UPPSALA UNIVERSITET

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1719

Alzheimer's Disease Neuropathological Change and neuronal and glial alterations in patients with idiopathic Normal Pressure Hydrocephalus

SYLWIA LIBARD

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2021

ISSN 1651-6206
ISBN 978-91-513-1132-6
urn:nbn:se:uu:diva-433963

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbeckslaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Saturday, 27 March 2021 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Professor Per Kristian Eide (Neurosurgery, Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Norway).

Online defence: https://uu-se.zoom.us/j/68503970438

Abstract

Libard, S. 2021. Alzheimer's Disease Neuropathological Change and neuronal and glial alterations in patients with idiopathic Normal Pressure Hydrocephalus. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1719. 76 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1132-6.

Alzheimer's disease Neuropathological Change (ADNC), i.e. amyloid β (Aβ) and hyperphosphorylated τ (HPτ), is seen in excess in the brains of subjects with AD. Idiopathic Normal Pressure Hydrocephalus (iNPH) lacks defined hallmark lesions, affects the elderly and leads to cognitive impairment, gait disturbance and urinary incontinence that can be treated with a ventriculoperitoneal shunt (VPS). A few centres around the world have obtained a brain biopsy from the area of VPS. It has been reported that the presence of ADNC in the biopsy is associated with progression to AD.

We confirm that majority of iNPH subjects display ADNC, and the ADNC increases in extent with age, in line with AD. The HPτ pathology is sparse in majority of cases. We observed remarkable neuronal survival and loss of matrix/synapses in subjects with iNPH (paper III).

When studying subjects with notable Aβ pathology (paper IV), we observed a stepwise increase of pyroglutamylated Aβ (pyAβ) and phosphorylated Aβ variants in iNPH. These two Aβ variants are associated with symptomatic AD and correlate with HPτ pathology. The pyAβ in the frontal cortex is a predictive marker for AD. Thus, notable Aβ pathology in presence of HPτ in iNPH is suggestive of a moderate level of ADNC.

When assessing changes in the extent of pathology occurring during 21 months in a frontal cortex of a subject with iNPH and AD (paper II), HPτ pathology increased in parallel with neuronal and synaptic loss, whereas Aβ pathology and astroglial activity were stable over time. In contrast, we observed reduction of microglial markers, which might explain why anti-inflammatory treatment is effective only at an early stage of AD.

When assessing brain tissue, the section thickness must be standardised, as it affects the staining outcome and diagnosis (paper I).

In conclusion, we have demonstrated a progressive neurodegeneration of ADNC type in a population of iNPH subjects, mimicking what is seen in subjects with AD. A brain biopsy obtained from subjects with iNPH should be obligatory. This is because when ADNC is present in the biopsy, representing prodromal AD, contact with memory clinic should be initiated.

Keywords: idiopathic Normal Pressure Hydrocephalus, Alzheimer's disease, amyloid β, hyperphosphorylated τ

Sylwia Libard, Department of Immunology, Genetics and Pathology, Clinical and experimental pathology, Rudbecklaboratoriet, Uppsala University, SE-751 85 Uppsala, Sweden.

ISSN 1651-6206
ISBN 978-91-513-1132-6
urn:nbn:se:uu:diva-433963 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-433963)

In memory of my grandmother Marianna

List of Papers

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

I Libard S., Cerjan D., Alafuzoff I. (2019) Characteristics of the tissue section that influence the staining outcome in immunohistochemistry. *Histochemistry and Cell Biology,* 151:91-96.

II Libard S., Laurell K., Cesarini K.G., Alafuzoff I. (2018) Progression of Alzheimer's Disease related pathology and cell counts in a patient with idiopathic Normal Pressure Hydrocephalus. *Journal of Alzheimer's Disease,* 61(4)1451-1462.

III Libard S., Alafuzoff I. (2019) Alzheimer's disease neuropathological change and loss of matrix/neuropil in patients with idiopathic Normal Pressure Hydrocephalus, a model of Alzheimer's disease. *Acta Neuropathologica Communications,* 7(1):98.

IV Libard S., Walter J., Alafuzoff I. (2021) In vivo characterization of biochemical variants of amyloid β in subjects with idiopathic Normal Pressure Hydrocephalus and Alzheimer's Disease Neuropathological change. *Journal of Alzheimer's Disease,* In press.

Contents

Introduction 11
Alzheimer's Disease 11
Clinical approach 12
Genetic abnormalities 12
Early Onset Alzheimer's Disease 12
Late Onset Alzheimer's Disease 13
Neuropathological diagnosis 13
Sampling 13
Alzheimer's Disease Neuropathological Change 14
The final diagnosis of Alzheimer's disease 17
Neuroinflammation 19
The neurodegenerative process of AD 20
Idiopathic Normal Pressure Hydrocephalus 21
Pathophysiology of iNPH 22
Clinical approach 23
Neuropathological findings 24
Ageing as a risk factor 26
Immunohistochemistry 26

Aims 29
Overall aims 29
Specific aims 29

Materials and methods 30
Patients 30
Clinical assessment 30
Sampling 31
Immunohistochemistry 32
Assessment strategies 35
Statistical analysis 36

Results and discussion 37
Paper I 37
Paper II 39
Neuropathological assessment 40
Comparison of pathology at two time points 42

Paper III 44
Paper IV 46

Strengths and limitations 51

Summary of results 52

General discussion 54

Future perspectives 58

Populärvetenskaplig sammanfattning på svenska 59
Delarbete I 60
Delarbete II 60
Delarbete III 61
Delarbete IV 61

Acknowledgements 62

References 64

Abbreviations

Aa	Amino acids
AA	Alzheimer's Association
Aβ	Amyloid-β
Ab	Antibody
AD	Alzheimer's Disease
ADNC	Alzheimer's Disease Neuropathological Change
Ag	Antigen
APOE	Apolipoprotein ε
APP	Amyloid precursor protein
AR	Antigen retrieval
BBB	Blood brain barrier
CAA	Cerebral amyloid angiopathy
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CI	Cognitive impairment
CSF	Cerebrospinal fluid
DS	Detection system
DSM	Diagnostic and Statistical Manual of Mental Disorders
FT	Fixation time
HPτ	Hyperphosphorylated τ
ICP	Intracranial pressure
IHC	Immunohistochemistry
iNPH	Idiopathic Normal Pressure Hydrocephalus
LOAD	Late onset Alzheimer's Disease
MCI	Mild cognitive impairment
MMSE	Mini Mental State Examination
MRI	Magnetic resonance imaging
NFT	Neurofibrillary tangles
NIA	National Institute of Aging
NP	Neuritic plaque
NT	Neuritic thread
p	Phosphorylated
PART	Primary Age Related Tauopathy
PET	Positron Emission Tomography
PM	Post mortem
PMD	Post mortem delay
PS	Presenilin

py	Pyroglumamylated
SAF	Stained Area Fraction
ST	Section thickness
TDP43	Transactive response DNA binding protein 43
UUH	Uppsala Univeristy Hospital
VPS	Ventriculoperitoneal shunt

Introduction

As the world's population becomes older, the number of individuals with age-associated diseases is increasing [1,2]. One of the main disorders among the elderly is dementia, accounting for 50 million affected in the world [3]. This number is estimated to double every 20 years [2]. The incidence is 3 per 1,000 persons at age 60 – 64 and doubles with every 6-year increase in age to 175/1,000 persons 95 years and older [2,4]. According to the Swedish Statistical Bureau, in 2019, there were 550,000 persons who were 80 years or older in Sweden; by 2030, this number is estimated to increase to 800,000 people and by 2040 to one million [5,6]. The prevalence of dementia in Sweden is 130,000 – 150,000 individuals and is expected to increase with 20,000 – 25,000 every year, according to estimates from the National Board of Health and Welfare [7].

Dementia is a progressive neurodegenerative condition causing disturbances in cognition such as memory, learning, speech, orientation, planning, reasoning and complex thinking. It also causes neuropsychiatric symptoms such as apathy, confusion, depression, personality- and behavioural changes. Moreover, it can result in physical disabilities, especially at the end-stage of the disease, affecting basic body functions e.g. breathing, swallowing or continence [4,8,9]. It is a progressive, disabling and, at the end, fatal condition affecting the individual patient but also his or her relatives and healthcare providers [4,8,9].

Alzheimer's Disease

Alzheimer's disease (AD) is the most common type of dementia, affecting 60 – 80% of patients with dementia [4,8,9]. In younger patients, AD is observed in its pure form; however, numerous concomitant pathologies are observed with increasing age, which alter the clinical presentation/symptomatology [10-14]. The prodromal phase of the disease is very long as AD related brain alterations can be detected decades before the symptom debut, as well as in individuals who never develop a dementing illness [15,16]. Mild cognitive impairment (MCI) is the first symptomatic phase of the disease, presenting with subtle memory deficits that can also be caused by other diseases. A substantial number of subjects with MCI develop dementia over time [9,17]. When AD is established, the subject undergoes progressive deterioration over the years regarding memory, thinking, behaviour, daily tasks and physiological functions. The

disease duration after diagnosis can vary between 4 and 20 years [9]. There are several risk factors associated with AD, i.e. age, genetics (Apolipoprotein ε4 isoform (APOE ε4)), sex, cardiovascular disease, education level and traumatic brain injury [3,9,15,18,19]. The mortality caused by AD is high, as it is estimated as the fifth leading cause of death in the world in subjects > 65 years [3].

Clinical approach

The clinical diagnosis of dementia is based on recommendations from the Diagnostic and Statistical Manual of Mental Disorders, fifth edition (DSM-5) by the American Psychiatric Association [20]. In DSM-5, the diagnosis of dementia, of AD type particularly, is harmonised with guidelines from National Institute of Neurological and Communicative Disorders and Stroke and the AD and Related Disorders Association from 1984, which were updated in 2011 by National Institute on Aging (NIA) and Alzheimer's Association (AA) [20-25].

The clinical assessment includes a thorough investigation of the medical and family history obtained from the patient, but also from a family member or another person close to the patient [8,20]. The patient should undergo a general physical and neurological examination and neuropsychological examination, including Mini Mental State Examination (MMSE) [8,20,26]. Extensive blood testing should be performed to rule out treatable conditions that could cause the symptoms [8,20]. Evaluation of the cerebrospinal fluid (CSF) biomarkers, especially Amyloid-β (Aβ), Tau (τ) and hyperphosphorylated τ (HPτ) should be routinely performed [8,20,27]. The assessment also includes imaging investigation with Magnetic Resonance Imaging (MRI) and if possible, Positron Emission Tomography (PET) [8,20,28]. In 2018, NIA and AA made additional updates to the 2011 guidelines focusing on research purposes, reflecting new insights in the biology of AD, new biomarkers, molecular tracers and imaging techniques [29]. In practice, the clinical investigation of the patient, medical- and social efforts varies depending on the patient's age, the severity of the disease and the individual's social support [7].

Genetic abnormalities

Early Onset Alzheimer's Disease

Only a fraction of patients affected by AD are due to inherited genetic abnormalities [8,30]. The most common are mutations of presenilin (PS) 1 and PS 2. Those two proteins are constituents of γ-secretase, an enzyme engaged in the cleavage of amyloid precursor protein (APP) to Aβ [31-34]. Patients with mutation in either of the PSs are prone to develop an early and aggressive form of AD, debuting before the age of 65, due to an increase in Aβ42 load [30,35,36].

There are also mutations in the Aβ domain within APP that cause increased production and improve aggregative properties of Aβ [8,30,33].

Individuals with Down Syndrome are at high risk of developing dementia, particularly of AD type [8]. The syndrome is caused by a third copy, full or fragment, of chromosome 21, where the APP gene is situated. There are several genetic alterations interacting with Aβ production, causing AD within this patient category. Subjects with Down Syndrome may suffer from AD early in life, as most of the patients display significant load of AD pathology within their brains already in their 40s [37,38].

Late Onset Alzheimer's Disease

The majority of AD population, over 95%, present with late onset AD (LOAD). Carrying the APOE ε4 gene allele is one of the main risk factors for developing LOAD [8,19,30]. APOE is produced in the brain by microglia, astrocytes, choroid plexus, pericytes and stressed neurons [19]. Furthermore, APOE is involved in lipid transport and metabolism and can be inherited in three isoforms ε2-4, where the ε3 isoform is the neutral and most common in humans. ε4 isoform is the most pathogenic in AD. Subjects carrying one APOEε4 allele have three to four-fold higher risk of developing AD and heterozygotes up to 15-fold risk compared to subjects with other isoforms of ε [8,19,30]. Interestingly, the APOEε2 isoform is associated with decreased risk of AD when compared to ε3 and ε4 [19,30]. However, APOEε4 is associated with greater extent of Aβ aggregation and increased longitudinal progression in Aβ pathology in subjects with AD, as well as in subjects with mild cognitive impairment and subjects with preserved cognitive functions [19]. Also, APOEε4 is primarily associated with Aβ pathology, affecting Aβ -production, aggregation and clearing but is also involved in τ phosphorylation, HPτ pathology, synaptic function, neuroinflammation and neurotoxicity [19,39,40].

Neuropathological diagnosis

A definitive diagnosis of AD is established by a neuropathological examination of post-mortem (PM) brain tissue. The diagnostic criteria are based on the NIA and AA guidelines for neuropathologic assessment of AD [41,42].

Sampling

The NIA and AA guidelines for neuropathologic assessment of AD recommend a sampling procedure, according to the BrainNet Europe Consortium and by the Consortium to Establish a Registry for AD (CERAD) [41-47].

The neuroanatomical areas that are sampled for microscopical investigation should cover areas that are affected by different pathologies and proteinopathies. In our clinical practice, the following areas are assessed microscopically in line with BNE recommendations: 1. Frontal cortex; 2. Temporal cortex,

including superior and middle temporal gyri; 3. Gyrus cinguli; 4. Parietal cortex; 5. Motor cortex; 6. Occipital cortex, including calcarine fissure; 7. Anterior hippocampus; 8. Posterior hippocampus; 9. Basal forebrain; 10. Striatum; 11. Thalamus; 12. Midbrain; 13. Pons, including locus coeruleus; 14. Medulla oblongata, including dorsal motor nucleus of the vagus nerve; 15. Vermis cerebelli, including the dentate nuclei and 16. Cerebellum [43-46].

Alzheimer's Disease Neuropathological Change

The AD neuropathological change (ADNC) includes the hallmark lesions of AD, i.e. extracellular aggregates of Aβ, as Aβ plaques, within the neuropil and HPτ accumulation within neurons and their processes [34,41,42,48]. AD neuropathology was first described by Aloes Alzheimer, who identified the pathological lesions applying silver stain [49]. One of the lesions that was used for several years as a diagnostic hallmark was neuritic plaques (NP), i.e. "NP counts", composed, as we know today, of Aβ aggregates and HPτ- or ubiquitin (Ubq) positive neurites [47,50,51]. The second hallmark lesion was neurofibrillary tangle (NFT), also seen in silver stain, and as we know today is composed of intracytoplasmatic perisomal aggregation of HPτ [52-55].

Amyloid β

Aβ is generated by a two-step enzymatic cleavage of APP, a transmembrane protein located in the cell membranes and within membranes of organelles with an extracellular/luminal amino-terminus and intracellular carboxyl terminus. The APP gene is located on chromosome 21, and different splicing sites of the gene generate several isoforms of the protein, with APP 695 being the most common in neurons [33,34,56]. There, APP can be degraded in two different pathways. In the anti-amyloidogenic pathway, APP is primarily cleaved by α-secretase within the Aβ domain of the protein, releasing a soluble sAPPα peptide into the extracellular space. The transmembrane domain that remains is processed by γ-secretase into a pathologically irrelevant, small, truncated 3p Aβ peptide [31,33,56]. This pathway interferes with and prevents the Aβ formation [33]. Within the amyloidogenic pathway, APP is primarily cleaved by β-secretase, also called β-site APP cleaving enzyme-1, before the Aβ domain, resulting in an extracellular soluble sAPPβ peptide and transmembrane APP carboxy-terminal fragment. The transmembrane protein is then processed by γ-secretase, releasing Aβ into the extracellular space [31,33,34,56]. The Aβ protein can be 37 – 43 amino acids (aa) long [31,34].

The most common Aβ peptide is 40 aa long and is the main constituent of cerebral amyloid angiopathy (CAA) seen in the vascular structures in meninges and cerebral cortex [34,57]. The Aβ42 is also seen in CAA, albeit to a lesser extent. Aβ42 together with Aβ40 are both components of amyloid aggregates, but the Aβ42 isoform is more prone to forming soluble and toxic oligomers, which assemble into insoluble fibrils that aggregate in the parenchyma.

These Aβ42 dominating aggregates are generally referred to as plaques [32,34,58]. The plaques vary in morphology and composition, i.e. can be diffuse or dense. When Aβ42 aggregate is seen together with HPτ pathology as neurites, the lesion is referred to as NP; a lesion that was already defined while applying various silver stains [47,51]. The diffuse plaques are only seen when applying the immunohistochemistry (IHC) method and are commonly seen in ageing individuals, whereas the dense- and the HPτ containing NPs are mainly observed in AD [51,59,60].

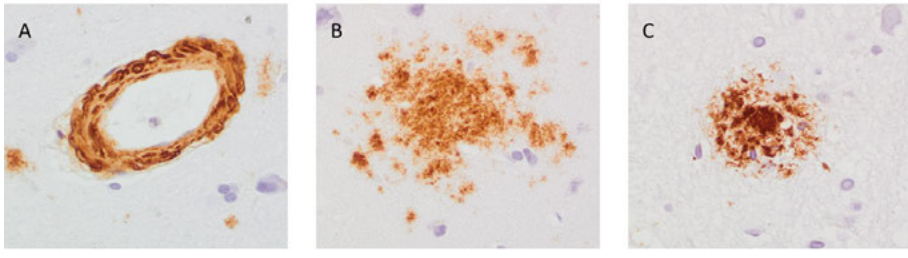

Figure 1. Amyloid β (Aβ) pathology. A. Cerebral amyloid angiopathy. B. Diffuse Aβ aggregate. C. Dense Aβ aggregate.

The Aβ pathology has been shown to progress through a distinctive pathway while applying IHC methodology, affecting specific neuroanatomical regions [48,61]. The current staging incorporates five different phases of the Aβ deposition, starting in the neocortical areas of the cerebrum as phase 1. In phase 2, the allocortical areas of medial temporal lobe (CA1 and entorhinal region), insular cortex, amygdala and gyrus cinguli are affected. In phase 3, the subcortical areas, i.e. striatum, basal forebrain nuclei, thalamus, hypothalamus and white matter, are affected. Within stage 4, the Aβ pathology is seen in the midbrain and brainstem structures. At the end stage, phase 5, the cerebellum is also affected [61].

The biochemical changes of Aβ

Besides the formation of Aβ protein of different lengths, the protein itself can undergo post- translational modifications. The altered biochemical properties can be a result of alternative primary cleavage of the protein already at the cell level, but it can also undergo secondary cleavages producing truncated Aβ peptides during the aggregate formation. Those changes can affect both the C-terminal and N-terminal of the protein and change the properties of the protein itself, resulting in Aβ peptides with increased toxicity, increased ability to aggregate and decreased ability to degradate. The post-translational modifications include, among others, pyroglutamylation, phosphorylation, nitration or oxidation [31,62].

The pyroglutamylated Aβ (pyAβ) is formed by releasing the first two amino acids at the N-terminus of the protein, and the terminal glutamate is

converted into pyroglutamate. This variant of Aβ is associated with increased ability to aggregate, increased neurotoxic properties and maintaining the inflammatory environment by promoting astrogliosis [31,62,63]. Recent studies on PM brain tissue revealed that the extent/level of pyAβ was significantly higher in AD in comparison with cognitively unimpared controls. Additionally, when detected in the cortical areas of the brain, the extent of pyAβ was significantly associated with HPτ pathology and the level of cognitive decline [64,65].

Phosphorylation of Aβ is another post-translational modification that has been extensively studied, especially the phosphorylated Aβ (pAβ) variant at serine 8 and to a lesser extent at serine 26. The pAβ is described to increase the formation of oligomeric assemblies which are the core of fibrillisation, increase neurotoxicity and is resistant to degradation [31,66,67]. The pAβ variant has been detected in mouse models of AD, PM brain tissue from subjects with AD and Down Syndrome, and was associated with symptomatic dementia [66-70].

Interestingly, recent studies have shown that during the formation of Aβ aggregates and CAA, different Aβ variants can be identified within the lesions in a stepwise order [68,70]. The suggested stage 1 corresponds with the presence of Aβ without the N-terminal truncated, pyAβ or pAβ species and was observed in brain tissue from patients with asymptomatic, preclinical AD. Within stage 2, the pyAβ was additionally seen within the aggregates; moreover, at stage 3, the pAβ subtype was also identified within the lesions. The biochemical Aβ stage 3 was associated with symptomatic dementing illness [68,70].

Hyperphosphorylated τ

The HPτ is the second main constituent of ADNC [48]. τ is a microtubule associated protein, a normal constituent within neurons, localised primarily in axons [71]. In its native form, the τ protein is an unfolded monomer, in the brain occurring in six different isoforms [71,72]. The τ protein is involved in stabilisation of the neuronal structures, i.e. by stabilising and inducing formation of microtubules, axonal transport and neurotransmission [72-74]. Those functions are controlled by phosphorylation of τ and are suppressed by hyperphosphorylation of the τ protein [55,73,75]. In AD, the τ protein is abnormally hyperphosphorylated, resulting in up to 3-fold higher phosphorylation levels than in healthy individuals [72,73]. The process of hyperphosphorylation changes the properties of the protein, promoting the formation of toxic, soluble oligomers and further into insoluble, straight filaments and paired helical filaments, which are more prone to aggregate within neurons [72,76,77]. The HPτ is predisposed to bind to normal τ protein, thereby interfering with the microtubule assembly and promoting the pathology further in a prion-like manner [55,72,75,78]. Other biochemical processes such as acetylation, ubiquitination, glycation and truncation additionally affect the properties of τ, thus further promoting the pathologic phosphorylation and aggregation of the protein [71-73].

When the HPτ accumulates in the neural soma, it is a main constituent of NFT and when affecting the neuronal processes, the pathology represents as neuritic threads (NT) [52,53]. When NT/neurites are incorporated into Aβ aggregates, the structure is called NP [51,53,54,79].

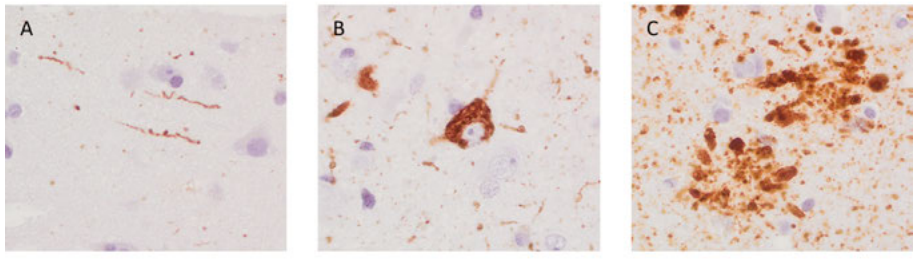

Figure 2. Neurofibrillary pathology when applying antibody towards hyperphosphorylated τ. A. Neuritic threads. B. Neurofibrillary tangle. C. Neuritic plaque.

The progression of HPτ pathology follows characteristic neuroanatomical regions [48,80]. Stages I and II progressively affect the entorhinal brain regions within the medial temporal lobe i.e. transentorhinal cortex, followed by the entorhinal cortex and CA1 and CA2 regions of the hippocampus. Within stages III and IV, the pathology within the primary areas increases and extends into the limbic allocortex and escalates largely into the adjoining neocortex. In stages V – VI, the pathology progresses within the neocortex, involving secondary and primary areas at the end stage, engaging the parastriate and striate areas of the occipital lobe [48,80]. Recently, it has been suggested that HPτ accumulation is initiated at the level of pons decades before the symptom debut, referred to as stages a to 1b [15].

During stages I – II, patients are usually asymptomatic; stages III – IV are associated with MCI , and final stages V – VI with full-blown dementia [42,48,81].

Within the scope of this thesis, the focus is on τ pathology in AD. However, it is worth noting that τ is involved in several neurodegenerative diseases as primary tauopathies, i.e. progressive supranuclear palsy, corticobasal degeneration, Pick disease or argyrophilic grain disease and aging-related tau astrogliopathy (ARTAG) [82,83].

The final diagnosis of Alzheimer's disease

Since 1991, the diagnosis of AD was based on the counts of NP assessed, proposed by CERAD. The recommendations included assessment of the amount of NP visualised histochemically in a set of neocortical sections [41,42,47]. Noteworthy, a CERAD NP stage can also be given applying IHC while using a combination of both Aβ and HPτ or Ubq stain; a lesion with concomitant Aβ and HPτ or Ubq is referred to as a NP [45,46,51,60,84]. Several studies have described that the number of Ubq NP exceeds the number of NP containing HPτ in brains of cognitively unimpaired and in AD. While NP with Ubq is

associated with brain ageing, the HPτ containing NP is associated with CI and AD [51,85].

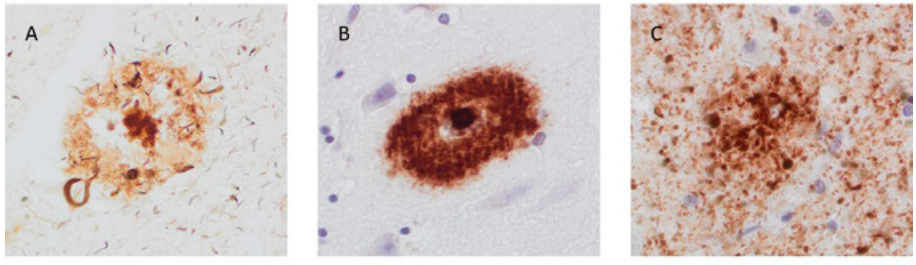

Figure 3. Neuritic plaque pathology. A. Bielchowsky silver stain. B. Immunohistochemical (IHC) staining applying antibody (Ab) towards Amyloid β. C. IHC staining applying Ab against HPτ.

The final neuropathological diagnosis of AD is an integrated diagnosis using the Thal phase of Aβ pathology (A), Braak stage of neurofibrillary change (B), and CERAD score of NP (C). These are summarised into ABC scores, which are then transformed into one of the four levels of ADNC: None, Low, Intermediate or High level of AD pathology [41,42].

ADNC can be present within the brain parenchyma several years before the onset of the symptoms. There are also individuals that never exhibit cognitive symptoms despite having AD pathology within their brain tissue [15,16,86]. HPτ pathology can be detected in the brainstem nuclei early on in life, and cortical Aβ aggregates can be seen in unimpaired elderly individuals [15,16,61,86].

The pre-symptomatic stages of the disease are very interesting, as several drug trials describe the most efficient effect of the substance in the prodromal stages of the disease [87-90]. The target of the treatment regiments is to stop the progressive nature of Aβ and HPτ and thereby slow or prevent the process of neurodegeneration.

Alzheimer's Disease Neuropathological Change		Hyperphosphorylated τ pathology (Braak stage)		
A = Amyloid β pathology (Thal phase)	C = Neuritic plaque pathology (CERAD)	B0 or B1 (0 or I-II)	B2 (III-IV)	B3 (V-VI)
A0 (0)	C0 none	Not	Not	Not
A1 (1-2)	C0-C1 (none-sparse)	Low	Low	Low
	C2-C3 (moderate-frequent)	Low	Intermediate	Intermediate
A2 (3)	Any C	Low	Intermediate	Intermediate
A3 (4-5)	C0-C1 (none-sparse)	Low	Intermediate	Intermediate
	C2-C3 (mederate-frequent)	Low	Intermediate	High

Figure 4. Grading scheme, according to NIA and AA guidelines for neuropathologic assessment of AD [41,42].

Neuroinflammation

Inflammation is a biological process for protection against foreign agents, controlling and confining injuries and repairing tissue damage. Neuroinflammation is closely associated with the neurodegenerative process of AD. In the brain, the main mediators of inflammation are astrocytes and microglia [91-93].

Astrocytes are specialised glial cells seen throughout the central nervous system, in both grey and white matter. During the last 20 years, a multifaceted role of astrocytes has been discovered. Except for the features commonly attributed to astrocytes, such as supporting cells and cells involved in tissue repair and scar formation, the astrocytes are acknowledged to play a role in the blood-brain barrier, water balance/glymphatic functions, metabolic signalling, and neuronal and synaptic support and functions [92,94,95].

Microglia are the macrophages of the brain, the first line of defence against changes in the local environment. They are highly specialised cells with cell processes that detect changes in the brain. They are able to change their morphology and biological properties depending on the cellular- or tissue changes, responding with inflammatory reaction by cytokine and chemokine release and phagocytosis. They interact closely with neurons/synapses and astrocytes, contributing to brain development, neuronal plasticity and repair and synaptic signalling [91,96,97].

The inflammatory environment is altered with ageing and increases due to the process of neurodegeneration [95,98]. Both astrocytes and microglia are significantly increased in AD [95,99,100]. This is because of a reaction towards Aβ and HPτ, trying to restore the homeostasis of the affected brain tissue [91,93]. It is acknowledged that microglia can phagocyte the soluble Aβ oligomers and/or degrade them enzymatically, a clearance mechanism that can be beneficial at the beginning of the neurodegenerative process. As the Aβ pathology

progresses, the microglia are unable to clear the Aβ and are activated into a chronic inflammatory state, changing their properties, promoting a pro-inflammatory neurotoxic milieu [101,102]. Astrocytes are activated in AD, by Aβ and HPτ pathology, and the inflammatory environment, in close collaboration with microglia, releasing cytotoxic proteins, resulting in maintaining the inflammatory, neurotoxic environment and promoting the progress of neurodegeneration [91,93,95,103]. Thus, in the setting of AD, the neuroinflammatory process contributes to and exacerbates the ADNC pathology as well as synaptic and neuronal damage and death [91,93].

This contribution to the AD pathology is interesting, as anti-inflammatory treatment is easy to apply. Subjects with regular administration of non-steroidal anti-inflammatory drugs displayed lower counts of astrocytic- and microglial-activation [104]. Several drug trials with regular anti-inflammatory treatment have been carried out with contradictory results. When the disease was already established, the effect was not acknowledged; nonetheless, subjects receiving treatment continuously while in the pre-symptomatic phase displayed some effect on cognition [90,105,106].

The neurodegenerative process of AD

In parallel with the progression of ADNC and neuroinflammation, there is a progressive neurodegenerative process that is ongoing, resulting in brain atrophy caused by synaptic and neuronal damage and finally, cell death [107-109].

Synaptic loss is an early event in AD, which progresses through the course of the disease [110,111]. The synaptic changes precede the neuronal loss and are closely associated with cognitive symptoms [112,113]. The synaptic network is also involved in propagation of pathological proteins, hence accelerating the neurodegenerative process [81,107,108]. ADNC causes the synaptic damage by different mechanisms. One of them is loss of pre-synaptic and post-synaptic proteins, crucial for synaptic function and thus leading to decreased function related indirectly to the cognitive impairment [114-116]. Dendritic spines, structures responsible for direct contact and propagation of excitatory impulses, are damaged and decreased in volume in AD and thereby directly decrease the signal transmission through synapses, leading to cognitive impairment [116,117]. Extracellular Aβ aggregates accumulate around synapses, influence the receptors localised on the cell-surface, impair the signal transmission and damage the dendritic spines [116]. HPτ is involved in dendritic pathology as it can redistribute from axons to dendrites, where it accumulates in the dendritic spines, affecting the transportation, synaptic plasticity and memory [116,117]. ADNC also affects the mitochondrial function in synapses. Synaptic function requires energy, which is why the mitochondria is transported to synaptic terminals to cater to the demands. Aβ impairs the mitochondrial function, causing mitochondrial fragmentation and an increase in free radicals and oxidative stress that is directly toxic, causing impaired transmission and synaptic damage [116].

The neuronal population is affected directly during the disease process by neurofibrillary pathology [53,76]. Within the medial temporal lobe, an area that is affected by AD early on, there is evidence of increasing HPτ pathology and neuronal loss within the entorhinal cortex, but the neuronal loss exceeds the HPτ pathology [118,119]. This indicates that there are other mechanisms for neuronal cell death; some studies refer to apoptosis mediated processes [120]. Additionally, the mitochondrial dysfunction, resulting in production of free radicals and oxidative stress, can cause cell death early on in the disease process [116]. During the disease progression, the increase in NFT is paralleled with progressive neuronal loss [121]. Contrary to the above, cortical areas, affected late in the course of AD, displayed remarkable preservation of neurons bearing intracellular NFT [122].

The most intriguing feature in the neurodegenerative process is the predisposed pattern of the neuroanatomical propagation of the altered proteins. Prion-like properties have been associated with both Aβ and HPτ as both proteins undergo post-translational changes, forming toxic variants and promoting further neurodegeneration [31,62,72,116,123,124]. Regarding Aβ, animal studies have described a seeding mechanism, first located in the site of injection, but lately engaging the axonally connected regions. Moreover, toxic Aβ oligomers promote the formation of new oligomers from monomers, potentiating the effect, where the pyβ variant was one of the most prominent, both regarding neuronal toxicity and prion-like behaviour [63,125]. HPτ is also described to display seeding properties in cell cultures and animal models, promoting the pathology through axonally connected regions [72,124,125]. That is in line with the propagation of HPτ pathology in human to distant structures, when starting in the brainstem nuclei and directly progressing into the transenthorinal region of the brain [15,126].

The effect of ADNC on the neuronal/synaptic population is intriguing and still not entirely clear despite decades of research; hence, further studies are needed.

Idiopathic Normal Pressure Hydrocephalus

The idiopathic Normal Pressure Hydrocephalus (iNPH) is a neurological condition presenting with ventriculomegaly on radiological examination and slowly developing classical symptoms of gait and balance disturbances, cognitive impairment (CI) and difficulties with urinary control [127-129]. The disorder principally affects the elderly. In Sweden, the prevalence ranges between 0.2 to 2.1% in individuals 65 – 79-years-old and 5.9 to 8.9% in subjects over age 80 [130,131]. Despite quite notable incidence of iNPH, it is suspected to be underdiagnosed due to multiple disorders and co-morbidities with similar symptoms that are more common in the older population [127,128,132]. This is unfortunate as the symp-

toms of iNPH can be reversed as a result of a treatment with a ventriculoperitoneal shunt (VPS) insertion [127-129,133,134]. When left untreated, patients with iNPH have a higher risk of developing dementia and have higher mortality rates than shunted iNPH subjects and age-matched controls [133,135-137].

Pathophysiology of iNPH

Pathophysiology of iNPH is currently not entirely understood. Abnormal CSF dynamics, vascular diseases, neurodegenerative diseases, inflammatory processes, metabolic factors and heredity are described to influence the development of this ailment [138-140].

INPH is a disorder where the water balance disturbance causes hydrocephaly and is associated with abnormal CSF homeostasis, blood-brain barrier (BBB) damage and glymphatic dysfunction [139-142].

The mechanism to maintain water balance in the brain and a clearance mechanism from its metabolites are not entirely understood. As the brain lacks a defined lymphatic system, the brain clearance and homeostasis are accounted for by intra- and extra- cellular degradation of proteins, through blood-brain barrier transport and by different mechanisms through CSF, the recently discovered glio-lymphatic (glymphatic) system as one of them [143]. It is a complex pathway where the aquaporin-4 (Aqp4), a water channel in the astrocytic end-feet, is an important player involved in both BBB function, glymphatic clearance and brain water balance [143-146]. During disease, as iNPH and AD, the Aqp4 is reduced, thus, impairing the clearing mechanism [141,144-147]. In iNPH, both glymphatic influx and efflux disturbances are described [141,144,148]. Furthermore, the reduced glymphatic function is directly associated with ventriculomegaly, the hallmark lesion of iNPH [144].

In AD, the glymphatic system is an important pathway of clearance of soluble, interstitial Aβ; however, when the function is impaired, the outcome is decreased clearance and increased Aβ aggregation [143-145]. A substantial number of iNPH subjects display Aβ in their brain, and thus are affected by impaired Aβ clearance and progressive ADNC disease [144,145,149].

BBB damage is another contributing factor to the impaired water balance in iNPH as leakage of plasma proteins has been described in subjects with iNPH in parallel with Aqp4 decrease [139,150].

These finding are in line with what has previously been described in AD and vascular dementia [151,152]. The mechanisms of impaired glymphatic clearance and BBB damage are thus similar in AD and iNPH [144-146].

In iNPH, the defective CSF homeostasis causes hypoperfusion of the parenchyma, inflammatory responses and altered metabolism contributing to white matter damage, affecting the neuronal tracts causing the symptom development and at the end stage affecting neuronal functions [140,142].

Clinical approach

There are international guidelines to be applied when diagnosing subjects with iNPH, and patients with suspected iNPH undergo a thorough examination including clinical history, physical and neurological examination, extensive blood testing, neuropsychological evaluation, brain imaging, as well as CSF- and CSF dynamic- tests [153,154]. For a diagnosis, the age of onset should exceed 40 years, and the symptoms should progress over a long time, at least 3 to 6 months. Other conditions that can cause the symptoms should be identified or excluded. Although the classical symptoms are part of the iNPH triad, all three factors are not required for a diagnosis.

The symmetric gait disturbance is described to be the earliest and most affected clinical feature of iNPH. The subject presents with short- and broad-based gait with decreased height and length. Additionally, balance abnormalities, difficulty in transitional movements and gait initiation, among others, can be noted [127-129,140,153-155]. There are several testing methods that can be applied, for instance, ten-metre walk test, Timed Up and Go test, walking backwards 3m test, and ordinal balance- and gait scales [156].

The CI and dementia indicate that prefrontal brain structures are involved, as the main symptoms include lack of attention and concentration, apathy, psychomotor slowing and memory deficits. The severity of CI varies in iNPH patients from mild CI to severe dementia. The neurological and neuropsychological evaluations are crucial during the diagnostic process as presence of other cognitive illnesses or co-morbidity with other dementias can affect the choice of treatment or the treatment outcome [127-129,140,153-155]. The cognitive function can be tested with MMSE and neuropsychological tests such as Grooved pegboard, the Rey Auditory Verbal Learning Test and the Swedish Stroop test [156].

Urinary symptoms can present early in the disease process, most commonly as increased urgency and frequency. Those symptoms can subsequently progress into incontinence [127-129,140,153-155]. Continence can be rated by an ordinal continence scale or urodynamic tests [140,156].

According to the guidelines, a radiological brain examination is required for diagnosis, either with computed tomography, or more preferred, MRI [140,153,154]. A communicating hydrocephalus, with enlarged lateral and third ventricles, is seen in subjects with iNPH. The ventricular enlargement can be measured by the Evans score, measuring a ratio between maximal diameter of frontal horns of the lateral ventricles and the maximal inner diameter of the skull. An Evans score exceeding 0.3 is a sign of ventriculomegaly. Other factors to be assessed are callosal angle, enlargement of temporal horns, any macroscopic obstruction of the CSF flow, periventricular signal changes, and disproportionately enlarged subarachnoid space hydrocephalus due to widened sylvian fissures [127-129,153,154,157].

CSF dynamic testing is recommended by the international guidelines to assess if a patient with iNPH has the potential to respond to shunt surgery. The most common is a large-volume lumbar puncture, also called the tap test, where 30 – 50 ml CSF is removed to temporarily create a shunt effect. The patient is examined just before the removal and 2 – 4 hours after the lumbar puncture. The symptoms are observed, tested and documented. Impaired gait is the symptom that is most likely to respond to the CSF removal [127-129,140,153-155]. Other accessible tests are CSF infusion testing, external lumbar drainage and intracranial pressure (ICP) monitoring [127-129,140,155].

When the tests above indicate a functional alteration, i.e. iNPH, then VPS insertion is the recommended treatment following the international guidelines [153,154]. During the shunting procedure, a catheter is placed through the brain parenchyma into the right frontal horn of the lateral ventricle. The distal end is positioned into the peritoneal cavity. Between the catheter ends, there is a shunt valve normalising the CSF flow within the brain by opening when the CSF pressure exceeds the physiological state [127-129,155].

According to the literature, 50 – 80% of patients improve in their initial symptoms after the shunt surgery [130,131,133-135,137,158]. When performed early in the disease process, it improves the long-term quality of life and increases survival [159,160]. Noteworthy, the incidence of iNPH, and thus hydrocephalus surgeries, is increasing even in the oldest population [130,131,133-135,137,158]. Despite advances in diagnostics and successful treatment outcome of iNPH patients, the mortality rate is still 2 – 4 times higher than in the general population [135,161].

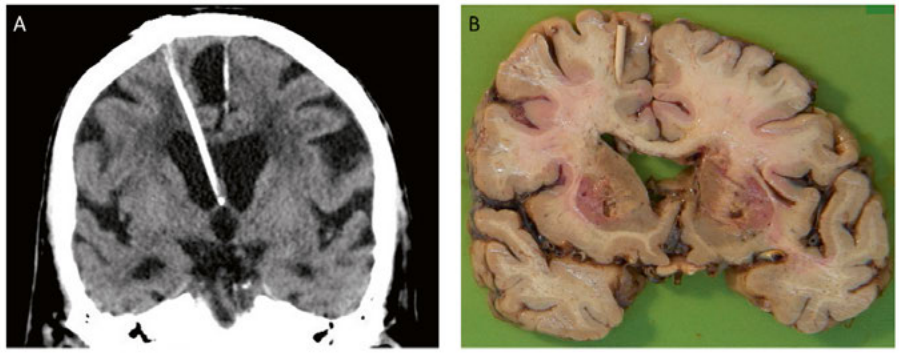

Figure 5. Ventriculoperitoneal shunt in the brain of a subject with idiopathic Normal Pressure Hydrocephalus. A. Radiology. B Post-mortem brain.

Neuropathological findings

During the last three decades, in a few centres around the world, tiny brain biopsies have been sampled from patients with iNPH within the area of the shunt channel, during the VPS surgery or ICP measurement [162-166]. There are also a few studies describing neuropathological findings in PM brain tissue

from patients with iNPH [167-169]. Up to date, no neuropathological lesion, which is characteristic of iNPH, has been identified. However, numerous publications describe ADNC within the brain tissue, surgical or PM, from patients with iNPH [149,162-169].

The presence of ADNC is associated with worse shunt response after the VPS surgery [164,170]. The patients presenting with ADNC in their biopsies had lower MMSE scores before surgery than patients without AD pathology [163,171]. Furthermore, subjects without ADNC in their brain biopsy, but presenting with cognitive impairment, displayed ADNC when re-biopsied [164]. Several studies have presented that a substantial number of subjects with ADNC develop clinical dementia over time, predominantly of AD type, and ADNC per se in the biopsy is the strongest predictive factor for developing AD [136,165,172,173]. A PM study revealed that as many as 56% of iNPH subjects displayed various levels of ADNC at the neuropathological examination [168]. In line with this finding, a few studies describe progression of ADNC over time in brain tissue, seen in the frontal cortical biopsies, followed by evaluation of the PM brain tissue from a subject with iNPH [167,169]. This is in line with the stepwise, architectural progression pattern of neurodegeneration seen in AD [41,44,48,61,80].

Although there are only few groups in the world assessing the brain tissue biopsies from patients with iNPH, they were able to produce valuable information, repetitively displaying presence of ADNC in patients with iNPH and progression of the pathology and clinical parameters of a dementing illness [136,162-167,169,173]. This indicates that substantial number of iNPH subjects also suffer from AD. Additionally, these results may indicate that iNPH can be considered as a model of early, prodromal stages of AD; consequently, patients affected would thereby benefit from early intervention not only with regard to the CSF-dynamics but also to stop the progress of neurodegeneration.

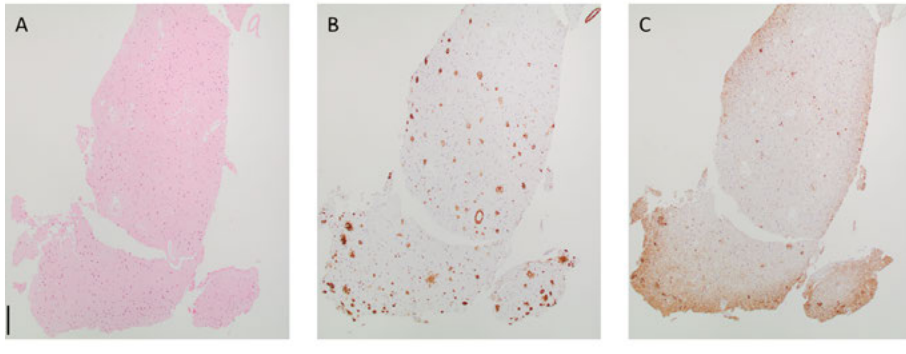

Figure 6. A frontal cortical biopsy from a subject with idiopathic Normal Pressure Hydrocephalus. A. Applying haematoxylin-eosin staining. B. Applying antibody (Ab) towards Amyloid β. C. Applying Ab towards hyperphosphorylated τ. Bar 200μm.

Ageing as a risk factor

In 2011, while assessing a large cohort of subjects, age ranging from 1 to 100 years, Heiko Braak and colleagues reported that ADNC indeed progresses with age and that virtually all individuals at the age of 40 display HPτ. They further reported that at the age of 80, this pathology has reached the neocortex in each individual. Regarding Aβ, only about 50 – 60% display this alteration in their brain [15]. In line with the above, several reports have shown that iNPH also increases with age [130,131]. This interpretation is further complicated by the insight that there are numerous ageing related alterations that might interfere with the clinical outcome in normal ageing and dementia [11,12,14,16].

Besides the established proteinopathies such as AD, primary tauopathies, synucleinopathies and transactive response DNA binding protein 43 causing diseases (Frontotemporal lobar degeneration and Amyotrophic lateral sclerosis), several new entities have been recognised during the last decade, i.e. Primary Age Related Tauopathy (PART), ARTAG, and the most recent Limbic predominate age-related TDP 43- encephalopathy (LATE) [82,174,175]. Some studies suggest that during ageing and development of a proteinopathy, an altered protein can further promote other concomitant pathologies [176].

Parallel with the proteinopathies, the cerebrovascular diseases and systemic diseases can also have an effect on cognition [13,14,177].

The complexity of ageing and dementia is acknowledged when performing PM neuropathological examination, as most of the aged individuals display two or more pathologies in their brains i.e. display mixed pathology and when cognitively impaired, suffer from mixed dementia [10,11,13,14,176]. This is indeed of interest in many aspects as the mixed pathologies can alter symptomatology, complicate the clinical assessment leading to a clinical diagnosis, and influence the choice of a treatment strategy.

The mechanisms of ageing and dementia are thus multifaceted and in need of further studies. This is because the incidence of dementia is increasing in the world, causing suffering at an individual level and raising concerns that the healthcare system has not been able to offer a cure or at least symptom relief treatment [3,9].

Immunohistochemistry

IHC is a technique used in pathology, routine diagnostics and research to visualise specific proteins within cells and tissues using an antibody (Ab) – antigen (Ag) mediated process [178-180]. Routinely, the most used fixative is 10% buffered formalin (4% formaldehyde) as it is the best preservative of morphology within the tissue [181,182]. During the fixation procedure, formalin penetrates the tissue, binds to proteins, i.e. the Ag of interest, and crosslinks these with unrelated proteins, thereby blocking the epitopes within the Ag and changing

their molecular structure [178]. When fixed, the tissue is embedded into paraffin blocks to facilitate storage and handling of the tissue [183]. The blocks are cut into 3 to 10 µm thick sections, which are de-paraffinised with xylene and re-hydrated through alcohol treatment. Antigen retrieval (AR) is performed to reverse the block of Ag caused by formalin fixation. There are both enzymatic- and heat-induced AR methods. The heat-induced AR is most common, where the tissue is heated while being treated in high or low pH buffers [178,182,184]. In some cases of manual staining, blocking of endogenous Fc-receptors can be performed to prevent non-specific binding of the Ab and thereby background staining [185]. The most important step in IHC is choice of a specific and reliable Ab. Abs can be monoclonal or polyclonal. Monoclonal Abs originate from one B-cell, recognise one epitope of an Ag that eventually has high specificity and display less background staining. Thus, generally, several commercial monoclonal Abs are tested prior to use. The polyclonal Abs are produced by several B-cells, recognising numerous epitopes of an Ag, thereby having higher affinity but also risk of unspecific binding due to reactivity with similar epitopes [178,182]. To visualise the Ab-Ag reaction, different detection systems (DS) can be used. Briefly, the Abs are labelled with a chromogen, which as a result of the Ab-Ag binding undergoes an enzymatic reaction and produces a colour signal. The DS can be one – three step reactions. The DS used in our studies is currently the most recommended, polymer-based 2-step method, where the primary Abs bind to secondary Abs, bound to a polymer structure with multiple chromogen molecules [178,186]. It is a simple method with high sensitivity and low risk of background staining.

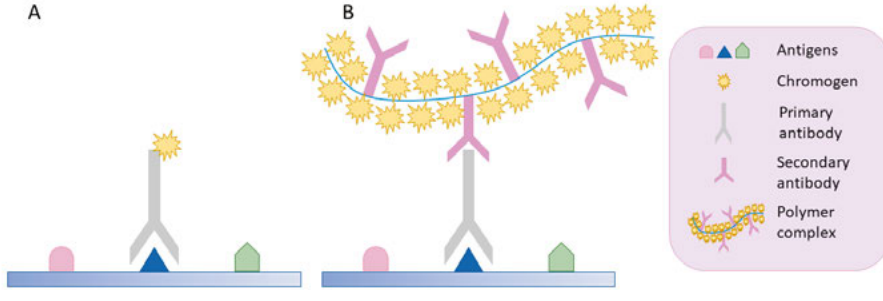

Figure 7. The process of immunohistochemistry. A. Direct method. B. Polymer-based method.

The outcome of IHC depends on all the steps mentioned above, but pre-analytical factors also have to be considered. Cold ischemia time i.e. time the tissue stays at room temperature before being put into a fixative, the fixation time (FT), type of fixative, type of paraffin i.e. melting temperature, storage time in blocks, the section thickness (ST), storage time of cut sections, AR technique, the choice of Ab used and DS are all crucial factors during the IHC process [178,182-184,186-191]. In the field of neurodegeneration, there are additional

factors, as the tissue is assessed PM, i.e. agonal state and post-mortem delay (PMD), which affect the quality of tissue, i.e. epitopes and thereby the outcome of the staining [187,192,193].

To produce reliable, reproducible, IHC results, the process should be performed using standardised protocols [179]. Automatic IHC platforms are gaining popularity within clinical practice due to consistent quality of staining when using manufacturer's settings for the apparatus, pre-fabricated buffering solutions and ready-to-use Abs. This mode of action is certainly helpful to eliminate human error; however, it lacks the transparency of the methodology that is desirable to reproduce a staining for research purposes, where the manual mode of action should be preferred.

One should be aware of eventually altering the staining protocol while assessing a surgical sample and PM material. Some epitopes of interest might be damaged by various pre-analytical variables, leading to false negative outcome and thus cannot be assessed in PM tissue. Contrary to the above, a surgical sample is well preserved and thus gives an option to assess a number of alterations previously not assessed in AD or iNPH, thus, eventually identifying a hallmark lesion of iNPH.

Aims

Overall aims

The purpose of this work was to characterise ADNP, neuronal population, glial- and inflammatory markers in surgically sampled brain tissue from patients with iNPH. Specifically, the aim was to investigate how these different variables influence one another and how they are affected by the patient's age and gender. An additional aim was to see how those parameters are affected by disease duration from the point of the biopsy until death. We also aimed to identify IHC manuals to enable the comparison of surgical and PM brain tissue samples needed to realise this project.

Specific aims

I	To assess how different ST influences the outcome of IHC applying Abs towards ADNC, neuronal, glial and inflammatory markers, on PM brain tissue.
II	To investigate changes in ADNC, neuronal-, glial- and inflammatory markers during a time frame of 21 months, within the brain parenchyma from a patient with clinical diagnosis of iNPH and AD.
III	To assess morphometrically and evaluate ADNC and the neuronal population in cortical brain biopsies, obtained during curative VPS insertion, from subjects treated for iNPH.
IV	To evaluate different variants of Aβ and their association to HPτ in brain biopsies from iNPH subjects and within a tissue micro array (TMA) containing brain tissue samples from subjects with different stages of AD.

Materials and methods

Patients

The project and thus all the included studies were authorised by the regional Ethical Committee in Uppsala, Sweden (#2013/176 updated 2016 and #2011/286 updated 2015). An informed consent to use the diagnostic tissue for scientific purposes had been given by the patients or their close relatives.

The studies were performed on brain tissue obtained surgically (study II, III and IV) or PM (study I, II and IV). The surgical biopsies were obtained from iNPH patients, treated with curative VPS insertion, at Uppsala University Hospital (UUH) during 2010 – 2018. The PM brain samples were obtained during neuropathological examination at the UUH during 2011 –2013.

The study in papers I and II was performed on brain tissue from the same subject, a female with a clinical diagnosis of iNPH and AD. In paper I, only the PM brain tissue, obtained during 2011, was assessed. In paper II, the pathology assessed was compared in both surgical biopsies, obtained in 2010 and PM brain tissue from the same subject.

In paper III, the cohort included 95 subjects out of 364 biopsied during the VPS insertion during 2010 and 2016. The selection criteria were age range between 75 – 79 years, independent of the pathology seen in their biopsies.

Paper IV included brain biopsy samples from 127 subjects out of 448 treated during curative VPS insertion at UUH. The selection criteria were notable Aβ pathology in the diagnostic biopsy and age 70 years or older (70 – 88 years) at the time of the biopsy.

Clinical assessment

The information regarding clinical assessment of the female patient described in papers I and II was obtained retrospectively. The patient underwent a standardised investigation at her local hospital, including clinical examination and assessment of her cognitive status as well as blood samples and imaging. The patient was investigated by a multidisciplinary iNPH team at UUH, which included a neurosurgeon, neurologist, physiotherapist and occupational therapist. The team assessed and evaluated her motoric and cognitive functions; beyond the CSF analysis, a CSF dynamic test was performed. According to local routines at UHH, the patient was additionally evaluated by the iNPH

team, 3 and 12 months after the shunt insertion to assess the treatment outcome and shunt function.

Sampling

In papers II, III and IV, brain biopsies were obtained during the VPS shunt insertion from an area of superior or medial gyri of the right frontal lobe, as previously described [163,165]. The biopsies were fixed in 10% buffered formalin (4% formaldehyde) for 24 hours at room temperature. Thereafter, the tissue samples were embedded into paraffin blocks (Histowax from Histolab Products), cut into 4µm thick sections, and placed on the Super Frost slides for HE and Super Frost Plus slides for IHC stainings.

In papers I, II, IV, PM brain tissue was also assessed. In papers I and II, the autopsy was carried out at a County hospital in Uppsala-Örebro region, Sweden. The PMD was four days. The brain was placed in 10% buffered formalin (4% formaldehyde) for 46 days, stored at room temperature and shipped to UUH for neuropathological examination.

In paper IV, the PM brain tissue was obtained from 19 subjects, whose brains underwent a neuropathological examination at UUH during 2011 – 2013. The brains were fixated in 10% buffered formalin (4% formaldehyde).

All PM brains (papers I, II and IV) were assessed macroscopically and were sampled according to a standardised procedure. The regions sampled were as follows: 1. Frontal cortex; 2. Temporal cortex; 3. Gyrus cingula; 4. Parietal cortex; 5. Motor cortex; 6. Occipital cortex; 7. Anterior hippocampal formation; 8. Posterior hippocampal formation; 9. Basal forebrain with amygdaloid body; 10. Striatum with insular cortex; 11. Thalamus; 12. Mesencephalon; 13. Pons; 14. Medulla oblongata; 15. Cerebellar vermis and nucleus dentatus and 16. Cerebellar cortex. In paper II, an additional region was included and sampled, namely, right frontal cortex with a shunt channel. The samples were processed into paraffin blocks and sectioned. All samples were primarily sectioned into diagnostic 7µm thick sections and placed on the Super Frost slides for HE and Super Frost Plus slides for IHC stainings.

For diagnostic purposes, all cases were assessed following international criteria regarding the Aβ-, HPτ-, α-synuclein and TDP43 pathology [41-43,194].

For scientific purposes, in paper I, the sample from the right frontal cortex without a shunt channel was sectioned into 4 µm and 7 µm thick sections and placed on Super Frost Plus slides for further IHC analyses.

In paper II, samples for the analysis were chosen from two adjacent regions, i.e. a sample from the frontal cortex without shunt channel and a sample from the frontal cortex with the shunt channel. The blocks were sectioned into 4µm thick sections and placed on Super Frost Plus slides for IHC stainings.

In paper IV, a tissue micro array (TMA) block was constructed, where each of the 19 subjects was represented by two cores, measuring 2 mm in diameter,

from the amygdaloid complex. The TMA block was then sectioned into 4µm thick sections and placed on Super Frost Plus slides for IHC stainings.

Immunohistochemistry

The IHC was performed both manually and using an automatic platform. Manual staining was applied in papers II and III using the BrightVision detection system (IL Immunologic, Duiven, Netherlands) with Romulin AEC for antigen detection (BioCare Medical, Pacheco, CA, USA). The automatic staining procedures applied in all papers were performed according to manufacturer's instructions on Dako Autostainer Plus or Dako OMNIS using Dako EnVision Flex detection system (Dako Cytomation, Glostrum, Denmark). The Abs used in the studies, the pretreatment regiments and the platform used are summarised in table 1.

In paper IV, two of the antibodies, namely the $pA\beta^{1E4E11}$ and $A\beta^{7H3D6}$, were generated by one of the co-authors as previously described [66]. These antibodies required additional treatment strategies to optimise the outcome of the staining. When applied on PM sample, the $pA\beta^{1E4E11}$ Ab was incubated overnight at 4°C; for the detection, a mouse linker was applied for 20 minutes. In contrast, when applied on a brain biopsy, the $pA\beta^{1E4E11}$ primary Ab was applied 2 x 30 minutes, followed by mouse linker for 20 minutes, followed by using Dako EnVision Flex Kit and 2 x 5 minutes DAB (Horseradish Peroxidase included in Dako Envision Flex Kit). When applying the $A\beta^{7H3D6}$ Ab, a rabbit anti-rat Ab was applied for 30 minutes before the Dako Envision Flex Kit.

Table 1. Immunohistochemical stainings

Antibody	Clone	Company/Code	Dilution	Pretreatment	Paper
Amyloid β (Aβ) 1-42*	12F4	Covance/SIG-39142	1:1000	80% FA – 1h	II
Aβ 1-40*	polyclonal	BioSource/44-348A	1:500	80% FA – 1h	II
Aβ aa8-17	6F/3D	Dako-Agilent/M0872	1:50	98-100% FA-2 min	I, II, III
Aβ aa8-17	6F/3D	Dako-Agilent/M0872	1:50	100% FA – 5 min	IV
Aβ aa17-24	4G8	Biolegend/800703	1:4000	100% FA – 5 min	IV
pyAβ N3pE	polyclonal	Tecan/JP18591	1:50	100% FA – 5 min	IV
pAβ S8	1E4E11	"In house" [66]	1:500	100% FA – 3 min	IV
umAβ	7H3D6	"In house" [66]	1:1000	100% FA – 5 min	IV
CD68(PGM1)*	PG-M1	Dako/M087629-2	1:200	ac, TE	II
CD68(KP1)	KP1	Dako/IR609		pH High	I
Glial fibrillary acidic protein (GFAP)	polyclonal	Dako/ Z0334		pH High	I, II
Embryonic lethal abnormal visual systemproteins 3 and 4 human homolog HuC/HuD *	16A11	ThermoFisher Scientific/A-21271	1:2000	ac, CB	II, III
Human leucocytic antigen -DR, α-chain (HLA-DR)	TAL.1B5	Dako/M0746	1:30	pH Low	I, II
Ionised calcium-binding adaptor molecule1(Iba1)	polyclonal	Wako/Nordic Biolabs 019-19741	1:5000	pH High	I, II

Microtubule associated protein 2 (MAP2)*	HM-2	Sigma Aldrich/M4403	1:500	ac, CB	II
NEUronal Nuclei (NeuN)	A60	Millipore/MAB377	1:2000	CB	III
Neurofilament H (SMI32)	SMI32	Sternberger/SMI32	1:1000	pH High	I, II
Synaptophysin (SYP1)	SP11	Abcam/ab16659	1:40	pH High	I, II
Synaptophysin (SYP2)	SY38	Dako-Cytomat-ion/M0776	1:50	CB	III
Hyperphosphorylated τ (HPτ)	PHF-TAU-AT8	Fisher Scientific-In-vitrogen/MN1020	1:1000		I, II, III, IV

* Stainings performed manually; Formic acid (FA), amino acid (aa), pyroglutamylated (py), phosphorylated (p), unmodified (um), autoclave (ac), Tris-EDTA buffer pH 9.0 (TE), citrate buffer pH6.0 (CB).

Assessment strategies

All samples were assessed using light microscopy (Olympus BX45) at x20 to x400 magnification. All slides included in the papers were then scanned into digital slides, in ScanScope virtual slide format, at x200 magnification using Aperio AT2 (Leica Biosystems, Inc)

In papers I – III, all the samples were morphometrically analysed, while in paper IV only a subset of samples was morphometrically analysed to harmonise different assessment strategies. The positive pixel count (PPC) algorithm (version 9.1) within the Aperio ImageScope software (Leica Biosystems) was applied for the morphometric image analyses. One parameter in the software, "The intensity threshold (Upper Limit) of WEAK positive pixels" was increased from 250 to 255 when performing an analysis. All other parameters remained pre-set in the software.

In papers I and II, the algorithm was applied on PM brain samples where two grey matter areas, measuring 4 mm^2, were analysed. In the biopsy samples in paper II, the whole grey matter area was assessed and measured in mm^2. In papers III and IV, the algorithm was applied on the grey matter in each biopsy, excluding the CAA and molecular layer when present in a biopsy.

A customised colour code visualised the pixels with different staining intensities within a sample. Negative pixels were blue, weak staining was marked in yellow, moderate in orange and strong stained pixels were brown. The IR pixels within the assessed area were counted and transformed into a stained area in mm^2. In papers I and II, all positive pixels within a sample, i.e. yellow, orange and brown were incorporated in the final analysis.

In papers III and IV, the number of pixels was chosen according to the intensity and quality of the staining, as well as the compartmentalisation of the protein of interest. The sum of all positive pixels was accounted for when assessing Aβ. The moderate and strong positive pixels were assessed in HPτ and embryonic lethal abnormal visual system proteins 3 and 4 human homolog HuC/HuD (HuC/HuD) stainings. Strong positive pixels were only assessed for Neuronal Nuclei (NeuN) and synaptofysin (SYP) stainings.

The final results are the ratio between the stained IR area per total area analysed, given as stained area fraction (SAF) x 100 in per cent. When two separate regions were analysed within a sample, as in papers I and II, a mean value of SAF was calculated for the given sample.

In paper IV, a semi-quantitative assessment strategy of the pathological alterations was used on the whole iNPH cohort and the TMA with samples from PM brains. The whole grey matter area was assessed in the biopsy samples, including molecular layer and CAA. In the TMA, the whole cores were assessed. When the extent of pathology differed between the two cores from the same subject, the core with highest extent of pathology was included in the final analysis. The extent of pathology was graded from 0 to 3, where 0 = no pathology, 1 = low level of pathology, 2 = moderate level of pathology and 3

= high level of pathology. When assessing the extent of HPτ pathology, as visualised in figure 8, grade 1 was assigned when a single or a few HPτ reactive granules or threads were present in the grey matter area. Scattered granules and threads and a few tangles were seen when grade 2 HPτ pathology was seen. When abundant number of neurites and several tangles were seen within a biopsy, the sample was deemed to be representing a grade 3 HPτ pathology. While assessing Aβ staining outcome, when a single – couple aggregates were seen in a biopsy, the sample was assigned as being grade 1. Grade 2 was assigned when scattered Aβ aggregates were noted in the grey matter. When abundant Aβ aggregates were present, the sample was assigned as being grade 3.

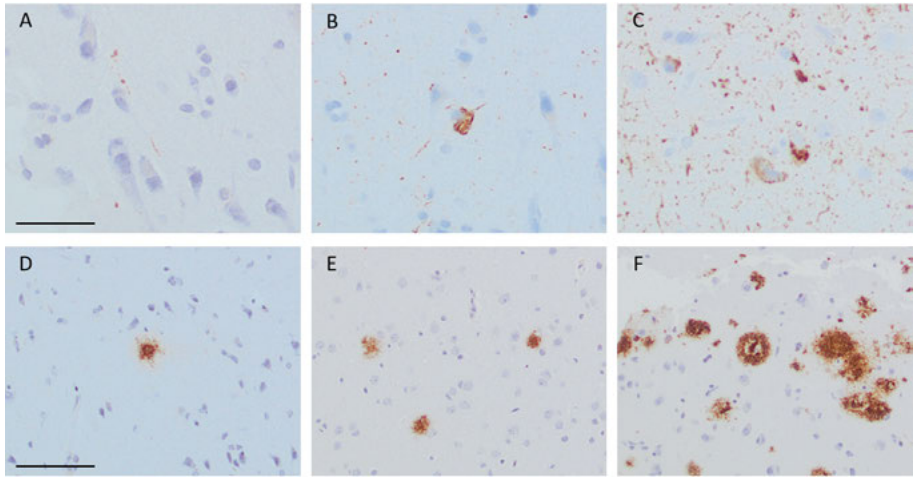

Figure 8. Grading of pathology in frontal cortical biopsies from subjects with iNPH (paper IV). In A-C, there are various grades of hyperphosphorylated τ pathology: A. grade 1, B. Grade 2 and in C grade 3. In D-E, there are various grades of amyloid β pathology: D. grade 1, E. Grade 2 and F. Grade 3. A-C bar 50µm and D-F bar 100 µm. Figure adapted from paper IV, Libard et al, 2021, in press in Journal of Alzheimer's Disease, with permission.

Statistical analysis

In papers I and II, the analysis is calculated in per cent. In papers III and IV, IBM SPSS statistics were used to perform the statistical analyses. To describe the cohort, means and standard error of means (M±SE) tests were applied. Non-parametric tests, such as Mann Whitney U test (MWU), Kruskal Wallis test (KWT) and Wilcoxon signed-rank test (WSRT) were used to evaluate differences between groups. The non-parametric Spearman's rho two-tail test was applied to define correlations between the studied variables.

Results and discussion

Paper I

In this paper, we studied how different section thicknesses affect an outcome of IHC when applying Abs commonly used while assessing brain alterations, both in surgical biopsies and PM. The results when assessing the performance of these Abs in 4 μm and 7μm thick sections from PM obtained frontal lobe of the brain are summarised in table 2. In addition to the SAF, we observed that the staining intensity was stronger in the thicker sections. The staining intensity did not affect the outcome of the SAF analysis as all positive pixels were included in the analysis, independent of their intensity.

IHC is a protein visualisation technique used in clinical pathology and in research [179,180]. Various characteristics of the tissue and the method applied for the analysis, i.e. cold ischemia time/PMD, fixative, FT, type of paraffin, storage time and temperature, choice of Ab, AR technique and DS, can alter the outcome of IHC [178,192,193]. There are, however, several differences in management of surgical brain biopsies and PM brain tissue for routine diagnostics; one of them is the ST used, i.e. 4μm for surgical samples and 7 – 15μm for PM brain tissue [44,179]. The effect of ST on IHC staining outcome has been previously described in peripheral organs, where cancer markers were studied in 2, 4, 6 and 8 μm thick sections; in those studies, a difference of 2μm in ST could influence the outcome [190].

In our setting, the expression of all the markers, routinely used in diagnostics of various CNS alterations, increased with ST (table 2).

In the context of neurodegeneration, the changes we observed regarding the extent of ADNC expression can affect the interpretation of the grade of the pathology and in worst case result in an inaccurate diagnosis. The Aβ pathology increased by 41% in the 3μm thicker section. This result can affect the Aβ pathology staging if the ST is not what is recommended, as the difference in staining outcome within the defined area can lead to incorrect, false negative outcome and thus lower Thal phase [61]. HPτ pathology increased with 15% in the thicker section. Thus, if the current recommendations are not followed, the outcome can be false negative in an area of interest and can affect the Braak staging [80]. Altogether, if the ST is not taken into consideration, the outcome might be false low ADNC staging; moreover, if the Aβ pathology is misinterpreted in the cortical areas, there may also be an incorrect diagnosis of PART [41,42,175].

Table 2. Stained area fraction (SAF) in per cent in 4µm and 7µm thick sections from the frontal cortex. Table adapted from Libard et al. 2019 [195] with permission.

	4µm	7µm
Protein	SAF	
Synaptophysin	86	91
Neurofilament H Sternberger-Meyer Immunocytochemicals 32 (SMI32)	5	10
Glial fibrillary acidic protein (GFAP)	78	86
CD68	2	4
Human leucocytic antigen -DR (HLA-DR)	6	9
Ionized calcium-binding adaptor molecule1(Iba1)	3	5
Amyloid β (Aβ)	16	27
Hyperphosphorylated τ protein (HPτ)	61	72

We assessed two different neuronal markers: SYP and neurofilament H 32 (SMI32). SYP is the most important synaptic marker routinely used in research and clinical practice, as it is a well-characterised protein located in the presynaptic vesicles [114]. SMI32 is a non-phosphorylated neurofilament protein, a neuronal cytoskeletal marker [121]. Both SYP and SMI32 increased with thickness section, which can affect the interpretation of synaptic damage and neuronal loss, commonly seen in AD [108].

Glial fibrillary acidic (GFAP) protein is an astroglial marker, extensively used in neuropathology [92]. Astrocytes have a multi-layered role in the brain and neurodegenerative disease, and a misinterpretation of the GFAP staining outcome can affect the assessment of the process [92,95].

We assessed three microglial markers: CD68, a transmembrane protein located in cytoplasmic granulae in macrophages and microglia; Human Leucocytic Antigen – DR (HLA-DR), a MHC class II cell surface receptor; and Ionised calcium binding adaptor molecule 1 (Iba1), an inflammatory factor located in the microglial cytoplasm [102]. All microglial markers increased with ST. In the setting of AD, inaccurate assessment of inflammatory markers can affect the interpretation of the inflammatory process closely associated with the neurodegenerative changes [102,104].

Standardised protocols when assessing pathological changes, in particular neurodegenerative alterations, are of great importance for intra- and inter-laboratory reproducibility, in clinical practice and research. As the extent of the pathology and neuroanatomical regions affected are of great importance when assessing neurodegenerative changes, a cooperation of 30 neuropathologists within the BrainNet Europe consortium assessed different proteinopathies in the brain. When the same section was assessed, the agreement rate was 80 – 90%; however, when stainings were performed in different centres, the agreement rates decreased to 50 – 80% [43,60]. Based on our findings, the result would drop even more if the ST were to vary.

Another factor for consideration, which has not previously been described, is the size of the Ab used. The most common Abs in IHC are of IgG type, with a weight of 150kDa and measuring 4x8.5x14.5 nm [178,196]. When constructing an Ab for IHC, other molecules are added to the IgG, thus changing the weight, molecular structure and probably also the ability to diffuse into a tissue sample [178]. We were not able to acquire information regarding the size of the commercial Abs used in our study.

A tissue section for IHC contains various cellular structures. When the thickness of the section increases, additional structures/epitopes are accessible for Abs in the three-dimensional tissue block and thus can influence the extent and intensity of the staining as seen here [191]. When performing an IHC staining, the manufacturer's recommendations regarding the ST should be considered as the changes in the IHC outcome with various ST, as seen in our study and by others, can affect the extent of pathology, number of IR cells and the definitive diagnosis [190].

In conclusion, IHC method is commonly used in diagnostics today; furthermore, automatic stainers are used in most larger laboratories to standardise the outcome. This might lead to a presumption that clinicians who assess the stained slides can be confident regarding the staining results. This is however a false interpretation of the situation, since the biological tissue is influenced by numerous factors as described above. In addition, there are numerous steps prior to the use of the automatic stainer that can alter the outcome. Nonetheless, these aspects stated above have not been fully acknowledged.

Paper II

Here, we investigated the changes in the expression of ADNC, neuronal-, glial- and inflammatory makers that occurred during 21 months in the brain of a female patient with concomitant iNPH and AD. The summary of the disease process, the clinical assessment, treatment and follow-up are summarised in figure 9.

Figure 9. Flowchart. Figure adapted from Libard et al. 2018 [169] with permission.

Neuropathological assessment

The stereotactic biopsy acquired from the right frontal lobe of the brain, during the curative VPS insertion, included both grey- and white matter. Within the grey matter, there were neurons with basophilic, intrasomatic aggregates; within the white matter, diffuse gliosis was seen. No other pathological changes were seen in the HE staining. When performing IHC analysis, HPτ reactive tangles were observed in the neurons and numerous neurites were seen in the neuropil. Diffuse and compact, extracellular, Aβ aggregates were seen in the grey matter. CAA was not detected (Figure 10). Additionally, one neuron contained intracytoplasmatic TDP43 IR granule.

The PM brain weight was 1140g in fixed condition. The shunt was located in the right frontal lobe and at sectioning the shunt tip was placed in the ventricular system. No macroscopic alteration has been noted. A standardised assessment of the neurodegenerative markers revealed Aβ pathology of Thal phase 3 and CAA type 2 [57,61]. HPτ pathology was consistent with Braak stage V, and ARTAG and concomitant TDP43 pathology corresponded to Josephs stage 3 [80,82,194]. Synuclein pathology was not detected in predilected areas [43]. The section from the area with the shunt revealed a 2mm wide channel, surrounded by 4 – 6mm area of reactive changes, including reactive gliosis and inflammatory response with foamy macrophages and scattered lymphocytes (Figure 10).

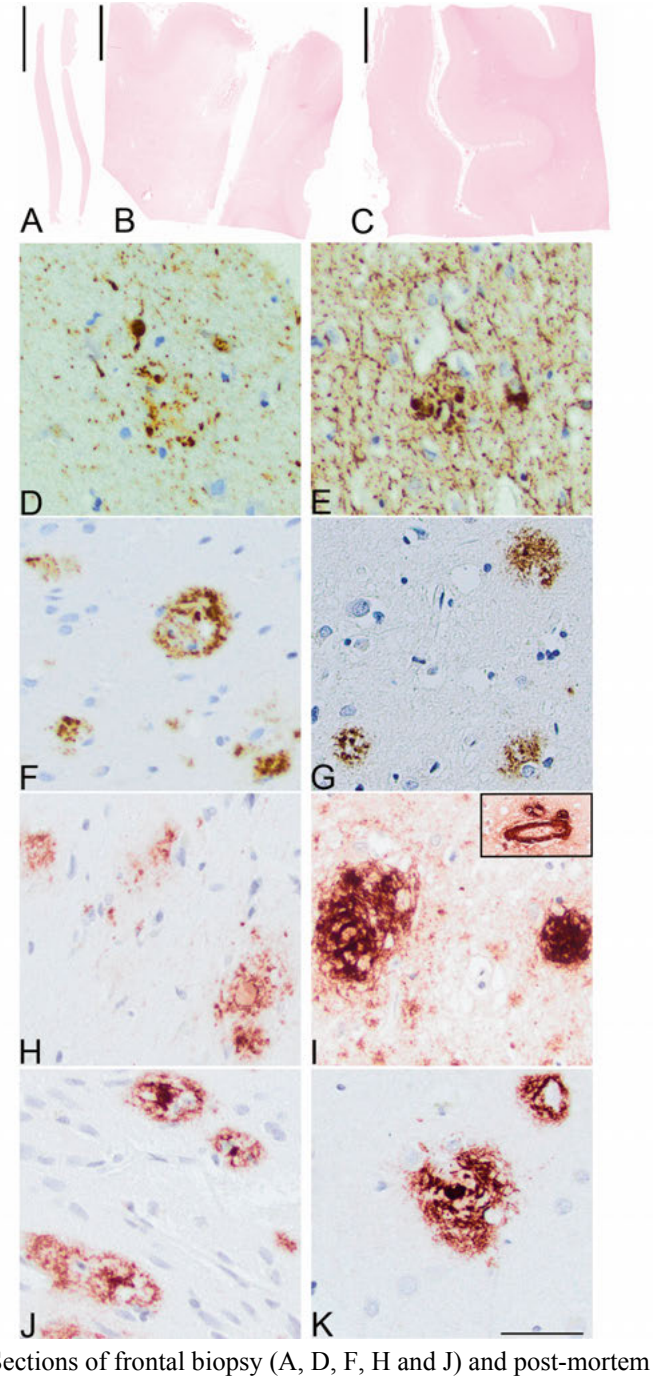

Figure 10. Sections of frontal biopsy (A, D, F, H and J) and post-mortem acquired samples from the frontal cortex without shunt channel (C, E, G, I and K) and with shunt channel (B) when applying haematoxylin-eosin staining (A-C) and antibodies towards hyperphosphorylated τ (D-E), amyloid β (Aβ) (F-G), Aβ40 (H-I) and Aβ42 (J-K). Cerebral amyloid angiopathy in I. Bars A-C 5mm, D-K 50µm. Figure adapted from Libard et al. 2018 [169] with permission.

Comparison of pathology at two time points

The performance of the Abs studied here as SAF, within the biopsy and the PM brain tissue from the frontal cortex, with and without shunt channel, is summarised in table 3.

Table 3. Stained area fraction (SAF) in per cent in frontal brain biopsy and post-mortem assessment of frontal cortex with and without shunt channel. Table adapted from Libard et al. 2018 [169] with permission.

	SAF in percent		
Protein	Biopsy specimen	Frontal cortex without SC	Frontal cortex with SC
Synaptophysin	95	86	88
Microtubule associated protein 2	85	35	84
Neurofilament H Sternberger-Meyer Immunocytochemicals 32	62	5	19
Embryonic lethal abnormal visual system proteins (nELAV) 3 and 4 human homolog HuC/HuD	26	10	19
Glial fibrillary acidic protein	79	78	74
CD68	22	3	3
Human leucocytic antigen -DR	9	6	6
Ionized calcium-binding adaptor molecule1	7	3	5
Amyloid β (Aβ)	18	16	12
Aβ40	30	49	46
Aβ42	37	25	13
Hyperphosphorylated τ protein	44	61	42

Shunt channel (SC).

All the neuronal markers assessed decreased significantly with time. Synaptic damage is an early event in the setting of AD, associated with the disease progression and cognitive symptoms and ongoing during the course of the disease [107,108,114,197]. This is in line with our results, as a decrease of SYP IR was detected during 21 months in a subject with high level of ADNC. MAP2 – a microtubule associated protein located primarily in the dendrites, SMI32 – a structural neurofilament protein and HuC/HuD – a protein involved in post-transcriptional neuronal differentiation, only located in the neurons, all decreased with time in the studied subject [121,198-200]. This correlates with our results reflecting the neurodegenerative process of AD, resulting in progressive neuronal loss until the end stage of the disease [108,119,197,201,202].

We observed that in parallel with the neuronal loss, the extent of HPτ pathology increased over time, a result that is well supported by the progressive

nature of the disease affecting different neuroanatomical regions in a predetermined order, constantly increasing in extent (figure 10)[15,48,80]. The increasing signs of neurodegeneration, as observed here, suggest that subjects with AD would benefit from anti-apoptotic/neuroprotective treatment even at the end-stage of the disease [203].

Aβ pathology was stable and Aβ42 decreased in our subject, probably due to the fact that cortical areas of the brain are the first to be affected in the predisposed propagation of Aβ; also, the cortical regions are probably saturated when reaching Thal phase 3, as previously described [61,110]. Aβ40 increased with time due to the CAA seen at PM examination but lacking in the biopsy (Figure 10). CAA primarily affects meningeal vessels, and meningeal coverings are seldom present in tiny iNPH biopsies [57].

GFAP IHC visualised extensive gliosis in the brain parenchyma of our patient, which was stable over time. Astrocytic activation in association with ADNC is an early event in AD, and most studies describe the increase of gliosis in parallel with the progression of AD [95,100,104]. To our knowledge, the process has not been studied longitudinally in human tissue specimen but was observed previously in an animal model, where the gliosis increased at the beginning of the neurodegenerative process but then levelled off [204]. Also, a longitudinal imaging study revealed that in sporadic AD, the glial activity was increased in AD subjects in comparison with controls, but stayed stable over time, further supporting our results [205].

We observed signs of microglial activation in our subject, both in the biopsy specimen and PM, but all the microglial markers decreased with time. This is contrary to previous studies, where the inflammatory markers mirrored the increase of ADNC [99,102,104,206]. However, to our knowledge, a longitudinal observation of microglial activity in the same subject has not been performed.

The inflammatory process in AD is well acknowledged, but trials of regular intake of anti-inflammatory treatment in AD subjects have not resulted in the anticipated effect [106]. Our observation here, in a subject at the end-stage of the disease, with decreasing microglial activity and rather stable astrocytosis, supports these trials. Neuroinflammatory responses seem to start early in the process of neurodegeneration, which is why early introduction of anti-inflammatory treatment could delay the event as observed in some studies [90,91,93,105].

VPS shunt insertion is the only treatment available in subjects with iNPH [127,155]. Here we were able to assess the changes occurring in the area surrounding the shunt channel, revealing tissue damage located directly in association with the channel and where reactive changes around it were sparse. This further motivates the treatment strategy as it increases the patient's quality of life [160,161]. The biopsy was taken directly from the area of the shunt channel, thus not causing any additional damage in the brain, which is why this diagnostic approach is highly motivated.

In conclusion, the neurodegenerative process of AD is progressing even at the end stage of the disease, suggesting that anti-apoptotic and neuroprotective

treatment would be beneficial. The Aβ pathology was stable in the frontal cortex, as the cortical areas are saturated at the end stage of the disease. The inflammatory response seems to decrease in the final stages of AD, as previously shown in several anti-inflammatory drug trials. Obtaining a diagnostic brain biopsy during symptom-relieving VPS insertion is recommended, as the tissue damage within the biopsied area is negligible. Moreover, the information obtained from the neuropathological assessment can offer the patient specialised care and targeted treatment options. PM examination of brain tissue from subjects with iNPH should be mandatory.

Paper III

In this paper, we studied expression of ADNC and neuronal markers in a cohort of 95 iNPH subjects, aged from 75 to 79 years old.

Substantial number of subjects displayed ADNC, as 63% displayed Aβ (SAF ranging from 1% to 27%), 61% displayed HPτ (SAF ranging 0.02% to 19%), and concomitant ADNC was seen in 49% of subjects. The span in the expression of ADNC markers is visualised in figure 11.

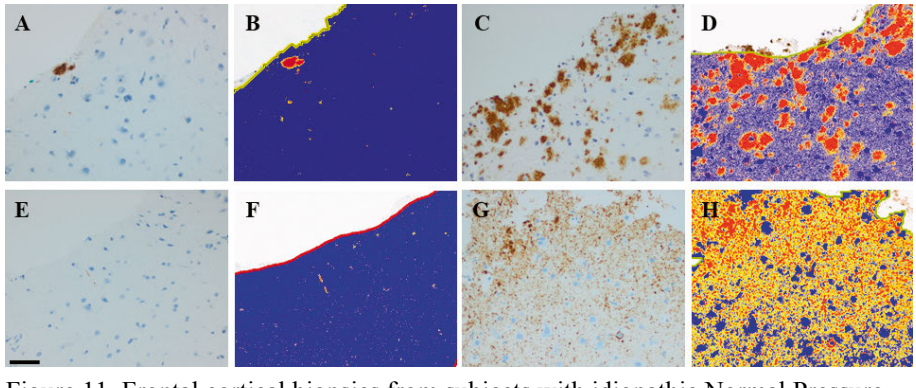

Figure 11. Frontal cortical biopsies from subjects with idiopathic Normal Pressure Hydrocephalus when applying antibodies towards Amyloid β (A-D) and towards hyperphosphorylated τ (E-H). Results of positive pixel counts algorithm as stained area fraction were as follows: in B 1%, D 27%, F, 0.05%, H 19%. Bar 50µm. Figure adapted from Libard et al. 2019 [149] with permission.

ADNC and Aβ pathology, in particular, are in line with previous observations in an iNPH population, contrary to HPτ pathology, which is significantly higher in our cohort [162,164-166]. This can be explained by the age of the subjects included in the studies. Our main inclusion criterion was age range between 75 and 79 years, an age group predilected to display ADNC in the brain, in line with the neurodegenerative process of the disease. Other publications, however, included subjects within an age range from 28 to 87 years [15,165]. Moreover, the assessment method we used has probably influenced the result.

We used a morphometric analysis, while others assessed the pathology semi-quantitatively; the digital method is more sensitive than the naked eye [191]. Here, we even assessed occurrence of small grains as a positive result, but the assessment criteria of neurofibrillary change have not been described in detail by others [164-166]. The extent of HPτ pathology was low in our cohort, as 81% of the biopsies displayed a SAF <1%; however, if those were to be excluded, our results would be comparable with others [165].

ADNC was observed in more females than males. The extent of the HPτ pathology and at a borderline level, also Aβ pathology, was higher in females. This is in line with previous observation in PM brains of AD subjects, as female sex is one of the risk factors of AD [8,18,207].

Both hallmark lesions of AD increased with age, correlated with each other and correlated with age at a borderline level as summarised in table 4. This is in line with the anatomical and age associated neurodegenerative process of AD and corresponds with the studies claiming that substantial number of iNPH subjects develop AD with time [15,61,80,136,173].

Table 4. Correlations [p] using Spearman's rho test, significance at the 0.01 level. Table adapted from Libard et al. 2019 [149] with permission.

	All	Female	Male
Number	96	45	50
Age/Aβ	$0.19^{0.07}$		$0.26^{0.07}$
Age/HPτ	$0.18^{0.08}$		
Age/NeuN	$\mathbf{0.49^{0.000}}$	$\mathbf{0.42^{0.004}}$	$\mathbf{0.51^{0.000}}$
Aβ/ HPτ	$\mathbf{0.58^{0.000}}$	$\mathbf{0.64^{0.000}}$	$\mathbf{0.47^{0.001}}$
Aβ/NeuN		$0.28^{0.06}$	
NeuN/HuC/HuD	$\mathbf{0.27^{0.010}}$		$0.26^{0.06}$

In our cohort, we observed notable neuronal survival but also loss of matrix/synaptic density in the grey matter. The two neuronal markers correlated with each other; one of them, NeuN, correlated and increased with age, while the synaptic marker seemed to diminish in association with higher neuronal density (table 4, figure 12).

Synaptic alterations are described in normal ageing and in various neurodegenerative diseases. Synaptic damage and loss occur early in AD, induced by ADNC and are associated with the propagation of altered proteins and cognitive symptoms and cerebral atrophy [107,108,116]. The depletion and loss of synapses in the setting of AD are well documented, and SYP is a reliable synaptic marker [114].

We assessed the synaptic density in a few samples only, disregarding the subject's age or ADNC pathology. The result suggests that synaptic alteration/loss precedes the neuronal damage also in our cohort of iNPH subjects,

as previously observed in AD [110,113]. The assessment of synaptic density in subjects with pure iNPH is, to our knowledge, not yet described.

Majority of the subjects studied here displayed a low extent of HPτ in their cortical brain biopsies, which, with concomitant Aβ pathology, is in line with low/intermediate level of ADNC, according to the international diagnostic criteria, i.e. early in the neurodegenerative process [41,42]. The early stage of neurodegeneration and signs of neuronal preservation in iNPH subjects suggest that these patients would benefit from HPτ targeting-, neuroprotective- and antiapoptotic therapies and initiation of contact at a memory clinic [88,203,208].

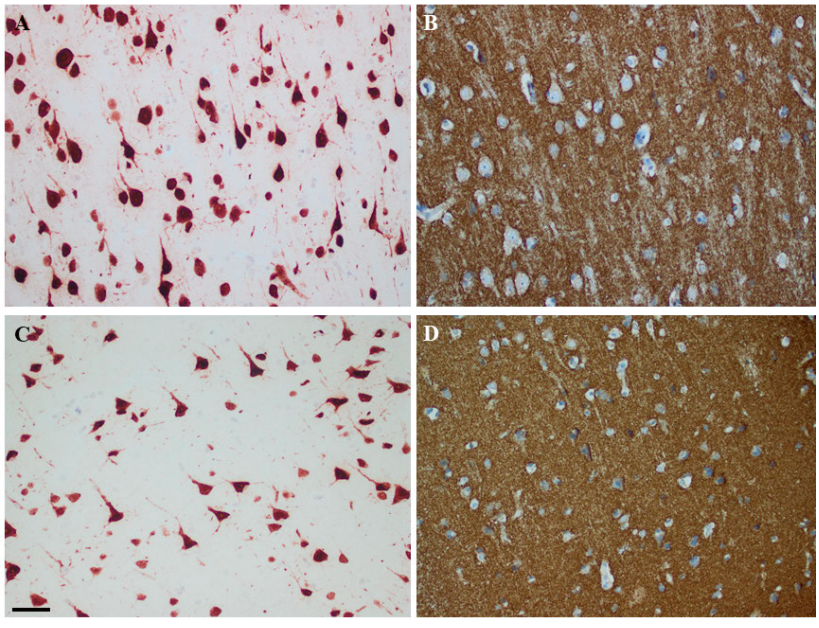

Figure 12 Frontal cortical biopsies from subjects with idiopathic Normal Pressure Hydrocephalus when applying antibodies towards NeuN in A (stained area fraction (SAF) 5%) and C (SAF 10%) and towards synaptophysin in B (SAF 86%) and D (SAF 90%). Bar 50µm. Figure adapted from Libard et al. 2019[149] with permission.

Paper IV

Recently, it has been described that biochemical changes of Aβ occur in a hierarchical manner in AD and are associated with cognitive decline [68,70]. Here we studied the presence of biochemical Aβ variants in 127 iNPH subjects, age range 70 to 88 years and notable Aβ pathology, as well as a TMA, including PM brain tissue from 19 subjects with various levels of ADNC.

We noted that in our cohort with notable Aβ pathology, 91% also displayed HPτ. As in paper III, the presence of neurofibrillary changes is higher than described by other groups [164-166]. Here, besides the age of the studied subjects

and methodology used, as discussed in paper III, one of the inclusion criteria, i.e. subjects with a notable level of Aβ pathology, could also affect this outcome. Based on the PM data, HPτ pathology is not expected to be seen in the cortical areas of the brain at early stages of ADNC, when the Aβ is sparse, i.e. Thal phase 1[61,80]. Thus, by excluding the cases with low level of Aβ, it probably also affected the prevalence of HPτ. It is worth noting, however, that previous reports did not describe in detail the extent of the Aβ pathology studied [164,165,172].

All proteins assessed in our cohort increased with age, in line with the biological process of ADNC (table 5) [15,16]. Unexpectedly, a slight decrease in the protein expression was seen between age group 2 and 3, but only in females. However, it is not known whether this is related to the fact that female sex is a risk factor to develop AD and in the oldest population, in our cohort, only the subjects with lower ADNC levels that were not prone to develop AD were biopsied [18]. In addition, other secondary processes in the brain, such as inflammation, vascular disease or effect of different medications, could affect the outcome [14,99,104].

In our study, all subjects displayed the pyAβ in their brain samples, and the pAβ was seen in 98% of the samples. Additionally, the extent of pyAβ was significantly higher in all age groups than pAβ (table 5). This is in line with the hierarchical process of Aβ biochemical changes, previously described in PM brain tissue from subjects with AD and DS [68-70]. We were not able to perform the biochemical staging in our study, as all samples with low Aβ pathology were excluded from the cohort. The biochemical Aβ variants studied here have been associated with neurotoxicity and increased ability to aggregate, and according to previous studies are associated with cognitive symptoms [31,62,68,70]. The high prevalence of pyAβ and pAβ in our cohort and the HPτ pathology seen within the same population indicate that the level of ADNC in iNPH subjects with notable Aβ pathology reaches rather an intermediate level, according to international criteria [41,42].

Table 5. Level of protein expression within different age groups given as mean ± standard error of means (m±SE). Significance level 0.05 in bold. Table adapted from paper IV, Libard et al, 2021, in press in Journal of Alzheimer's Disease, with permission.

Age	Age group	Number	Gender	HPτ	Aβ				
			F/M		6F/3D	4G8	um7H3D6	*pyN3pE*	*p1E4E11*
70-88	All	127	64/63	1.35±0.80	2.71±0.46	2.79±0.41	2.48±0.60	*2.50±0.65*	*1.37±0.56*
70-74	1	43	23/20	1.21±0.11	2.58±0.08	2.67±0.07	2.28±0.10	*2.35±0.11*	*1.30±0.08*
75-79	2	45	23/22	1.51±0.13	2.84±0.06	2.93±0.04	2.62±0.08	*2.60±0.09*	*1.40±0.08*
80-88	3	39	18/21	1.33±0.12	2.69±0.08	2.74±0.07	2.54±0.09	*2.54±0.10*	*1.41±0.10*
Statistics /KWT				ns	**p=0.025**	**p=0.009**	**p=0.032**	*ns*	*ns*

Hyperphosphorylated τ (HPτ), Amyloid β (Aβ), females (F), males (M), unmodified (um), pyroglutamylated (py), phosphorylated (p), Kruskal-Wallis Test (KWT).

All the proteins assessed here correlated with each other in the whole cohort. In the two oldest groups, where the ADNC pathology was more advanced, a significant association was seen between the HPτ and the modified Aβ variants, contrary to the two general Aβ markers. This is in line with a previous study, where an association of pyAβ and HPτ was observed in frontal cortical areas of the brain in a PM brain cohort and the pyAβ, in particular, associated with AD [64].

We also assessed the prevalence of Aβ variant without py or p modifications at the N-terminus, i.e. $Aβ^{7H3D6}$ (umAβ) [66]. This variant increased with age and correlated with other Aβ clones and variants as well as with HPτ. This reflects the complexity in the composition of Aβ aggregates in iNPH and AD, when different post-translational modifications alter the properties of the protein and its ability to participate in the disease process as well as the possibility to find new treatment targets [31,62].

The composition of Aβ aggregates is quite interesting when observed in tiny brain biopsies from iNPH subjects and indicates that the ongoing ADNC process is indeed the same as in AD. This is in line with, as previously described, the observation that Aβ is the strongest factor for worse shunt response and development of AD [170,173].

In addition to the iNPH cohort, we assessed all the proteins in a reference TMA, containing samples from 19 PM brains with various levels of ADNC (table 6).

In line with previous studies, we have seen an increase of pyAβ and pAβ with increasing level of ADNC, the extent of the pyAβ always being higher than pAβ, thus confirming the hierarchical changes of Aβ aggregates as previously described in PM brain tissue of AD and DS and in our iNPH cohort [68-70].

In summary, our results regarding the biochemical variants of Aβ in iNPH links the pathology in iNPH with AD. Some 60% of all iNPH subjects do display Aβ in their brain, and these subjects should, based on our results, be considered as suffering from AD in various clinical stages. This observation suggests that maybe all iNPH subjects should also be assessed by applying PIB analysis (those not been biopsied) to facilitate referral to the most competent caregiver.

Table 6. The level of protein expression in brain samples from subjects with different levels of Alzheimer's disease neuropathological change. Significance, 0.05 given in bold. Table adapted from paper IV, Libard et al, 2021, in press in Journal of Alzheimer's Disease, with permission.

Level of ADNC	**Number**	**Value**	**HPτ**	**Aβ**				
				6F/3D	**4G8**	**um7H3D6**	***pyN3pE***	***p1E4E11***
All	19	m±SE	2.32±0.89	1.89±0.81	2.42±0.69	1.26±0.99	*1.84±0.90*	*1.16±1.17*
Low	6	m±SE	1.33±0.82	1,17±0.75	2.17±0.98	0.67±0.82	*1.33±1.03*	*0.50±0.84*
Intermediate	7	m±SE	2.57±0.54	2.00±0.58	2.43±0.54	1.00±0.82	*1.71±0.76*	*0.86±1.22*
High	6	m±SE	3.00±0.00	2.50±0.55	2.67±0.52	2.17±0.75	*2.50±0.55*	*2.17±0.75*
Statistics/KWT			**p=0.005**	**p=0.015**	ns	**p=0.024**	*ns*	***p=0.031***

Hyperphosphorylated τ (HPτ), Amyloid β (Aβ), unmodified (um), pyroglutamylated (py), phosphorylated (p), mean ± standard error of means (m±SE), Kruskal-Wallis Test (KWT).

Strengths and limitations

The main limitation in papers I and II is the fact that only one case was studied. Despite this weakness, the information acquired was of high value in paper I, as we could show the importance of standardised procedures when handling a tissue for an analysis, to provide a correct assessment and diagnosis. In neuropathology, the surgical samples are routinely 4µm and the assessment of PM brain tissue is performed on 7µm thick sections. To compare the extent of pathology in these two sample types, a uniform ST is warranted.

In paper II, the diagnostic PM brain tissue was only available in one case. Unfortunately, the autopsy rates have been decreasing in the industrial world and especially when there is a known underlying disease in older population, the autopsy is considered by some as not of interest [209]. Nonetheless, the information acquired in paper II is a unique insight into an ongoing disease process at the end-stage of AD, valuable for consideration when critically reading the literature, planning future studies or considering a treatment strategy in clinical practice.

In papers III and IV, we studied representative cohorts of iNPH subjects, homogenous regarding the age and gender. The main strength was that we could assess ADNC and neuronal markers in surgical samples, as almost all research in the field of neurodegeneration is performed on cell cultures, animal models or PM brain tissue. The strength in using surgical biopsies as opposed to PM brain tissue is that we were able to avoid factors associated with a state of death, PMD and long fixation time [192,193]. It also makes it possible to study the neurodegenerative processes prospectively [167,169]. The main limitation in studying surgical biopsies is the size of the tissue sample, i.e. sampling deficit. The small biopsy, even if representative, represents only a minimal portion of one area of the brain, thus not reflecting the complexity of the brain regarding different regions and pathologies. Additionally, as the tissue sample is small, so is the possibility to perform more extensive protein analyses.

Summary of results

I All Abs assessed increased in SAF when increasing ST from 4μm to 7μm. When performing IHC analysis, there are several confounding factors that have to be considered, and ST is certainly one of them. Additionally, the molecular size of the Ab should be considered as it influences the penetration of the Ab into the tissue section.

II During the end stage of AD, the Aβ pathology was stable, but there were signs of neurodegeneration, in terms of progressive HPτ pathology and loss of synaptic and neuronal markers, indicating that neuroprotective drugs should be considered. The gliosis seemed to be stable, but there were signs of decreased microgliosis, i.e. the inflammatory response in the final stages of the disease seems to level off. The tissue damage within the biopsied area around the shunt channel was scanty, which is why this diagnostic and symptom-relieving procedure is recommended. PM examination of brain tissue from subjects with iNPH is warranted.

III Significant number of iNPH subjects display ADNC in their brain tissue; females seem to be more affected than males. The AD pathology seems to be increasing with age, in line with the biology of AD observed in numerous studies. Within our cohort, there were remarkable signs of neuronal preservation and loss of matrix and synaptic marker. This can mirror early stages of neurodegeneration, where the neuronal processes are primarily affected. Based on our results, the cohort of iNPH incorporates a substantial number of subjects with ADNC in their brain biopsy, i.e. subjects at an ealy stage of AD, which indicates that subjects with a clinical diagnosis of iNPH are a reliable model for AD.

IV We could for the first time exhibit modified Aβ variants in an iNPH population and confirm the findings previously seen in AD, specifically that pyAβ precedes pAβ in the aggregate for-

mation throughout the ongoing neurodegenerative process. Additionally, the modified Aβ variants correlated with HPτ in the brain biopsies from the right frontal cortex. This is a finding, previously described in a PM cohort, where the pyAβ in the frontal cortex was a predictive factor of AD. We could establish that similar to AD, the Aβ aggregates in iNPH are complex in their composition. Our results were confirmed in a TMA with brain tissue from subjects with various levels of ADNC, displaying the hierarchical nature of Aβ modifications, in parallel with the increasing level of pathology.

General discussion

iNPH is a disease affecting a substantial number of aged individuals, and also increases with age [130,131]. The main symptoms, i.e. gait disturbance, urinary urgency and cognitive impairment, are caused by a CSF balance disturbance causing hydrocephaly [127,129]. Thus, although extensively studied, the pathophysiological mechanisms causing this condition are not entirely understood [138,140,142]. The symptoms can be relieved by a VPS insertion, normalising the CSF flow in the brain [127,129]. Most subjects respond to this treatment, especially regarding the gait, and the prognosis is best when an intervention is performed early in the disease process [159,161]. Unfortunately, the cognitive symptoms seem to progress over time [135,136,171,173].

During the last 20 years, a few centres around the world have taken diagnostic biopsies from the area of the shunt channel, opening a new window to study different processes within the brain of aged individuals, in vivo [162-166]. This has resulted in a large body of literature studying mainly ADNC, the association with risk factors of AD and prognostic values in iNPH [132,136,170,171,173]. The studies revealed that a substantial number of iNPH subjects, indeed, display ADNC, and many of them progress to dementia of AD type [135,136,162-166,168,171,173]. Aβ is associated with worse shunt-response and an individual prognostic marker to develop AD [170,173].

In the frame of this thesis, we wanted to further study the ADNC and the neurodegenerative process associated with this pathology, as well as to bring more understanding regarding the ADNC in iNPH subjects and its association to AD.

To do so, we had to standardise the method in IHC to optimise the assessment strategy of surgical iNPH samples and PM brain tissue, as a surgical specimen in neuropathology is 4µm and PM brain tissue samples 7µm. We noted, in paper I, that the pathology, studied with IHC, is affected by 3µm ST, in both the intensity and extent. To be able to compare pathology in a biopsy and PM brain, the specimen's ST should be the same.

In line with previous studies, also in our cohort, paper III, a substantial number of iNPH subjects displayed ADNC [162,164-166]. Surprisingly, the prevalence of both the hallmark lesions of AD, i.e. Aβ and HPτ in particular, was higher in our cohort than in other published studies. Several factors have influenced the outcome, for instance, the age of the cohort, the methodology used, i.e. morphometrical analysis versus a semi-quantitative assessment and last but not least, the assessment strategy applied [162,164-166]. When assessing

HPτ pathology, where the difference compared to others was most pronounced, in our setting even the smallest IR lesions, i.e. grains and small treads were accounted for as positive. We have discussed this aspect with other research groups in the field, and it has become clear that the grains we assessed have not been assessed by others. The information regarding the assessment of IR positive lesions has not been described in detail by others; however, when excluding samples with low level of HPτ pathology (SAF <1%), the results were comparable. The extent of HPτ pathology in the frontal cortical brain biopsies was sparse, as 80% displayed SAF <1%, indicating early stages of neurofibrillary pathology within this area of the brain, which, in association with Aβ pathology seen, designates a low-intermediate level of ADNC according to international criteria [41,42,80]. The ADNC increased with age, both in papers III and IV, in line with the age associated progression of the pathology well described in AD [15].

Simultaneously with the increase in ADNC and age, we noted an increase of neurons within the analysed region of interest. As it seemed impossible to be caused by formation of new neuronal cells, this observation indirectly indicated the loss of matrix between the cells of interest, causing atrophy of affected brain tissue [110,116]. This fact was further supported by the loss of synaptic marker in the neuropil, in parallel with the increase in expression of neuronal markers. Synaptic loss is detected early in AD and progresses over the course of the disease, as it is initiated and maintained by the ADNC. In addition, synapses are described to be involved in the propagation of the altered proteins and highly associated with the progressive cognitive impairment seen in AD [107,108,110]. Neuronal loss is an inevitable event in the course of AD, occurring successively and affecting cortical areas later in the disease process at the same time as the increasing ADNC pathology [80,110,122]. Thus, the cohort of iNPH subjects, in paper III, with low level of ADNC and early signs of neurodegeneration, mainly by synaptic loss, mirrors early stages of AD in this population.

Further, we studied the biochemical properties of Aβ aggregates in a cohort of iNPH subjects with notable Aβ pathology, in paper IV. We could, in our study, for the first time, provide evidence that biochemical changes occur in Aβ aggregates in the brains of iNPH subjects in the same hierarchical manner as previously described in AD, i.e. pyAβ precedes the pAβ [68,70]. In AD, the presence of these modified Aβ variants is associated with cognitive symptoms and the pAβ, in particular, with a dementing illness [68,70]. In addition, in our cohort, both pyAβ and pAβ correlated with the extent of HPτ pathology. This is in line with a previous PM study, where the association between pyAβ and HPτ was detected in AD and the presence of pyAβ in the frontal cortex was a single strongest indicator for development of AD [64]. This is quite intriguing, as the presence of notable Aβ pathology in iNPH subjects, including both pyAβ and pAβ variants, in association with HPτ, indicates a moderate to high level of ADNC by the diagnostic criteria applied [41,42]. Such findings, when

observed in the frontal cortex, as in our studies, indicate a symptomatic disease eventually progressing into full-blown AD [64,68,70].

Beyond the N-terminal truncated Aβ variants, for the first time, we could assess, in an iNPH cohort, the presence of $A\beta^{7H3D6}$, a Aβ without py or p at the N terminus [66]. This variant was present in the Aβ aggregates of iNPH subjects and increased with age. This is evidence of the diversity of the Aβ pathology at a biochemical level that can be used for the benefit of the patient. There are commercial Abs that can detect both pyAβ and pAβ applying IHC that could be of value for iNPH subjects with cognitive symptoms and ADNC to further stage the pathology. This plethora of Aβ variants is also promising for the future because treatment targets as up to date as the Aβ targeting treatment strategies applied have not been efficient [87]

In paper II, we were able to study changes in the protein expession, which occurred during 21 months, in a brain biopsy and PM brain tissue, from a subject with concomitant iNPH and end stage AD. We observed that the HPτ pathology increases at the same time as a neuronal loss in this individual, in line with the progressive nature of AD throughout the disease [80,110,122]. This indicates that treatment strategies targeting HPτ, anti apoptotic- and neuroprotective drugs could be considered even at the end-stage of the disease [88,203,208]. In contrast, the Aβ seemed to be stable over time. The Aβ pathology is seen in the cortical areas early in the disease process; however, in advance phase, the tissue is saturated with Aβ, which is why the extent of pathology remained stable from the time of biopsy to death, in line with what has previously been described in AD [61,110]. We assessed the inflammatory process by studying expression of an astroglial marker and microglial markers. The GFAP remained stable, but the extent of microglial markers declined with time. The neuroinflammation in AD is extensively studied, associated with ADNC and involved in the disease progression [91,93,206]. To our knowledge, this process has not been described in the same subject, which is why our results are not comparable with others. There are several trials describing positive effect of anti-inflammatory treatment in AD [90,104]. Unfortunately, when applied at the end stage of the disease, the anti-inflammatory treatment does not have an effect, which can be explained by our findings [106].

In conclusion, our studies on an iNPH population could follow a progressive path of neurodegeneration. The path started with a low level of ADNC pathology and early signs of synaptic damage in paper III. It moved to more advanced ADNC at moderate to high level with presence of pyAβ and pAβ, both associated with cognitive symptoms and progression to AD, in paper IV. Finally, in paper II, we ended with a prospective assessment of pathology at the end stage of AD, presenting with ongoing HPτ pathology and neuronal loss. Altogether, our findings support that substantial number of iNPH subjects indeed also suffer from AD, consequently making them a reliable model

of AD. This would also explain why Aβ in iNPH subjects is a strong prognostic marker for AD, and why substantial number develop AD with time and display AD pathology at PM neuropathological examination [136,168,171,173].

Whether iNPH subjects are predisposed to developing AD or AD subjects are predisposed to developing iNPH is not yet acknowledged. A few groups have assessed the inflammatory process and glymphatic dysfunction associated with iNPH, which also seems to be impaired in AD and may be a bridge to understand these two diseases [145,210,211]. Another explanation is also the fact that in elderly and aged individuals, several concomitant pathologies can contribute to the pathology causing loss of the cognition [10,14].

The assessment of neurodegenerative markers in brain biopsies from subjects still alive is perceived by some as quite controversial, as curative treatment is lacking for neurodegenerative diseases. I personally think that the diagnostic biopsies taken in the mode of action as done here are surely motivated. The biopsy is taken from the area of VPS, the only symptom-relieving treatment in iNPH, and the tissue damage, mostly caused by the shunt itself, is negligible. The biopsy is primarily a diagnostic tool; thus, when ADNC is detected, it should initiate contact with specialised memory clinics to establish a contact and be able to consider the treatment arsenal available for subjects in the early stage of a dementing illness.

As the population in the world becomes older and the number of individuals with a dementing illness increases, it is of utmost importance to study the pathological mechanisms promoting the pathologies related to ageing, i.e. proteinopathies, neuroinflammation, CSF water balance, glymphatic functions, cerebrovascular disease and systemic illness. Assessment of brain biopsies from iNPH subjects can give valuable insight into the disease process. In addition, PM brain assessment is important in order to study disease processes in the whole brain, which is why autopsy and PM neuropathological examinations should be promoted and warranted.

I strongly believe that a competent assessment of the PM brain obtained from a subject with diagnosis of iNPH is informative and thus needed. Up to date, we have analysed about 500 biopsies during a period of more than 10 years, whereas the number of PM brains obtained from subjects that have gone through a biopsy is extremely low, i.e. only three cases. How can the medical community treat these patient properly if an intensive search for causative mechanism is not facilitated?

Future perspectives

Aβ is often identified in biopsies of iNPH subjects, where it is associated with worse shunt response and cognitive decline [41,42,163,165,170,171].

The characteristic Aβ aggregates in AD are based on light microscopy located extracellularly within the neuropil [48,51,61]. However, a decade ago, a few studies demonstrated intraneuronal presence of both APP and Aβ in PM brain tissue, a biopsy specimen from a patient with iNPH and cell lines visualised by IHC or immuno electron microscopy (EM) [212,213]. These results were revolutionary; however, it has become known that the antibodies used do not differentiate APP from Aβ, thus suggesting that only APP is seen intracellularly. One of the future aims is to assess compartmentalisation of APP and different biochemical species of Aβ in cortical biopsies from iNPH patients with immuno EM technique.

Inflammatory processes are described as affecting and promoting the neurodegenerative course of AD [90,91]. Microglia activations are described as supporting the progression of both Aβ and HPτ pathology [99,102,206]. Astrocytes are also involved in the inflammatory process as activation due to ADNC, maintaining the pathological course of the disease [91,100,104,214]. As the AD manifests itself within the grey matter, the inflammatory changes are most relevant around the ADNC. In the setting of iNPH, no association of pro-inflammatory cytokines and ADNC or shunt response was noted in CSF from patients with iNPH [215]. Several recent papers have acknowledged the role of astrocytes and glymphatic pathway also in the pathogenesis of iNPH [147,210,216]. It should be noted that the areas analysed in our study were within the cortex, region of interest in AD. However, as described in imaging studies, periventricular and white matter changes are frequently seen in patients with iNPH [157]. It is of utmost importance to study the inflammatory process in iNPH, especially within the white matter of the biopsy specimen from iNPH patients. It is also important to further study different inflammatory markers within the grey matter, associating with different loads of ADNC.

Populärvetenskaplig sammanfattning på svenska

Demens är ett tillstånd som drabbar äldre och yttrar sig i störningar av minne, tänkande och uppfattningsförmåga, personlighets- och beteendeförändringar och slutligen störningar i basala fysiologiska funktioner. Den absolut vanligaste kliniska demensdiagnosen är Alzheimers sjukdom (AS), som beskrivs hos 60–80% av patienter som drabbats av demens [4,8,9].

AS orsakas av att två förändrade kroppsegna proteiner, s.k. AS patologiska förändringar (ASPF), lagras in i hjärnan, hyperfosforylerat τ (HPτ) i nervcellerna och amyloid β (Aβ) i hjärnvävnaden. Dessa sprider sig i hjärnan enligt ett förutbestämt mönster och engagerar successivt allt större hjärnområden [48,61,80]. Under den pågående processen förstörs nervceller och deras utskott vilket stoppar de impulser som förmedlas via nervsystemet och därmed förstörs hjärnans funktioner [107]. Detta kan pågå i många år då HPτ kan ses i hjärnan redan hos unga individer och Aβ kan ses hos individer i 40 års ålder [15]. En del av personer med ASPF kommer aldrig utveckla demens och en minoritet av patienter som har genetiska anlag kommer att utveckla sjukdomen tidigt [8,16]. Patienterna försämras över tid och sjukdomsförloppet, som kan pågå i 4-20 år, är förknippad med lidande för den enskilda patienten och dess familj[9]. För närvarande finns inget botemedel men läkemedelsindustrin utvecklar nya läkemedel med mål att stoppa eller fördröja processen. Demens utgör den femte vanligaste dödsorsaken hos individer äldre än 65 år [3].

Idiopatisk normaltryckshydrocefalus (iNPH) är ett tillstånd hos äldre orsakad av störningar i cerebrospinalvätskebalansen som ger upphov till vattenskalle och symtom i form av minnesstörning, gångrubbning och urininkontinens. Den enda behandlingen som finns tillgänglig för att häva detta tillstånd är kirurgisk, genom att avleda cerebrospinalvätskan från hjärnan med en shunt till bukhålan [127]. I fåtal centra i världen har man i samband med shuntoperationen tagit ett litet vävnadsprov från hjärnan från området precis där shunten ska sitta. Flera studier har visat att i dessa vävnadsprover från iNPH-patienter har man funnit ASPF och när dessa förekommit har patienterna svarat sämre på behandling och senare utvecklat en demenssjukdom [136,164,165,170,173].

Vi har i detta projekt studerat ASPF, nervceller, hjärnans stödjeceller, s.k. astrocyter och inflammatoriska celler i vävnadsprover tagna i samband med kurativ shuntinsättning hos patienter med iNPH.

Delarbete I

För att vid patologisk undersökning påvisa proteiner används en metod som kallas immunohistokemi. Metoden används sedan 40 år tillbaka både för rutindiagnostik av förändringar i patientprover och i forskning. Den är säker om man tar hänsyn till parametrar som kan påverka dess utfall [178].

En av parametrarna är tjocklek av vävnadsbiten som färgas för att senare undersökas mikroskopiskt [190,191]. I neuropatologi används 4µm snittjocklek vid undersökning av biopsiprover från operation av levande patienter, som vid iNPH, och 7µm tjocka snitt när man undersöker hjärnvävnad från avlidna, som är den snittjocklek som i majoritet beskrivs i AS-forskning [46].

Vi har i detta arbete påvisat att skillnader i snittjocklek på 3µm påverkar utfallet av färgningen med alla markörer vi undersökt. I neuropatologi kan detta påverka bedömning av nervcellskada, nivå av AS-patologi och även leda till feldiagnos. Snittjocklek vid jämförelse av kirurgiskt uttagen hjärnvävnad och hjärnvävnad från avlidna bör standardiseras.

Delarbete II

I detta arbete har vi undersökt hur proteinuttryck av ASPF, nervceller, astrocyter och hjärnans inflammatoriska celler ändras under en period av 21 månader, från biopsi i samband med shuntinsättning till död och neuropatologisk undersökning av hjärnan, hos en och samma individ med iNPH och AS.

Vi har noterat att HPτ ökade med tiden samtidigt som markörer för nervceller minskade. Detta stämmer med det progressiva sjukdomsförloppet och förlusten av nervceller som ses vid AS [80,121]. Aβ-proteinet har inte förändrats nämnvärt över tid, vilket kan förklaras genom att den ses i det berörda hjärnområdet tidigt i sjukdomsprocessen och vid avancerad sjukdom, som hos denna patient, är patologin mättad [61]. Även astrocytmarkören förblev stabil över tid men denna beskrivs reagera huvudsakligen till Aβ vilket kan förklara våra fynd [95]. De neuroinflammatoriska markörerna minskade över tid, vilket var överraskande då inflammatoriska processer är associerade med ASPF [91]. Man har i olika studier noterat att antiinflammatorisk behandling kan ha effekt på ASPF tidigt i sjukdomsförloppet men inte vid avancerad sjukdom som hos vår patient, vilket är i linje med våra resultat [90,106].

Att kunna jämföra patologi på detta sätt är unikt och mycket informativt och motiverar ökat antal hjärnundersökningar på avlidna med iNPH med tidigare biopsiuttag vid demenssymtom.

Delarbete III

Här undersökte vi förekomsten av ASPF och dess association till nervceller i 95 iNPH-patienter i åldrarna 75 till 79 år.

Vi påvisade att ca. 60% av iNPH patienter uppvisar ASPF i sin hjärnvävnad. Mängden HPτ-protein var mycket låg i majoriteten av fallen talande för tidig nivå av AS-processen [41,42,80]. Därutöver noterade vi att med stigande ålder och ökad mängd ASPF minskade nervcellernas utskott [107,110]. Nervcellskropparna var emellertid bevarade. Dessa fynd tillsammans talar för att majoriteten av iNPH-patienter är i ett mycket tidigt skede av sjukdomsförloppet av sin demenssjukdom. Alla dessa patienter skulle gynnas av kontakt med minnesklinik samt eventuell behandling som stoppar eller fördröjer framfarten av ASPF och den parallellt förekommande degenerationen av nervceller.

Delarbete IV

I detta arbete undersöktes förekomst av specifika varianter av Aβ-protein hos iNPH-patienter med måttligt förhöjd och hög mängd Aβ-protein i hjärnvävnaden. Varianter vi studerade, pyroglutamylerat Aβ (pyAβ) och fosforylerat Aβ (pAβ), är associerade med symtom givande AS [68,70]. PyAβ, när detekterat i hjärnans framlob, är en stark markör för utveckling av AS [64]. Tidigare studier har även noterat att dessa varianter kan påvisas i kronologisk ordning, där Aβ utan py eller p ses i början av sjukdomen, pyAβ noteras när förändringarna blir mer avancerade och pAβ ses vid symtom givande sjukdom [68,70].

Vi har kunnat bekräfta detta även i hos iNPH-patienter där pyAβ föregick pAβ och båda dessa varianter var associerade med HPτ. Detta talar för att iNPH-patienter vars Aβ-patologi passerat låg nivå har nått måttlig-hög nivå av ASPF och således representerar redan en mer allvarlig fas av AS.

Sammanfattningsvis har vi genom våra studier visat att majoriteten av iNPH-patienter uppvisar ASPF i sin hjärnvävnad, att omfattningen av ASPF ökar med ålder och parallellt förändras den biokemiska sammansättning av ASPF och skadar nervcellernas utskott, som på sikt leder till nervcellsförlust [149,169]. Denna progressiva process är identisk i AS och talar snarast för att ett antal av iNPH-patienterna även lider av AS. Dessa patienter gynnas av kontakt med minnesklinik och eventuell skyddande behandling.

Att undersöka dessa hjärnbiopsier från iNPH-patienter har varit kontroversiellt då det idag saknas behandling som motverkar utvecklingen av ASPF. Personligen anser jag dock att vävnaden som tidigare kasserades bör tas till vara, undersökas och diagnosticeras. Informationen är till gagn för patienten och leder till att de drabbade patienterna får sakkunnig, stödjande vårdkontakt som eventuellt kan förebygga snabb sjukdomsprogress i den mån det går, stödja deras vårdbehov och främja välbefinnande.

Acknowledgements

I would like to express my gratitude and appreciation to all the patients included in my studies as well as their relatives.

I would like to thank:

Irina Alafuzoff, my main supervisor, boss, mentor, role model and friend. I am grateful for the knowledge, inspiration, support and guidance in my professional career, scientific path and personal life. You are the reason I continued my career in pathology, and you sowed a seed of science in me that will grow forever. You made me smarter, wiser and stronger! I am so grateful that you are a part of my life.

Anna Dimberg, my co supervisor, for your support, positive energy, valuable discussions, interesting collaborations and last but not least, for help with the administrative machinery to make this thesis possible.

My co-authors, for their efforts, contributions and support, resulting in the publications that I am so proud of**. Dijana Cerjan**, for skilful laboratory work and expertise in immunohistochemistry. **Kristina Giuliana Cesarini**, for sharing your knowledge in assessing and operating on patients with iNPH, and giving me clinical insights that I could not obtain otherwise. **Katarina Laurell**, for clinical input in my work. **Jochen Walter**, for sharing your knowledge regarding Aβ variants and the antibodies we could use.

To the neuropathology group at Uppsala University Hospital, **Olivera Casar-Borota**, **Fredrik Tamsen**, **Maud Salomonsson**, **Karin Staxäng**, **Pere Giné Gras**, **Vidar Skulerud fd Andersson**, **Fabiola Xheka**, **Elin Berglund**, **Nuno Reinaldo** and **Julia Flodström** and at Uppsala University: **Svetlana Popova**, **Adila Elobeid** and **Marina Leino**; for the collaboration, skilful work, support and great atmosphere along the way.

To the **FoUU** at the Pathology department at Uppsala University Hospital for all the testing of antibodies, stainings and discussions and help with the administration of my research.

Meena Strömqvist, for critical reading of all manuscripts.

Hans-Gabriel and Alice Trolle-Wachtmeisters foundation for medical research, and Uppsala University Hospital ALF grants for the generous financial support for my research work.

All my colleagues at the Pathology Department at Uppsala University Hospital for everyday collaboration in my clinical practice, collegiality, a pleasant atmosphere and your support. I am privileged to work with all 100 of you!

To my family and friends, for endless support in all aspects of my life, which keeps me going. Life is a journey, and I am grateful we are travelling together.

To my mum **Danuta**, my sister **Maja** and my grandmother **Marianna**. For the endless love, the happy upbringing, values, support and always believing in me. You formed me to become the person I am today, always by my side with love in your hearts. To my nephew **Marcus**, for being a sunshine in our lives. I love you all!

Last but not least, the two greatest loves of my life, my daughters **Stella** and **Matilda**. For your love and for all the happy moments we share. For the scones on Sundays and sausage stroganoff you make for me when I am tired. For the cosy moments we have in the evenings, when we share our day while watching "Veterinärerna". For the road trips, listening to the best DJ in the world. For making me feel alive. Everything I do, I do it for you!

References

1. Centers for Disease C, Prevention. Trends in aging--United States and worldwide. *MMWR Morb Mortal Wkly Rep.* 2003;52(6):101-104, 106.
2. Prince MJ, Wu F, Guo Y, et al. The burden of disease in older people and implications for health policy and practice. *Lancet.* 2015;385(9967):549-562.
3. Collaborators GBDD. Global, regional, and national burden of Alzheimer's disease and other dementias, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet Neurology.* 2019;18(1):88-106.
4. International WHOaAsD. *Dementia: a public health priority.* WHO Press, World Health Organization, 20 Avenue Appia, 1211 Geneva 27, Switzerland: World Health Organization; 2012.
5. *Sveriges framtida befolkning 2016-2060 [Elektronisk resurs]*. Statistiska centralbyrån; 2016.
6. *Regional befolkningsframskrivning 2020-2030 [Elektronisk resurs]*. Stockholm: Statistiska centralbyrån; 2020.
7. *Nationella riktlinjer för vård och omsorg vid demenssjukdom [Elektronisk resurs]*. Socialstyrelsen; 2017.
8. Alzheimer's A. 2016 Alzheimer's disease facts and figures. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2016;12(4):459-509.
9. 2020 Alzheimer's disease facts and figures. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2020.
10. Kovacs GG, Alafuzoff I, Al-Sarraj S, et al. Mixed brain pathologies in dementia: the BrainNet Europe consortium experience. *Dementia and geriatric cognitive disorders.* 2008;26(4):343-350.
11. Rahimi J, Kovacs GG. Prevalence of mixed pathologies in the aging brain. *Alzheimer's research & therapy.* 2014;6(9):82.
12. Nelson PT, Trojanowski JQ, Abner EL, et al. "New Old Pathologies": AD, PART, and Cerebral Age-Related TDP-43 With Sclerosis (CARTS). *Journal of neuropathology and experimental neurology.* 2016;75(6):482-498.
13. Robinson JL, Corrada MM, Kovacs GG, et al. Non-Alzheimer's contributions to dementia and cognitive resilience in The 90+ Study. *Acta Neuropathol.* 2018;136(3):377-388.
14. Alafuzoff I, Libard S. Mixed Brain Pathology Is the Most Common Cause of Cognitive Impairment in the Elderly. *Journal of Alzheimer's disease : JAD.* 2020.
15. Braak H, Thal DR, Ghebremedhin E, Del Tredici K. Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. *Journal of neuropathology and experimental neurology.* 2011;70(11):960-969.
16. Elobeid A, Libard S, Leino M, Popova SN, Alafuzoff I. Altered Proteins in the Aging Brain. *Journal of neuropathology and experimental neurology.* 2016;75(4):316-325.
17. Mufson EJ, Binder L, Counts SE, et al. Mild cognitive impairment: pathology and mechanisms. *Acta Neuropathol.* 2012;123(1):13-30.

18. Oveisgharan S, Arvanitakis Z, Yu L, Farfel J, Schneider JA, Bennett DA. Sex differences in Alzheimer's disease and common neuropathologies of aging. *Acta Neuropathol.* 2018;136(6):887-900.
19. Yamazaki Y, Zhao N, Caulfield TR, Liu CC, Bu G. Apolipoprotein E and Alzheimer disease: pathobiology and targeting strategies. *Nat Rev Neurol.* 2019;15(9):501-518.
20. Hugo J, Ganguli M. Dementia and cognitive impairment: epidemiology, diagnosis, and treatment. *Clin Geriatr Med.* 2014;30(3):421-442.
21. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology.* 1984;34(7):939-944.
22. McKhann GM, Knopman DS, Chertkow H, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011;7(3):263-269.
23. Jack CR, Jr., Albert MS, Knopman DS, et al. Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011;7(3):257-262.
24. Albert MS, DeKosky ST, Dickson D, et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011;7(3):270-279.
25. Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011;7(3):280-292.
26. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. *Journal of psychiatric research.* 1975;12(3):189-198.
27. Blennow K, Hampel H. CSF markers for incipient Alzheimer's disease. *The Lancet Neurology.* 2003;2(10):605-613.
28. Klunk WE, Engler H, Nordberg A, et al. Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Annals of neurology.* 2004;55(3):306-319.
29. Jack CR, Jr., Bennett DA, Blennow K, et al. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2018;14(4):535-562.
30. Schellenberg GD, Montine TJ. The genetics and neuropathology of Alzheimer's disease. *Acta Neuropathol.* 2012;124(3):305-323.
31. Kummer MP, Heneka MT. Truncated and modified amyloid-beta species. *Alzheimer's research & therapy.* 2014;6(3):28.
32. Walsh DM, Selkoe DJ. A beta oligomers - a decade of discovery. *J Neurochem.* 2007;101(5):1172-1184.
33. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med.* 2012;2(5):a006270.
34. Storey E, Cappai R. The amyloid precursor protein of Alzheimer's disease and the Abeta peptide. *Neuropathol Appl Neurobiol.* 1999;25(2):81-97.

35. Kumar-Singh S, Theuns J, Van Broeck B, et al. Mean age-of-onset of familial alzheimer disease caused by presenilin mutations correlates with both increased Abeta42 and decreased Abeta40. *Hum Mutat.* 2006;27(7):686-695.
36. Bentahir M, Nyabi O, Verhamme J, et al. Presenilin clinical mutations can affect gamma-secretase activity by different mechanisms. *J Neurochem.* 2006;96(3):732-742.
37. Lott IT, Head E. Dementia in Down syndrome: unique insights for Alzheimer disease research. *Nat Rev Neurol.* 2019;15(3):135-147.
38. Carmona-Iragui M, Videla L, Lleo A, Fortea J. Down syndrome, Alzheimer disease, and cerebral amyloid angiopathy: The complex triangle of brain amyloidosis. *Dev Neurobiol.* 2019.
39. Yu JT, Tan L, Hardy J. Apolipoprotein E in Alzheimer's disease: an update. *Annu Rev Neurosci.* 2014;37:79-100.
40. Huang Y, Mahley RW. Apolipoprotein E: structure and function in lipid metabolism, neurobiology, and Alzheimer's diseases. *Neurobiol Dis.* 2014;72 Pt A:3-12.
41. Montine TJ, Phelps CH, Beach TG, et al. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol.* 2012;123(1):1-11.
42. Hyman BT, Phelps CH, Beach TG, et al. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2012;8(1):1-13.
43. Alafuzoff I, Ince PG, Arzberger T, et al. Staging/typing of Lewy body related alpha-synuclein pathology: a study of the BrainNet Europe Consortium. *Acta Neuropathol.* 2009;117(6):635-652.
44. Alafuzoff I, Thal DR, Arzberger T, et al. Assessment of beta-amyloid deposits in human brain: a study of the BrainNet Europe Consortium. *Acta Neuropathol.* 2009;117(3):309-320.
45. Alafuzoff I, Pikkarainen M, Al-Sarraj S, et al. Interlaboratory comparison of assessments of Alzheimer disease-related lesions: a study of the BrainNet Europe Consortium. *Journal of neuropathology and experimental neurology.* 2006;65(8):740-757.
46. Alafuzoff I, Arzberger T, Al-Sarraj S, et al. Staging of neurofibrillary pathology in Alzheimer's disease: a study of the BrainNet Europe Consortium. *Brain Pathol.* 2008;18(4):484-496.
47. Mirra SS, Heyman A, McKeel D, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology.* 1991;41(4):479-486.
48. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol.* 1991;82(4):239-259.
49. Hippius H, Neundorfer G. The discovery of Alzheimer's disease. *Dialogues Clin Neurosci.* 2003;5(1):101-108.
50. Khachaturian ZS. Diagnosis of Alzheimer's disease. *Archives of neurology.* 1985;42(11):1097-1105.
51. Dickson DW. The pathogenesis of senile plaques. *Journal of neuropathology and experimental neurology.* 1997;56(4):321-339.
52. Terry RD. The Fine Structure of Neurofibrillary Tangles in Alzheimer's Disease. *Journal of neuropathology and experimental neurology.* 1963;22:629-642.

53. Braak H, Braak E, Grundke-Iqbal I, Iqbal K. Occurrence of neuropil threads in the senile human brain and in Alzheimer's disease: a third location of paired helical filaments outside of neurofibrillary tangles and neuritic plaques. *Neuroscience letters.* 1986;65(3):351-355.
54. Bancher C, Brunner C, Lassmann H, et al. Accumulation of abnormally phosphorylated tau precedes the formation of neurofibrillary tangles in Alzheimer's disease. *Brain Res.* 1989;477(1-2):90-99.
55. Iqbal K, Alonso AC, Gong CX, Khatoon S, Singh TJ, Grundke-Iqbal I. Mechanism of neurofibrillary degeneration in Alzheimer's disease. *Mol Neurobiol.* 1994;9(1-3):119-123.
56. Wilkins HM, Swerdlow RH. Amyloid precursor protein processing and bioenergetics. *Brain Res Bull.* 2017;133:71-79.
57. Thal DR, Ghebremedhin E, Rub U, Yamaguchi H, Del Tredici K, Braak H. Two types of sporadic cerebral amyloid angiopathy. *Journal of neuropathology and experimental neurology.* 2002;61(3):282-293.
58. Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB, Teplow DB. Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. *Proc Natl Acad Sci U S A.* 2003;100(1):330-335.
59. Nelson PT, Alafuzoff I, Bigio EH, et al. Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. *Journal of neuropathology and experimental neurology.* 2012;71(5):362-381.
60. Alafuzoff I, Pikkarainen M, Arzberger T, et al. Inter-laboratory comparison of neuropathological assessments of beta-amyloid protein: a study of the BrainNet Europe consortium. *Acta Neuropathol.* 2008;115(5):533-546.
61. Thal DR, Rub U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. *Neurology.* 2002;58(12):1791-1800.
62. Dunys J, Valverde A, Checler F. Are N- and C-terminally truncated Abeta species key pathological triggers in Alzheimer's disease? *J Biol Chem.* 2018;293(40):15419-15428.
63. Nussbaum JM, Schilling S, Cynis H, et al. Prion-like behaviour and tau-dependent cytotoxicity of pyroglutamylated amyloid-beta. *Nature.* 2012;485(7400):651-655.
64. Mandler M, Walker L, Santic R, et al. Pyroglutamylated amyloid-beta is associated with hyperphosphorylated tau and severity of Alzheimer's disease. *Acta Neuropathol.* 2014;128(1):67-79.
65. Neddens J, Daurer M, Flunkert S, et al. Correlation of pyroglutamate amyloid beta and ptau Ser202/Thr205 levels in Alzheimer's disease and related murine models. *PloS one.* 2020;15(7):e0235543.
66. Kumar S, Wirths O, Theil S, Gerth J, Bayer TA, Walter J. Early intraneuronal accumulation and increased aggregation of phosphorylated Abeta in a mouse model of Alzheimer's disease. *Acta Neuropathol.* 2013;125(5):699-709.
67. Kumar S, Wirths O, Stuber K, et al. Phosphorylation of the amyloid beta-peptide at Ser26 stabilizes oligomeric assembly and increases neurotoxicity. *Acta Neuropathol.* 2016;131(4):525-537.
68. Gerth J, Kumar S, Rijal Upadhaya A, et al. Modified amyloid variants in pathological subgroups of beta-amyloidosis. *Ann Clin Transl Neurol.* 2018;5(7):815-831.
69. Kumar S, Lemere CA, Walter J. Phosphorylated Abeta peptides in human Down syndrome brain and different Alzheimer's-like mouse models. *Acta Neuropathol Commun.* 2020;8(1):118.

70. Rijal Upadhaya A, Kosterin I, Kumar S, et al. Biochemical stages of amyloid-beta peptide aggregation and accumulation in the human brain and their association with symptomatic and pathologically preclinical Alzheimer's disease. *Brain : a journal of neurology.* 2014;137(Pt 3):887-903.
71. Bakota L, Brandt R. Tau Biology and Tau-Directed Therapies for Alzheimer's Disease. *Drugs.* 2016;76(3):301-313.
72. Chu D, Liu F. Pathological Changes of Tau Related to Alzheimer's Disease. *ACS Chem Neurosci.* 2019;10(2):931-944.
73. Iqbal K, Liu F, Gong CX. Tau and neurodegenerative disease: the story so far. *Nat Rev Neurol.* 2016;12(1):15-27.
74. Drubin DG, Kirschner MW. Tau protein function in living cells. *J Cell Biol.* 1986;103(6 Pt 2):2739-2746.
75. Alonso AC, Zaidi T, Grundke-Iqbal I, Iqbal K. Role of abnormally phosphorylated tau in the breakdown of microtubules in Alzheimer disease. *Proc Natl Acad Sci U S A.* 1994;91(12):5562-5566.
76. Grundke-Iqbal I, Vorbrodt AW, Iqbal K, Tung YC, Wang GP, Wisniewski HM. Microtubule-associated polypeptides tau are altered in Alzheimer paired helical filaments. *Brain Res.* 1988;464(1):43-52.
77. Alonso Adel C, Li B, Grundke-Iqbal I, Iqbal K. Polymerization of hyperphosphorylated tau into filaments eliminates its inhibitory activity. *Proc Natl Acad Sci U S A.* 2006;103(23):8864-8869.
78. Iqbal K, Zaidi T, Bancher C, Grundke-Iqbal I. Alzheimer paired helical filaments. Restoration of the biological activity by dephosphorylation. *FEBS Lett.* 1994;349(1):104-108.
79. Braak E, Braak H, Mandelkow EM. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol.* 1994;87(6):554-567.
80. Braak H, Alafuzoff I, Arzberger T, Kretzschmar H, Del Tredici K. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. *Acta Neuropathol.* 2006;112(4):389-404.
81. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. *Cold Spring Harb Perspect Med.* 2011;1(1):a006189.
82. Kovacs GG, Ferrer I, Grinberg LT, et al. Aging-related tau astrogliopathy (ARTAG): harmonized evaluation strategy. *Acta Neuropathol.* 2016;131(1):87-102.
83. Castellani RJ. The Significance of Tau Aggregates in the Human Brain. *Brain Sci.* 2020;10(12).
84. Boras E, Slevin M, Alexander MY, et al. Monomeric C-reactive protein and Notch-3 co-operatively increase angiogenesis through PI3K signalling pathway. *Cytokine.* 2014;69(2):165-179.
85. Elobeid A, Rantakomi S, Soininen H, Alafuzoff I. Alzheimer's disease-related plaques in nondemented subjects. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2014;10(5):522-529.
86. Braak H, Del Tredici K. The pathological process underlying Alzheimer's disease in individuals under thirty. *Acta Neuropathol.* 2011;121(2):171-181.
87. Panza F, Lozupone M, Seripa D, Imbimbo BP. Amyloid-beta immunotherapy for alzheimer disease: Is it now a long shot? *Annals of neurology.* 2019;85(3):303-315.
88. Congdon EE, Sigurdsson EM. Tau-targeting therapies for Alzheimer disease. *Nat Rev Neurol.* 2018;14(7):399-415.

89. van Dyck CH. Anti-Amyloid-beta Monoclonal Antibodies for Alzheimer's Disease: Pitfalls and Promise. *Biol Psychiatry.* 2018;83(4):311-319.
90. McGeer PL, Rogers J, McGeer EG. Inflammation, Antiinflammatory Agents, and Alzheimer's Disease: The Last 22 Years. *Journal of Alzheimer's disease : JAD.* 2016;54(3):853-857.
91. Heneka MT, Carson MJ, El Khoury J, et al. Neuroinflammation in Alzheimer's disease. *The Lancet Neurology.* 2015;14(4):388-405.
92. Sofroniew MV, Vinters HV. Astrocytes: biology and pathology. *Acta Neuropathol.* 2010;119(1):7-35.
93. Bronzuoli MR, Iacomino A, Steardo L, Scuderi C. Targeting neuroinflammation in Alzheimer's disease. *J Inflamm Res.* 2016;9:199-208.
94. Marina N, Turovsky E, Christie IN, et al. Brain metabolic sensing and metabolic signaling at the level of an astrocyte. *Glia.* 2018;66(6):1185-1199.
95. Garwood CJ, Ratcliffe LE, Simpson JE, Heath PR, Ince PG, Wharton SB. Review: Astrocytes in Alzheimer's disease and other age-associated dementias: a supporting player with a central role. *Neuropathol Appl Neurobiol.* 2017;43(4):281-298.
96. Augusto-Oliveira M, Arrifano GP, Lopes-Araujo A, et al. What Do Microglia Really Do in Healthy Adult Brain? *Cells.* 2019;8(10).
97. Wolf SA, Boddeke HW, Kettenmann H. Microglia in Physiology and Disease. *Annu Rev Physiol.* 2017;79:619-643.
98. Conde JR, Streit WJ. Microglia in the aging brain. *Journal of neuropathology and experimental neurology.* 2006;65(3):199-203.
99. Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen P, Sr., Alafuzoff I. Reactive microglia in aging and dementia: an immunohistochemical study of postmortem human brain tissue. *Acta Neuropathol.* 1999;97(4):383-392.
100. Overmyer M, Helisalmi S, Soininen H, Laakso M, Riekkinen P, Sr., Alafuzoff I. Astrogliosis and the ApoE genotype. an immunohistochemical study of postmortem human brain tissue. *Dementia and geriatric cognitive disorders.* 1999;10(4):252-257.
101. Webers A, Heneka MT, Gleeson PA. The role of innate immune responses and neuroinflammation in amyloid accumulation and progression of Alzheimer's disease. *Immunol Cell Biol.* 2020;98(1):28-41.
102. Minett T, Classey J, Matthews FE, et al. Microglial immunophenotype in dementia with Alzheimer's pathology. *Journal of neuroinflammation.* 2016;13(1):135.
103. Serrano-Pozo A, Mielke ML, Gomez-Isla T, et al. Reactive glia not only associates with plaques but also parallels tangles in Alzheimer's disease. *The American journal of pathology.* 2011;179(3):1373-1384.
104. Alafuzoff I, Overmyer M, Helisalmi S, Soininen H. Lower Counts of Astroglia and Activated Microglia in Patients with Alzheimer's Disease with Regular Use of Non-Steroidal Anti-Inflammatory Drugs. *Journal of Alzheimer's disease : JAD.* 2000;2(1):37-46.
105. Wang J, Tan L, Wang HF, et al. Anti-inflammatory drugs and risk of Alzheimer's disease: an updated systematic review and meta-analysis. *Journal of Alzheimer's disease : JAD.* 2015;44(2):385-396.
106. Aisen PS, Schafer KA, Grundman M, et al. Effects of rofecoxib or naproxen vs placebo on Alzheimer disease progression: a randomized controlled trial. *Jama.* 2003;289(21):2819-2826.
107. Spires-Jones TL, Hyman BT. The intersection of amyloid beta and tau at synapses in Alzheimer's disease. *Neuron.* 2014;82(4):756-771.

108. Spires-Jones TL, Attems J, Thal DR. Interactions of pathological proteins in neurodegenerative diseases. *Acta Neuropathol.* 2017;134(2):187-205.
109. Rajmohan R, Reddy PH. Amyloid-Beta and Phosphorylated Tau Accumulations Cause Abnormalities at Synapses of Alzheimer's disease Neurons. *Journal of Alzheimer's disease : JAD.* 2017;57(4):975-999.
110. Ingelsson M, Fukumoto H, Newell KL, et al. Early Abeta accumulation and progressive synaptic loss, gliosis, and tangle formation in AD brain. *Neurology.* 2004;62(6):925-931.
111. Masliah E, Mallory M, Hansen L, DeTeresa R, Alford M, Terry R. Synaptic and neuritic alterations during the progression of Alzheimer's disease. *Neuroscience letters.* 1994;174(1):67-72.
112. DeKosky ST, Scheff SW, Styren SD. Structural correlates of cognition in dementia: quantification and assessment of synapse change. *Neurodegeneration.* 1996;5(4):417-421.
113. Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ. Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. *Neurology.* 2007;68(18):1501-1508.
114. Masliah E, Terry RD, DeTeresa RM, Hansen LA. Immunohistochemical quantification of the synapse-related protein synaptophysin in Alzheimer disease. *Neuroscience letters.* 1989;103(2):234-239.
115. Callahan LM, Vaules WA, Coleman PD. Progressive reduction of synaptophysin message in single neurons in Alzheimer disease. *Journal of neuropathology and experimental neurology.* 2002;61(5):384-395.
116. John A, Reddy PH. Synaptic basis of Alzheimer's disease: Focus on synaptic amyloid beta, P-tau and mitochondria. *Ageing Res Rev.* 2021;65:101208.
117. Dorostkar MM, Zou C, Blazquez-Llorca L, Herms J. Analyzing dendritic spine pathology in Alzheimer's disease: problems and opportunities. *Acta Neuropathol.* 2015;130(1):1-19.
118. Gomez-Isla T, Price JL, McKeel DW, Jr., Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci.* 1996;16(14):4491-4500.
119. Gomez-Isla T, Hollister R, West H, et al. Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Annals of neurology.* 1997;41(1):17-24.
120. Su JH, Zhao M, Anderson AJ, Srinivasan A, Cotman CW. Activated caspase-3 expression in Alzheimer's and aged control brain: correlation with Alzheimer pathology. *Brain Res.* 2001;898(2):350-357.
121. Thangavel R, Sahu SK, Van Hoesen GW, Zaheer A. Loss of nonphosphorylated neurofilament immunoreactivity in temporal cortical areas in Alzheimer's disease. *Neuroscience.* 2009;160(2):427-433.
122. Bussiere T, Gold G, Kovari E, et al. Stereologic analysis of neurofibrillary tangle formation in prefrontal cortex area 9 in aging and Alzheimer's disease. *Neuroscience.* 2003;117(3):577-592.
123. Bloom GS. Amyloid-beta and tau: the trigger and bullet in Alzheimer disease pathogenesis. *JAMA Neurol.* 2014;71(4):505-508.
124. Walker LC. Prion-like mechanisms in Alzheimer disease. *Handb Clin Neurol.* 2018;153:303-319.
125. Nussbaum JM, Seward ME, Bloom GS. Alzheimer disease: a tale of two prions. *Prion.* 2013;7(1):14-19.
126. Braak H, Del Tredici K. Alzheimer's pathogenesis: is there neuron-to-neuron propagation? *Acta Neuropathol.* 2011;121(5):589-595.

127. Williams MA, Malm J. Diagnosis and Treatment of Idiopathic Normal Pressure Hydrocephalus. *Continuum (Minneap Minn).* 2016;22(2 Dementia):579-599.
128. Williams MA, Relkin NR. Diagnosis and management of idiopathic normal-pressure hydrocephalus. *Neurology Clinical practice.* 2013;3(5):375-385.
129. Ghosh S, Lippa C. Diagnosis and prognosis in idiopathic normal pressure hydrocephalus. *American journal of Alzheimer's disease and other dementias.* 2014;29(7):583-589.
130. Jaraj D, Rabiei K, Marlow T, Jensen C, Skoog I, Wikkelso C. Prevalence of idiopathic normal-pressure hydrocephalus. *Neurology.* 2014;82(16):1449-1454.
131. Andersson J, Rosell M, Kockum K, Lilja-Lund O, Soderstrom L, Laurell K. Prevalence of idiopathic normal pressure hydrocephalus: A prospective, population-based study. *PloS one.* 2019;14(5):e0217705.
132. Allali G, Laidet M, Armand S, Assal F. Brain comorbidities in normal pressure hydrocephalus. *Eur J Neurol.* 2018;25(3):542-548.
133. Toma AK, Papadopoulos MC, Stapleton S, Kitchen ND, Watkins LD. Systematic review of the outcome of shunt surgery in idiopathic normal-pressure hydrocephalus. *Acta neurochirurgica.* 2013;155(10):1977-1980.
134. Eide PK, Sorteberg W. Diagnostic intracranial pressure monitoring and surgical management in idiopathic normal pressure hydrocephalus: a 6-year review of 214 patients. *Neurosurgery.* 2010;66(1):80-91.
135. Jaraj D, Wikkelso C, Rabiei K, et al. Mortality and risk of dementia in normal-pressure hydrocephalus: A population study. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2017.
136. Koivisto AM, Kurki MI, Alafuzoff I, et al. High Risk of Dementia in Ventricular Enlargement with Normal Pressure Hydrocephalus Related Symptoms1. *Journal of Alzheimer's disease : JAD.* 2016;52(2):497-507.
137. Sundstrom N, Malm J, Laurell K, et al. Incidence and outcome of surgery for adult hydrocephalus patients in Sweden. *British journal of neurosurgery.* 2017;31(1):21-27.
138. Brautigam K, Vakis A, Tsitsipanis C. Pathogenesis of idiopathic Normal Pressure Hydrocephalus: A review of knowledge. *J Clin Neurosci.* 2019;61:10-13.
139. Eide PK, Hansson HA. Blood-brain barrier leakage of blood proteins in idiopathic normal pressure hydrocephalus. *Brain Res.* 2020;1727:146547.
140. Skalicky P, Mladek A, Vlasak A, De Lacy P, Benes V, Bradac O. Normal pressure hydrocephalus-an overview of pathophysiological mechanisms and diagnostic procedures. *Neurosurg Rev.* 2020;43(6):1451-1464.
141. Eide PK, Ringstad G. In Vivo Imaging of Molecular Clearance From Human Entorhinal Cortex: A Possible Method for Preclinical Testing of Dementia. *Gerontol Geriatr Med.* 2019;5:2333721419889739.
142. Wang Z, Zhang Y, Hu F, Ding J, Wang X. Pathogenesis and pathophysiology of idiopathic normal pressure hydrocephalus. *CNS neuroscience & therapeutics.* 2020;26(12):1230-1240.
143. Tarasoff-Conway JM, Carare RO, Osorio RS, et al. Clearance systems in the brain-implications for Alzheimer disease. *Nat Rev Neurol.* 2015;11(8):457-470.
144. Reeves BC, Karimy JK, Kundishora AJ, et al. Glymphatic System Impairment in Alzheimer's Disease and Idiopathic Normal Pressure Hydrocephalus. *Trends Mol Med.* 2020;26(3):285-295.
145. Rasmussen MK, Mestre H, Nedergaard M. The glymphatic pathway in neurological disorders. *The Lancet Neurology.* 2018;17(11):1016-1024.

146. Mader S, Brimberg L. Aquaporin-4 Water Channel in the Brain and Its Implication for Health and Disease. *Cells.* 2019;8(2).
147. Hasan-Olive MM, Enger R, Hansson HA, Nagelhus EA, Eide PK. Loss of perivascular aquaporin-4 in idiopathic normal pressure hydrocephalus. *Glia.* 2019;67(1):91-100.
148. Eide PK, Valnes LM, Pripp AH, Mardal KA, Ringstad G. Delayed clearance of cerebrospinal fluid tracer from choroid plexus in idiopathic normal pressure hydrocephalus. *J Cereb Blood Flow Metab.* 2020;40(9):1849-1858.
149. Libard S, Alafuzoff I. Alzheimer's disease neuropathological change and loss of matrix/neuropil in patients with idiopathic Normal Pressure Hydrocephalus, a model of Alzheimer's disease. *Acta Neuropathol Commun.* 2019;7(1):3.
150. Hasan-Olive MM, Hansson HA, Enger R, Nagelhus EA, Eide PK. Blood-Brain Barrier Dysfunction in Idiopathic Intracranial Hypertension. *Journal of neuropathology and experimental neurology.* 2019;78(9):808-818.
151. Alafuzoff I, Adolfsson R, Grundke-Iqbal I, Winblad B. Perivascular deposits of serum proteins in cerebral cortex in vascular dementia. *Acta Neuropathol.* 1985;66(4):292-298.
152. Alafuzoff I, Adolfsson R, Grundke-Iqbal I, Winblad B. Blood-brain barrier in Alzheimer dementia and in non-demented elderly. An immunocytochemical study. *Acta Neuropathol.* 1987;73(2):160-166.
153. Mori E, Ishikawa M, Kato T, et al. Guidelines for management of idiopathic normal pressure hydrocephalus: second edition. *Neurol Med Chir (Tokyo).* 2012;52(11):775-809.
154. Relkin N, Marmarou A, Klinge P, Bergsneider M, Black PM. Diagnosing idiopathic normal-pressure hydrocephalus. *Neurosurgery.* 2005;57(3 Suppl):S4-16; discussion ii-v.
155. Isaacs AM, Williams MA, Hamilton MG. Current Update on Treatment Strategies for Idiopathic Normal Pressure Hydrocephalus. *Curr Treat Options Neurol.* 2019;21(12):65.
156. Hellstrom P, Klinge P, Tans J, Wikkelso C. A new scale for assessment of severity and outcome in iNPH. *Acta neurologica Scandinavica.* 2012;126(4):229-237.
157. Virhammar J, Laurell K, Cesarini KG, Larsson EM. Preoperative prognostic value of MRI findings in 108 patients with idiopathic normal pressure hydrocephalus. *AJNR American journal of neuroradiology.* 2014;35(12):2311-2318.
158. Klinge P, Hellstrom P, Tans J, Wikkelso C. One-year outcome in the European multicentre study on iNPH. *Acta neurologica Scandinavica.* 2012;126(3):145-153.
159. Andren K, Wikkelso C, Hellstrom P, Tullberg M, Jaraj D. Early shunt surgery improves survival in idiopathic Normal Pressure Hydrocephalus. *Eur J Neurol.* 2020.
160. Israelsson H, Eklund A, Malm J. Cerebrospinal Fluid Shunting Improves Long-Term Quality of Life in Idiopathic Normal Pressure Hydrocephalus. *Neurosurgery.* 2020;86(4):574-582.
161. Andren K, Wikkelso C, Sundstrom N, et al. Survival in treated idiopathic normal pressure hydrocephalus. *J Neurol.* 2020;267(3):640-648.
162. Savolainen S, Paljarvi L, Vapalahti M. Prevalence of Alzheimer's disease in patients investigated for presumed normal pressure hydrocephalus: a clinical and neuropathological study. *Acta neurochirurgica.* 1999;141(8):849-853.

163. Elobeid A, Laurell K, Cesarini KG, Alafuzoff I. Correlations between mini-mental state examination score, cerebrospinal fluid biomarkers, and pathology observed in brain biopsies of patients with normal-pressure hydrocephalus. *Journal of neuropathology and experimental neurology.* 2015;74(5):470-479.
164. Pomeraniec IJ, Bond AE, Lopes MB, Jane JA, Sr. Concurrent Alzheimer's pathology in patients with clinical normal pressure hydrocephalus: correlation of high-volume lumbar puncture results, cortical brain biopsies, and outcomes. *Journal of neurosurgery.* 2016;124(2):382-388.
165. Leinonen V, Koivisto AM, Savolainen S, et al. Amyloid and tau proteins in cortical brain biopsy and Alzheimer's disease. *Annals of neurology.* 2010;68(4):446-453.
166. Bech-Azeddine R, Hogh P, Juhler M, Gjerris F, Waldemar G. Idiopathic normal-pressure hydrocephalus: clinical comorbidity correlated with cerebral biopsy findings and outcome of cerebrospinal fluid shunting. *Journal of neurology, neurosurgery, and psychiatry.* 2007;78(2):157-161.
167. Leinonen V, Koivisto AM, Savolainen S, et al. Post-mortem findings in 10 patients with presumed normal-pressure hydrocephalus and review of the literature. *Neuropathol Appl Neurobiol.* 2012;38(1):72-86.
168. Cabral D, Beach TG, Vedders L, et al. Frequency of Alzheimer's disease pathology at autopsy in patients with clinical normal pressure hydrocephalus. *Alzheimer's & dementia : the journal of the Alzheimer's Association.* 2011;7(5):509-513.
169. Libard S, Laurell K, Cesarini KG, Alafuzoff I. Progression of Alzheimer's Disease-Related Pathology and Cell Counts in a Patient with Idiopathic Normal Pressure Hydrocephalus. *Journal of Alzheimer's disease : JAD.* 2018;61(4):1451-1462.
170. Abu Hamdeh S, Virhammar J, Sehlin D, Alafuzoff I, Cesarini KG, Marklund N. Brain tissue Abeta42 levels are linked to shunt response in idiopathic normal pressure hydrocephalus. *Journal of neurosurgery.* 2018;130(1):121-129.
171. Koivisto AM, Alafuzoff I, Savolainen S, et al. Poor cognitive outcome in shunt-responsive idiopathic normal pressure hydrocephalus. *Neurosurgery.* 2013;72(1):1-8;discussion 8.
172. Leinonen V, Koivisto AM, Alafuzoff I, et al. Cortical brain biopsy in long-term prognostication of 468 patients with possible normal pressure hydrocephalus. *Neuro-degenerative diseases.* 2012;10(1-4):166-169.
173. Luikku AJ, Hall A, Nerg O, et al. Predicting Development of Alzheimer's Disease in Patients with Shunted Idiopathic Normal Pressure Hydrocephalus. *Journal of Alzheimer's disease : JAD.* 2019;71(4):1233-1243.
174. Nelson PT, Dickson DW, Trojanowski JQ, et al. Limbic-predominant age-related TDP-43 encephalopathy (LATE): consensus working group report. *Brain : a journal of neurology.* 2019;142(6):1503-1527.
175. Crary JF, Trojanowski JQ, Schneider JA, et al. Primary age-related tauopathy (PART): a common pathology associated with human aging. *Acta Neuropathol.* 2014;128(6):755-766.
176. Power MC, Mormino E, Soldan A, et al. Combined neuropathological pathways account for age-related risk of dementia. *Annals of neurology.* 2018;84(1):10-22.
177. Jellinger KA, Attems J. Prevalence of dementia disorders in the oldest-old: an autopsy study. *Acta Neuropathol.* 2010;119(4):421-433.
178. Ramos-Vara JA, Miller MA. When tissue antigens and antibodies get along: revisiting the technical aspects of immunohistochemistry--the red, brown, and blue technique. *Vet Pathol.* 2014;51(1):42-87.

179. Vyberg M, Nielsen S. Proficiency testing in immunohistochemistry--experiences from Nordic Immunohistochemical Quality Control (NordiQC). *Virchows Arch.* 2016;468(1):19-29.
180. Uhlen M, Zhang C, Lee S, et al. A pathology atlas of the human cancer transcriptome. *Science.* 2017;357(6352).
181. Paavilainen L, Edvinsson A, Asplund A, et al. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. *J Histochem Cytochem.* 2010;58(3):237-246.
182. Leong TY, Cooper K, Leong AS. Immunohistology--past, present, and future. *Adv Anat Pathol.* 2010;17(6):404-418.
183. Engel KB, Moore HM. Effects of preanalytical variables on the detection of proteins by immunohistochemistry in formalin-fixed, paraffin-embedded tissue. *Arch Pathol Lab Med.* 2011;135(5):537-543.
184. Boenisch T. Effect of heat-induced antigen retrieval following inconsistent formalin fixation. *Appl Immunohistochem Mol Morphol.* 2005;13(3):283-286.
185. Buchwalow I, Samoilova V, Boecker W, Tiemann M. Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. *Scientific reports.* 2011;1:28.
186. Skaland I, Nordhus M, Gudlaugsson E, et al. Evaluation of 5 different labeled polymer immunohistochemical detection systems. *Appl Immunohistochem Mol Morphol.* 2010;18(1):90-96.
187. Pikkarainen M, Martikainen P, Alafuzoff I. The effect of prolonged fixation time on immunohistochemical staining of common neurodegenerative disease markers. *Journal of neuropathology and experimental neurology.* 2010;69(1):40-52.
188. Karlsson C, Karlsson MG. Effects of long-term storage on the detection of proteins, DNA, and mRNA in tissue microarray slides. *J Histochem Cytochem.* 2011;59(12):1113-1121.
189. Grillo F, Bruzzone M, Pigozzi S, et al. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? *J Clin Pathol.* 2017;70(11):988-993.
190. McCampbell AS, Raghunathan V, Tom-Moy M, et al. Tissue Thickness Effects on Immunohistochemical Staining Intensity of Markers of Cancer. *Appl Immunohistochem Mol Morphol.* 2017.
191. Leong AS. Quantitative immunohistology: tissue section thickness, another glitch in the path to standardization. *Appl Immunohistochem Mol Morphol.* 2009;17(6):465-469.
192. Lundstrom Y, Lundstrom P, Popova SN, Lindblom RPF, Alafuzoff I. Detection of Changes in Immunohistochemical Stains Caused by Postmortem Delay and Fixation Time. *Appl Immunohistochem Mol Morphol.* 2019;27(3):238-245.
193. Monoranu CM, Apfelbacher M, Grunblatt E, et al. pH measurement as quality control on human post mortem brain tissue: a study of the BrainNet Europe consortium. *Neuropathol Appl Neurobiol.* 2009;35(3):329-337.
194. Josephs KA, Murray ME, Whitwell JL, et al. Updated TDP-43 in Alzheimer's disease staging scheme. *Acta Neuropathol.* 2016;131(4):571-585.
195. Libard S, Cerjan D, Alafuzoff I. Characteristics of the tissue section that influence the staining outcome in immunohistochemistry. *Histochem Cell Biol.* 2019;151(1):91-96.
196. Tan YH, Liu M, Nolting B, Go JG, Gervay-Hague J, Liu GY. A nanoengineering approach for investigation and regulation of protein immobilization. *ACS Nano.* 2008;2(11):2374-2384.

197. Di J, Cohen LS, Corbo CP, Phillips GR, El Idrissi A, Alonso AD. Abnormal tau induces cognitive impairment through two different mechanisms: synaptic dysfunction and neuronal loss. *Scientific reports.* 2016;6:20833.
198. Deschenes-Furry J, Perrone-Bizzozero N, Jasmin BJ. The RNA-binding protein HuD: a regulator of neuronal differentiation, maintenance and plasticity. *BioEssays : news and reviews in molecular, cellular and developmental biology.* 2006;28(8):822-833.
199. Ashford JW, Soultanian NS, Zhang SX, Geddes JW. Neuropil threads are collinear with MAP2 immunostaining in neuronal dendrites of Alzheimer brain. *Journal of neuropathology and experimental neurology.* 1998;57(10):972-978.
200. Adlard PA, Vickers JC. Morphologically distinct plaque types differentially affect dendritic structure and organisation in the early and late stages of Alzheimer's disease. *Acta Neuropathol.* 2002;103(4):377-383.
201. Knowles RB, Gomez-Isla T, Hyman BT. Abeta associated neuropil changes: correlation with neuronal loss and dementia. *Journal of neuropathology and experimental neurology.* 1998;57(12):1122-1130.
202. Duong T, Gallagher KA. Immunoreactivity patterns in neurofibrillary tangles of the inferior temporal cortex in Alzheimer disease. *Mol Chem Neuropathol.* 1994;22(2):105-122.
203. Alafuzoff I, Helisalmi S, Heinonen EH, et al. Selegiline treatment and the extent of degenerative changes in brain tissue of patients with Alzheimer's disease. *European journal of clinical pharmacology.* 2000;55(11-12):815-819.
204. Zhu S, Wang J, Zhang Y, et al. The role of neuroinflammation and amyloid in cognitive impairment in an APP/PS1 transgenic mouse model of Alzheimer's disease. *CNS neuroscience & therapeutics.* 2017;23(4):310-320.
205. Rodriguez-Vieitez E, Saint-Aubert L, Carter SF, et al. Diverging longitudinal changes in astrocytosis and amyloid PET in autosomal dominant Alzheimer's disease. *Brain : a journal of neurology.* 2016;139(Pt 3):922-936.
206. Zotova E, Bharambe V, Cheaveau M, et al. Inflammatory components in human Alzheimer's disease and after active amyloid-beta42 immunization. *Brain : a journal of neurology.* 2013;136(Pt 9):2677-2696.
207. Filon JR, Intorcia AJ, Sue LI, et al. Gender Differences in Alzheimer Disease: Brain Atrophy, Histopathology Burden, and Cognition. *Journal of neuropathology and experimental neurology.* 2016;75(8):748-754.
208. Wicinski M, Wodkiewicz E, Slupski M, et al. Neuroprotective Activity of Sitagliptin via Reduction of Neuroinflammation beyond the Incretin Effect: Focus on Alzheimer's Disease. *Biomed Res Int.* 2018;2018:6091014.
209. Latten BGH, Overbeek LIH, Kubat B, Zur Hausen A, Schouten LJ. A quarter century of decline of autopsies in the Netherlands. *Eur J Epidemiol.* 2019;34(12):1171-1174.
210. Eide PK, Hansson HA. Astrogliosis and impaired aquaporin-4 and dystrophin systems in idiopathic normal pressure hydrocephalus. *Neuropathol Appl Neurobiol.* 2018;44(5):474-490.
211. Eide PK, Ringstad G. Delayed clearance of cerebrospinal fluid tracer from entorhinal cortex in idiopathic normal pressure hydrocephalus: A glymphatic magnetic resonance imaging study. *J Cereb Blood Flow Metab.* 2019;39(7):1355-1368.
212. Aho L, Pikkarainen M, Hiltunen M, Leinonen V, Alafuzoff I. Immunohistochemical visualization of amyloid-beta protein precursor and amyloid-beta in extra- and intracellular compartments in the human brain. *Journal of Alzheimer's disease : JAD.* 2010;20(4):1015-1028.

213. Hansson Petersen CA, Alikhani N, Behbahani H, et al. The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae. *Proc Natl Acad Sci U S A.* 2008;105(35):13145-13150.
214. Medeiros R, LaFerla FM. Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. *Experimental neurology.* 2013;239:133-138.
215. Pyykko OT, Lumela M, Rummukainen J, et al. Cerebrospinal fluid biomarker and brain biopsy findings in idiopathic normal pressure hydrocephalus. *PloS one.* 2014;9(3):e91974.
216. Eide PK, Hasan-Olive MM, Hansson HA, Enger R. Increased occurrence of pathological mitochondria in astrocytic perivascular endfoot processes and neurons of idiopathic intracranial hypertension. *Journal of neuroscience research.* 2021;99(2):467-480.

Acta Universitatis Upsaliensis

Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 1719

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title "Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine".)

Distribution: publications.uu.se
urn:nbn:se:uu:diva-433963

ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2021