By using a biomarker index model, we tested if CSF Ng could be used in order to distinguish between different neurodegenerative disorders. When Ng was added to the model, this led to an increase in accuracy in distinguishing between controls and bvFTD (24%), MCI vs. bvFTD (3.6%), and PD vs. bvFTD (9.5%). By including Ng, there was also an increase in accuracy in distinguishing between MCI and PD (7.7%), MCI vs. PD MCI (6.4%), and MCI vs. PDD (4.3%).

There were strong correlations between both t-tau and p-tau with CSF Ng in several of the groups (Table 1), but no associations were found between $A\beta_{1\text{-}42}$ and CSF Ng.

Throughout all subjects included in the study, there was a significant correlation between Ng and longitudinal decline in MMSE scores per year (r_s=0.17, p = 0.001). However, when dividing all subjects into groups, the only significant relationship was in the MCI group (r_s = 0.45, p = 0.001).

Clinical diagnosis	t-tau	p-tau
Control	0.61***	0.39***
sAD	0.77***	0.56***
MCI	0.79***	0.60***
PD	0.75***	0.14
PD MCI	0.51*	0.52*
PDD	0.48**	0.18
PCA	0.3	− 0.3
DLB	0.77***	0.56**
CBS	0.87***	0.2
PSP	0.37	− 0.26
ALS	0.48***	− 0.097
bvFTD	0.57***	0.49**
lvPPA	0.64	0.33
nfvPPA	0.76**	0.19
svPPA	0.80**	0.35

Table 1. Correlations between CSF Ng and tau*. The values are Spearman's rank coefficient. *p < 0.05, **p < 0.01, ***p < 0.001*

In order to investigate the association between CSF Ng and *APOE ε4*, all subjects were divided into three groups based on the number of *ε4* alleles, *i.e.* zero, one, or two copies. There was a gene dose-dependent increase in CSF Ng with individuals that had two copies of the *ε4* allele having the highest concentrations. There were significantly higher concentrations of CSF Ng in this group compared to subjects with both one (p = 0.02) and no (p < 0.0001) *ε4* allele. There was also a significant difference between the groups with one or no *ε4* allele (p < 0.0001). When grouping clinically diagnosed sAD subjects according to *ε4* status, individuals with no copies had lower CSF Ng compared to sAD patients with one (p = 0.007) and two (p = 0.002) *ε4* alleles.

When looking at the cases confirmed by neuropathology, CSF Ng was increased in sAD compared to ALS (p = 0.03), FTLD (p = 0.006), and DLB (p = 0.03), but not PSP. Upon dividing the sAD subjects into four groups according to the severity of pathological changes as measured by CERAD (neuritic Aβ plaques) and Braak staging

(spread of NFTs), from no AD to high-level AD pathology, we found that CSF Ng increased with the degree of pathological changes. For both Braak stages and CERAD scores, there was a clear trend of increasing CSF Ng concentrations with an increased degree of AD pathology. However, the only significant differences were between the high-level AD pathology and the no AD pathology groups ($p = 0.0007$ and $p = 0.0002$ for Braak stages and CERAD scores respectively) and moderate vs. no AD pathology for Braak stage ($p = 0.02$). Furthermore, CSF Ng concentrations were positively associated with plaque load in both the amygdala ($p < 0.0001$) and hippocampus ($p = 0.0006$) as well as neuronal loss in the hippocampus ($p = 0.04$). When examining the relationship between CSF Ng and the different components of the ABC score, *i.e.* Thal stage, Braak stage, and CERAD score, stages 0 and 3 ($p = 0.0003$), and stages 1 and 3 ($p = 0.02$) in Thal staging had significantly different concentrations of CSF Ng. Moreover, Braak stage 3 had significantly higher CSF Ng compares to stages 0 ($p < 0.0001$) and 1 ($p = 0.002$). Lastly, there was a significant difference between CERAD score 0 and 3 ($p < 0.0001$).

In this paper, we showed that sAD have significantly increased concentrations of CSF Ng compared to six of the seven other investigated major neurodegenerative diseases, with the exception of PCA, and lvPPA when dividing FTD subjects into clinical subgroups. These results could be explained by the fact that both PCA and the majority of lvPPA cases have AD neuropathology. Furthermore, not only was increased CSF Ng specific for sAD across clinically diagnosed cases, but it was also increased in autopsy-confirmed cases which is important because of the relatively common occurrence of neuropathological comorbidity. The results also showed that addition of CSF Ng into the biomarker profile of AD would be useful since it decreased the probability of misdiagnosing bvFTD as healthy control or MCI, which would be likely to have a negative impact on study results.

CSF Ng concentrations increased in sAD subjects with one or two copies of the *APOE ε4* allele, and a similar trend was found in MCI. As homozygous *ε4* carriers have been found to have an increased plaque load, our results might be linked to increased synaptic damage due to plaques. When an association between AD pathology and CSF Ng was investigated, the results did indeed show a strong correlation between plaque load and CSF Ng in both the hippocampus and amygdala. Furthermore, both plaque and NFT spreading were significantly correlated with CSF Ng thus indicating a close relationship between Ng and the major aspects of AD neuropathology. A direct link between CSF Ng and synaptic function was also found since CSF Ng was associated with the rate of cognitive decline.

The key findings in Paper III were that CSF Ng is specific for sAD compared to most other major neurodegenerative diseases and that CSF Ng correlated very well with multiple aspects of AD neuropathology, including neuronal loss.

4.4 PAPER IV

Here, the aim was to perform a more thorough characterisation of Ng in brain tissue as well as quantify both full-length and endogenous Ng peptides in post-mortem brain tissue. Ng was quantified in sAD, fAD, healthy controls, and CU-AP subjects. The CU-AP individuals have both plaques and tangles, but without any cognitive deficits, which makes them very interesting to compare with the other groups.

We generated a new antibody towards Ng, Ng36, which was raised against aa 63-75 and developed a new sandwich ELISA using Ng36 as capture antibody and Ng2 as detection antibody. Ng36 was characterised by HI-MS and found to detect the same peptides, as well as full-length Ng, in human brain tissue as Ng2 and Ng3.

Endogenous Ng was characterised using HI-MS with Ng2 and Ng3. In total, 39 endogenous peptides were identified by high-resolution LC-MS/MS, and most of them ended at aa 75, 76, or 78 and started between aa 42 and 54 in a step-wise manner (Figure 9). In addition, 15 of these endogenous peptides were also identified with MALDI-TOF/TOF (Figure 10A).

Figure 9. Endogenous neurogranin peptides in brain tissue identified using HI-MS.

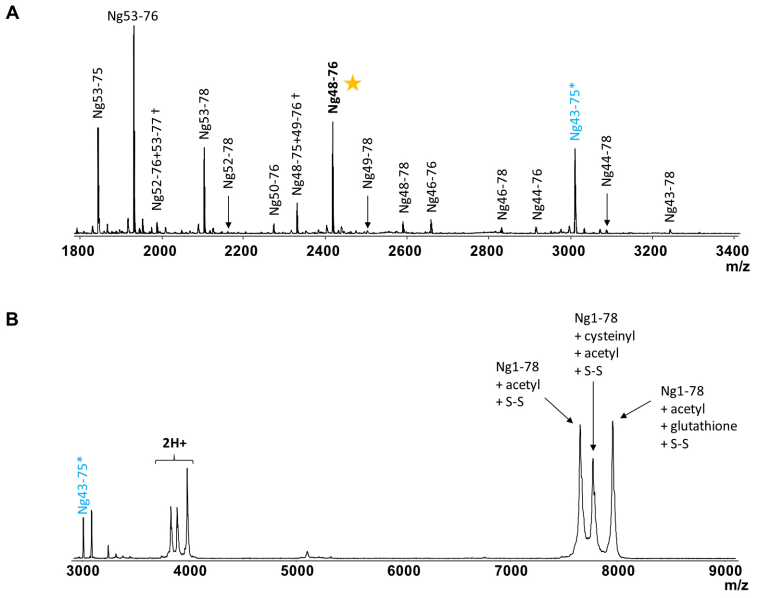

Figure 10. MALDI-TOF spectra of neurogranin in brain tissue. ***(A)*** *MALDI spectrum displaying several endogenous C-terminal neurogranin peptides.* ***(B)*** *MALDI spectrum displaying a cluster of peaks representing full-length neurogranin with different sets of PTMs. Ng43-75* represents the heavy isotope-labelled peptide that was used as an internal standard during quantification. Ng48-76, which is marked by a star, was found to be significantly increased in sAD CSF in Paper I. † signifies peptides with identical mass that can therefore not be distinguished during MALDI-TOF analysis.*

The PTMs glutathione (GSH), phosphorylation, disulfide bridge, cysteinyl, acetylation, and oxidation were also identified on full-length Ng (Figure 10B). Interestingly, the placement of the PTMs affected retention time during nano-LC-MS.

Full-length and peptide Ng were relatively quantified using HI-MS in a pilot study on superior parietal gyrus of 10 sAD subjects and 10 healthy controls. In this study, 14 endogenous peptides, as well as all species of full-length Ng, were quantified in all samples. After analysing the ratio between each of the peptides and the sum of all full-length Ng species, *i.e.* total full-length Ng, we found that eight peptide-to-total full-length Ng ratios were significantly increased in sAD compared to controls ($p < 0.05$ for all ratios), thus signifying decreased concentrations of full-length Ng compared to these peptides.

A second study, including temporal cortex from 9 sAD, 10 fAD, 13 CU-AP, and 9 controls, was analysed by HI-MS in order to validate the results from the pilot cohort. Ten endogenous Ng peptides, as well as all species of full-length Ng, were quantified in all samples of the second cohort. Here, nine of the quantified peptides showed significant differences across groups in peptide-to-total full-length Ng ratios. The most pronounced difference was seen in Ng53-78, for which the peptide-to-total full-length ratio was significantly reduced in sAD and fAD compared to controls ($p = 0.004$ for sAD; $p = 0.0005$ for fAD), and compared to CU-AP ($p = 0.007$ for sAD; $p = 0.002$ for fAD). When comparing the concentrations of total full-length Ng between the groups fAD had significantly lower levels than sAD ($p = 0.03$), CU-AP ($p = 0.0003$), and controls ($p = 0.01$). There was also a trend towards reduced total full-length Ng in sAD compared to controls and CU-AP, although it failed to reach statistical significance.

Next, the second cohort was analysed by WB (antibody Ng36), which most likely quantifies full-length and nearly full-length Ng, and fAD had reduced levels compared to CU-AP ($p = 0.008$), and controls ($p = 0.0002$). Once again there was a trend, which failed to reach statistical significance, of lower levels in sAD compared to CU-AP and controls. The results from ELISA analysis were similar to WB, with significantly lower concentrations in fAD compared to CU-AP ($p = 0.035$), and controls ($p = 0.003$), and significantly lower concentrations in sAD compared to CU-AP ($p = 0.017$). While ELISA most likely detects Ng in both peptide and intact form, the peptides probably exit the gel in WB due to their small size, meaning that this method mainly quantifies full-length, or nearly full-length, Ng.

In order to investigate any associations between brain Ng and degree of neuropathological changes, all subjects from the second cohort were divided according to the degree of neuropathological changes, as estimated by Braak stage (0-I, II-IV, V-VI), Thal phase (0-1, 2-3, 4-5), and CERAD score (0, A, B, C) separately. We discovered that brain Ng, irrespective of the method of quantification, was significantly higher in individuals with moderate and high-levels of NFTs and plaque load (*i.e.* Braak stage and CERAD score). For Thal phase, *i.e.* spreading of plaques, only the peptide-to-total full-length ratio of Ng53-78 displayed a significant difference between low and moderate pathology.

In this paper, full-length protein and endogenous Ng peptides were for the first time quantified in human brain tissue. In two independent cohorts, the ratios of peptide-to-total full-length Ng were increased for several peptides in sAD compared to healthy controls, which indicate increased concentrations of these peptides compared to full-length Ng. Moreover, in the second cohort, the ratios for several peptides were also increased in sAD compared to CU-AP and in fAD compared to both controls and CU-AP. In other words, there seems to be a shift from full-length protein towards peptides in both forms of AD. The results from WB and ELISA also supports this theory, as

fAD had decreased concentrations compared to both controls and CU-AP in those analyses and there was a trend of lower concentrations for sAD compared to controls and CU-AP as well. As previously mentioned, WB most likely detects mainly full-length Ng, while the ELISA most likely quantifies a mixture of both peptides and full-length Ng. However, the concentrations of the peptides were fairly low compared to the full-length Ng species, which probably is why the ELISA results were similar to WB. Thus, all three methods point to full-length Ng being decreased in both sAD and fAD. Although calpain-1 and prolyl endopeptidase were recently shown to be capable of cleaving Ng *in vitro* to generate several Ng peptides[283], further studies are needed since the reported cleavages do not account for all the peptides identified here.

Results from Paper III showed that CSF Ng quantified by ELISA is strongly correlated with the degree of neuropathological changes, which here was confirmed for brain Ng as well. As CERAD scores were more strongly associated with Ng than Thal phases, it seems that Ng is more closely associated with the number of plaques rather than the degree of spreading.

Strikingly, the CU-AP group did not have increased peptide-to-total full-length Ng ratios but were rather very similar to healthy controls, even though most subjects had a moderate degree of both plaques and NFTs. This indicates that the CU-AP individuals might have some mechanism that is either capable of protecting synapses from the damage normally induced by the pathological changes, or that synaptic damage still occurs, but they are somehow able to compensate for it.

We have previously shown that Ng is present in CSF as many different endogenous peptides (Paper I) and that there seems to be a CNS Ng pool comprised of CSF and brain tissue (Paper II). Since it was demonstrated here that full-length Ng most likely is degraded into peptides to a greater degree in sAD and fAD, it is tempting to speculate that the increased presence of peptides in sAD CSF presumably originates from the brain. Thus, CSF Ng is likely to mirror the ongoing synaptic degeneration in the brain.

The most important findings in Paper IV were that Ng levels were strongly associated with the degree of neuropathological changes regardless of group and that there was a shift from full-length to peptide Ng in both sAD and fAD compared to controls and CU-AP. As this shift was not present in healthy controls or CU-AP individuals, of which the latter have both plaque and tangle pathology but no cognitive impairment, the results indicate that Ng is a biomarker for AD-related synaptic degeneration leading to cognitive decline.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The work presented in this thesis includes characterisation of Ng in the CNS and periphery as well as evaluation of Ng as a biomarker in AD.

Using HI-MS, we were able to perform a detailed characterisation of both full-length Ng in brain tissue, where several PTMs were identified for the first time, and of endogenous Ng peptides that were present in CSF, plasma, and brain tissue. We showed that peptide profiles are distinctly different; some Ng peptides were ubiquitously present while others seemed to be associated with either the periphery or CNS, indicating the presence of two separate Ng pools with very little or no exchange between them. Most noticeably, many peptides ending at aa 76 were present in both brain tissue and CSF while no such peptides were detected in plasma (Figure 11). In addition, the endogenous peptide Ng48-76 was significantly increased in sAD CSF.

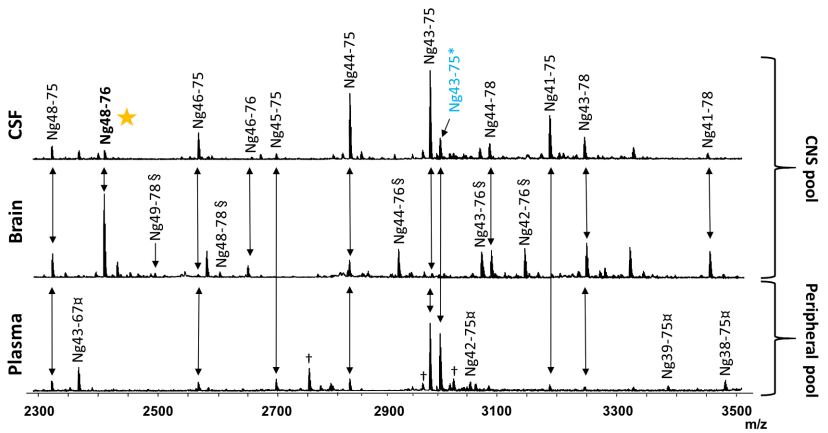

Figure 11. MALDI-TOF spectra displaying C-terminal neurogranin peptides found in cerebrospinal fluid, brain tissue, and plasma. *The peptide profiles of CSF and brain tissue were very similar compared to plasma, especially with consideration to peptides ending at aa 76. These peptides were abundant in both brain tissue and CSF, but no such peptides were identified in plasma. We therefore suggest that there are two separate Ng pools; the CNS pool consisting of brain tissue and CSF and the peripheral pool comprised of plasma. As the peptide profiles of the two pools are quite different, there appears to be very little or no exchange between them. Ng48-76, which is marked by a star, was found to be significantly increased in sAD CSF in Paper I. Ng43-75* represents the heavy isotope-labelled peptide that was used as an internal standard during quantification, but was not added in the brain tissue spectrum shown here. § and ¤ signify peptides that were found exclusively in brain tissue and plasma, respectively. † denotes peaks caused by non-specific binding of other plasma proteins to the magnetic beads used in HI-MS.*

Taking these results into consideration, our data indicate that the processes leading to the formation of peptides ending at aa 76 are specific to the CNS and might reflect ongoing neurodegeneration in AD. Since investigation of the ratio between Ng peptides and full-length Ng revealed a shift from intact Ng towards peptides in both sAD and fAD, it seems that the degradation of full-length Ng somehow is connected to neurodegenerative processes in AD, but as of now it is not known if that connection is direct, or if it is more of a secondary effect. As mentioned, two enzymes that together are capable of generating some of the endogenous peptides have been discovered[283], but more studies on the mechanisms behind the endogenous peptides might shed some light on the generation of peptides and the significance of increased peptides in terms of disease progress or stage.

In order to get a better understanding of the neurodegenerative processes that might affect or influence the formation of Ng peptides, especially those ending at aa 76, one might consider performing a detailed peptide characterisation in both brain tissue and CSF of other neurodegenerative diseases, such as those included in Paper III, as well. Such studies might give insight to if the formation of certain peptides might be unique for AD and thus could be relevant for the neurodegenerative process. However, it should be noted that even though researchers have identified numerous endogenous peptides from Aβ and APP[37, 60, 284], the physiological function is still unknown for the majority of these peptides. If a certain Ng peptide, or peptides, was found to have a role in disease development or reflect the progression of AD especially well, one might consider designing neo-specific antibodies towards this peptide in order to construct a new immunoassay, just as there are specific assays for $Aβ_{1-38}$, $Aβ_{1-40}$, and $Aβ_{1-42}$. If such a specific immunoassay would be developed, and of course depending on what peptide that should be quantified, it might perhaps be possible to revisit plasma Ng. The difference in Ng concentration between plasma and CSF, both measured by MSD and HI-MS, appears to be quite big, meaning that if CNS-specific peptides possibly were present at very low concentrations, they would be very difficult to detect. Although we did not detect any CNS-specific peptides in plasma using HI-MS, it is not impossible that such a feat could be achieved with a highly sensitive neo-specific immunoassay, such as the Single Molecule Array (Simoa) technology[285]. Regarding the identified PTMs, more work is needed in order to find out what they mean and if they might have some effect on the function of Ng.

CSF Ng measured by different immunoassays was increased in both sAD and MCI compared to controls. In fact, numerous independent studies have now shown increased CSF Ng in both sAD[251-255] and MCI[254, 255, 286] compared to healthy controls. A comparison of three assays for CSF Ng also showed very similar performance between them, even though they were all based on different antibodies and epitopes, indicating that CSF Ng has a robust performance as a biomarker[282]. One hypothesis that might explain the increase of Ng in CSF is that elevated concentrations of Ng

peptides in brain tissue might be reflected in CSF. Detailed HI-MS analysis of paired CSF and brain tissue samples from individuals with sAD and controls could perhaps shed some light on the possible connection between Ng in CSF and brain tissue. In addition, we also showed that increased CSF Ng is specific for sAD compared to a diverse set of other common neurodegenerative diseases. As already mentioned, other groups have also shown that CSF Ng is specific for sAD[279-282], but never before in such a large material or using neuropathologically confirmed cases. Thus, the increase in CSF Ng for sAD and MCI suggests that CSF Ng is a useful biomarker for neurodegenerative processes that are specific for AD, even at early stages. The fact that Ng is specific for AD is very important as it means it may be used as inclusion, or exclusion, criteria in clinical trials as well as used for differential diagnosis in clinics. However, what CSF Ng actually reflects, and the exact reasons for why increased CSF Ng is specific for AD, remains to be identified.

Considering that HI-MS analysis revealed that at least one Ng peptide, Ng48-76, is increased in sAD CSF and that peptide-to-total full-length Ng ratios were increased in both sAD and fAD brain tissue, it might be of interest to characterise the CSF Ng peptide profile of fAD using HI-MS as well. To our knowledge, no such papers have been published. However, immunoassay analysis indicates that CSF Ng also is increased in fAD[279]. It would also be very informative to follow fAD individuals longitudinally, with respect to CSF Ng levels, especially before the age of onset. Such studies are ongoing and may reveal how early CSF Ng starts to increase.

The finding that Ng measured in both CSF and brain tissue correlated very well with the degree of neuropathological changes speaks for that Ng indeed reflects these changes. Both CSF and brain Ng were closely associated with tau, as a CSF biomarker as well as in the form of NFTs. In contrast, there was very little or no association between CSF Ng and $A\beta_{1-42}$ while brain Ng correlated well with plaque load but not with degree of spreading. Therefore, it might be of interest to investigate both CSF and brain Ng in patients with primary tauopathies in order to gain further insights into the link between Ng and tau.

What does analysis of CSF Ng contribute with compared to the core AD biomarkers alone? There are studies which have shown that including CSF Ng also increase diagnostic accuracy[286] and, as shown in Paper III, it might decrease the risk of misdiagnosing bvFTD as healthy control or MCI. Furthermore, since we and others have shown that CSF Ng correlates well with both tau neuropathology and tau measured in CSF, it might be possible to use CSF Ng as a surrogate marker to monitor the effect of drugs targeting tau pathology.

Overall, larger studies and analysis of longitudinal samples, preferably from subjects who are followed for at least 5 years, are needed in order to investigate if CSF Ng indeed is capable of detecting pathological changes earlier than tau and $A\beta_{1-42}$. Also, PET ligands against the synaptic protein SV2A, allowing for monitoring of synaptic density in living patients, are currently being developed and evaluated[287, 288]. It would therefore be of great interest to investigate how CSF Ng correlates with SV2A PET or other future PET ligands for synaptic proteins.

In conclusion, the results from the papers included in this thesis have shown that CSF Ng is a novel biomarker for AD, even in early stages of the disease, and that CSF Ng can be used to differentiate between AD and other neurodegenerative diseases. Thus, Ng is indeed a new, and useful, item in the AD biomarker toolbox.

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