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Altered proteins in the aging brain

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Abstract
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The classification of neurodegenerative disorders is based on the major component of the protein aggregates in the brain. The most common altered proteins associated with neurodegeneration are Hyperphosphorylated tau (HPr), beta amyloid (Aβ), alpha-synuclein (aS) and transactive response DNA binding protein 43 (TDP43). In this study we assessed the incidence and the neuroanatomical distribution of proteins associated with neurodegeneration in the brain tissue of cognitively unimpaired subjects.

We demonstrated the early involvement of the Locus Coeruleus (LC) with HPr pathology in cognitively unimpaired mid aged subjects, a finding which supports the notion that LC is an initiation site of HPr pathology. This may suggest that development of clinical assessment techniques and radiological investigations reflecting early LC alterations may help in identifying subjects with early stages of neurodegeneration.

Furthermore, we studied a large cohort of cognitively unimpaired subjects with age at death ≥50 years and we applied the National Institute on Aging – Alzheimer’s disease (AD) Association (NIA-AA) guidelines for the assessment of AD related neuropathological changes. Interestingly, a considerable percentage of the subjects were classified as having an intermediate level of AD pathology. We also showed that the altered proteins; HPr, Aβ, αS, and TDP43 are frequently seen in the brain of cognitively unimpaired subjects with age at death ≥50 years, the incidence of these proteins increased significantly with age. This finding suggests that neurodegeneration has to be extensive to cause functional disturbance and clinical symptoms. Moreover, we investigated the correlation between AD related pathology in cortical biopsies, the AD / cerebrospinal fluid (CSF) biomarkers and the Mini Mental State examination (MMSE) scores in a cohort of idiopathic Normal Pressure Hydrocephalus (iNPH) patients. We demonstrated that AD/CSF biomarkers and MMSE scores reflect AD pathology in the cortical biopsies obtained from iNPH patients.

In conclusion, this study shows that the altered proteins associated with neurodegeneration are frequently seen in the brain tissue of cognitively unimpaired aged subjects. This fact should be considered while developing diagnostic biomarkers for identification of subjects at early stages of the disease, in order to introduce therapeutic intervention prior to the occurrence of significant cognitive impairment.

Keywords: Cognitively unimpaired subjects, Hyperphosphorylated tau, Beta amyloid, Alphasynuclein, Transactive response DNA binding protein 43

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To My Dear Aunt Adila
List of Papers

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

I  Elobeid A, Soininen H, Alafuzoff I. Hyperphosphorylated tau in young and middle-aged subjects. Acta Neuropathol. 2012; 123(1):97-104.

II Elobeid A, Rantakömi S, Soininen H, Alafuzoff I. Alzheimer's disease-related plaques in nondemented subjects. Alzheimers Dement. 2014;10(5):522-9.

III 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. J Neuropathol Exp Neurol. 2015; 74(5):470-9.

IV Elobeid A, Libard S, Leino M, Popova S, Alafuzoff I. Altered proteins in the aging brain. Accepted for publication in the Journal of Neuropathology and Experimental Neurology.

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Abbreviations

αS  α synuclein
Aβ  β amyloid
AD  Alzheimer’s disease
AGD  Argyrophilic Grain Disease
ALS  Amyotrophic lateral sclerosis
CAA  Cerebral Amyloid Angiopathy
CBD  Cortico Basal Degeneration
CERAD  The Consortium to Establish Registry of Alzheimer’s Disease
CSF  Cerebrospinal Fluid
DLB  Dementia with Lewy Bodies
FTLD  Frontotemporal lobar degeneration
FUS  Fused in Sacroma,
HPτ  Hyperphosphorylated τ
IHC  Immunohistochemistry
iLBRP  Incidental Lewy Body related pathology
LB  Lewy Body
LC  Locus Coeruleus
MSA  Multiple System Atrophy
NFTs  Neurofibrillary Tangles
NIA-RI  The National Institute of Aging and the Regan Institute
NIA-AA  The National Institute on Aging –Alzheimer’s Association
NPs  Neuritic Plaques
NTs  Neuropil Threads
PART  Primary Age Related Tauopathy
PD  Parkinson’s Disease
PDD  Parkinson’s Disease with Dementia
PiD  Pick’s Disease
PSP  Progressive Supranuclear Palsy
SOD1  Superoxidase dismutase-I
TDP43  Transactive response DNA binding protein 43
Introduction

The incidence of neurodegenerative disorders increases with age. During life, the diagnosis of these disorders is based on certain clinical presentation; however, diagnostic biomarkers are available for some diseases. The definite diagnosis is based on the results obtained from a neuropathological assessment of the brain tissue that is carried out post mortem. The neuropathological criteria have evolved since the 1990s due to the development of assessment strategies such as immunohistochemistry (IHC), a method that visualizes altered proteins in the brain tissue. Thus, many neurodegenerative diseases are currently referred to as proteinopathies.

Neurodegenerative proteinopathies

The current classification of age-related neurodegenerative disorders is based on the altered proteins that aggregate in the brain. The most common altered proteins found in age-related neurodegenerative proteinopathies are hyperphosphorylated \( \tau \) (HP\( \tau \)), \( \beta \) amyloid (A\( \beta \)), \( \alpha \) synuclein (\( \alpha \)S), and trans active response DNA binding protein 43 (TDP43)[1,2,3,4,5,6,7,8]. The most common proteinopathies are listed in Table 1.

Tauopathies

Tauopathy is a term used to describe a group of neurodegenerative disorders, characterized by the aggregation of altered \( \tau \) protein. In 1986, aggregated microtubule associated protein \( \tau \) was visualized in the brain of subjects with Alzheimer’s disease (AD) [9, 10]. The classification of tauopathies is based on the affected cell type (neuron or glia or both), the \( \tau \) isoform (3R\( \tau \), 4R\( \tau \)), and the neuroanatomical localization and distribution of the pathological deposits [11, 12,13,14].

**Tauopathies with either 3R\( \tau \) or 4R\( \tau \)**

*Progressive Supranuclear Palsy (PSP)*

PSP is a neurodegenerative disorder of middle and late age. The clinical characteristics of PSP include Parkinsonism, supranuclear gaze palsy, pseudobulbar palsy, frontotemporal dementia, and progressive aphasia [13, 15].
Table 1. Classification of neurodegenerative proteinopathies

| Proteinopathies | Protein involved | Disorder |
|-----------------|------------------|----------|
| Tauopathies     | 3Rτ              | Pick’s Disease |
|                 | 4Rτ              | Argyrophilic Grain Disease |
|                 |                  | Corticobasal Degeneration |
|                 |                  | Progressive Supranuclear palsy |
|                 | 3Rτ+4Rτ          | Alzheimer’s Disease |
|                 |                  | Neurofibrillary tangle only dementia |
| α synucleinopathies | αS          | Parkinson’s Disease |
|                  |                  | Parkinson’s Disease with Dementia |
|                  |                  | Dementia with Lewy bodies |
|                  |                  | Multiple System Atrophy |
| TDP43 proteinopathies | TDP-43   | Amyotrophic lateral sclerosis |
|                  |                  | Frontotemporal lobar degeneration |
| Aβ proteinopathies | Aβ           | Alzheimer’s Disease |
| PrP proteinopathies | PrP       | Creutzfeldt-Jakob disease |
| FUS proteinopathies | FUS       | Amyotrophic lateral sclerosis |
|                  |                  | Frontotemporal lobar degeneration |
| SOD-1 proteinopathies | SOD-1   | Amyotrophic lateral sclerosis |

HPτ, Hyperphosphorylated τ; Aβ, β- amyloid; αS, α- synuclein; TDP43, transactive response DNA binding protein 43 kDa; FUS, fused in sarcoma; SOD-1, superoxide dismutase-1

PSP is a sporadic disease; however, familial cases with PSP like phenotype and MAPT gene mutations have been reported [16]. Neuropathologically, PSP is characterized by atrophy of the basal ganglia, subthalamic nucleus, and the brain stem. The microscopic characteristics include glial alterations such as tufted astrocytes and oligodendroglial coiled bodies and also neuronal alterations such as round/globose neurofibrillary tangles (NFTs) and neuropil threads (NTs). All these lesions are IR for HPτ isoform 4Rτ. In addition, neuronal loss and gliosis are observed. These lesions are primarily seen in the central brain structures such as striatum, pallidum, subthalamic nucleus, substantia nigra, basis pontis, superior colliculi, and dentate nucleus [17, 18, 19,20].

Cortico-Basal Degeneration (CBD)

CBD is a rare neurological disorder affecting the aged. The clinical characteristics of CBD include Parkinsonism, cortical sensory loss, alien limb phenomenon, frontal lobe behavioral changes, dementia, and progressive aphasia [13, 21]. Most of the reported CBD cases are sporadic; however, cases
with MAPT gene mutations and CBD like phenotype have been reported [22, 23]. Neuropathologically, CBD is characterized by asymmetric frontoparietal atrophy that is most severe in the pre- and post-central regions, in addition to the depigmentation of the substantia nigra. The microscopic characteristics include glial alterations such as astrocytic plaques, neuropil threads, and oligodendroglial coiled bodies that are IR for HP\(\tau\), isoform 4R\(\tau\). In addition, gliosis, neuronal loss, spongiosis, and ballooned cells are seen [14, 24, 25, 26].

Arterial Grain Disease (AGD)
AGD is a late onset sporadic disorder clinically characterized by progressive cognitive decline and personality changes [27]. Neuropathologically, AGD is characterized by argyrophilic grains that are IR for HP\(\tau\) isoform 4R\(\tau\). These lesions are seen in both the cortical and the subcortical structures, being most frequent in the entorhinal and transentorhinal regions. These lesions are frequently seen concomitant with AD related pathology [11, 12, 27].

Pick’s Disease (PiD)
PiD is a rare disorder clinically characterized by behavioral frontotemporal dementia, and progressive aphasia; the motor symptoms are less common [28, 29]. Mutations in the MAPT gene have been suggested to be associated with this disorder [30]. Neuropathologically, PiD is characterized by frontotemporal atrophy and neuronal pick bodies that are IR for HP\(\tau\) isoform 3R\(\tau\). In addition, neuronal loss, spongiosis, gliosis, and ballooned cortical neurons are seen. HP\(\tau\)/IR Pick bodies are most abundant in the temporal cortex (neocortical layers II and VI) and in the hippocampus (granule cells of the dentate gyrus) [14, 31, 32, 33, 34, 35].

Tauopathies with both 3R\(\tau\) and 4R\(\tau\)
There are three defined disorders that display both 4R\(\tau\) and 3R\(\tau\): Primary Age Related Tauopathy (PART), Neurofibrillary tangle only dementia, and AD.

Progression and distribution of NFTs and NTs
In these tauopathies (PART, NFT only dementia, and AD), the neuroanatomical distribution and progression of the NFTs and NTs follows the pattern that has been described by Braak and Braak in 1991[36]. The Braak staging is based on the topographical distribution of the silver stained NFTs and NTs. Six stages were identified in 1991, i.e., the transentorhinal and entorhinal (stages I and II), limbic (III and IV), and finally, the isocortical stages (stages V and VI) that are sequentially involved. In 2011, Braak and colleagues updated their original work, stating that the locus coeruleus (LC) is probably the initiation site for the HP\(\tau\) pathology rather than the entorhinal...
cortex and most importantly that this LC alteration is seen in young subjects [37, 38]. In 2008, the Brain Net Europe (BNE) consortium reported that a consensus was reached in an inter-laboratory setting, including up to 30 neuropathologists in more than 15 centers, while applying IHC and the described staging criteria for Braak stages I to VI [1].

Primary Age Related Tauopathy (PART)
In 2014, a new entity with 4 and 3 Rτ was defined, i.e., PART. This entity incorporates the cognitively unimpaired aged subjects and subjects with mild cognitive impairment that display NFTs and NTs in the hippocampus and in the medial temporal lobe, displaying AD like distribution with a progression less or equal to stage IV. The neuropathological criteria also require that no or minimal Aβ pathology is observed [39].

Neurofibrillary tangle only dementia
This is a late onset dementia characterized neuropathologically by NFTs that follow the Braak staging [36], usually in stage IV and above, with the absence or scarcity of Aβ aggregates [14, 40].

Alzheimer’s disease
AD is the most common cause of dementia. Based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), and the National Institute of Neurological Disorders and Stroke-Alzheimer’s Disease and related Disorders (NINCDS-ADRDA) diagnostic criteria[41, 42], the clinical diagnosis of AD requires that two or more cognitive domains being affected, including memory impairment and at least one cognitive or behavioral deficits, which include visuospatial function, abstract reasoning, executive function, mood, personality, and language abnormalities. Neuropathologically, AD is characterized by identification of two hallmark altered proteins, i.e., HPτ and Aβ [1, 2, 43].

Progression and distribution of the Aβ pathology in AD
Already in 1991, Braak and Braak defined three stages ranging from A to C [36]. Thereafter, in 2002[2], Thal and colleagues, implementing the IHC technique, proposed a five stage assessment of Aβ/IR: stage 1 where IR is seen in the neocortex; stage 2 in the allocortex; stage 3 in the diencephalic nuclei, the striatum, and the cholinergic nuclei of the basal forebrain; stage 4 in the subcortical nuclei, and stage 5 in the cerebellum. In 2009, the BNE reported a high agreement rate while assessing the phases of Aβ as proposed by Thal and colleagues [44].

Cerebral Amyloid Angiopathy (CAA)
Aβ can also be seen in the vessel walls of the leptomeningeal and cortical vessels; moreover, based on the type of the vessel involved, two types of
CAA are defined. In type II, the arteries, arterioles, veins, and venules are affected, whereas in Type I in addition, deposition of Aβ in the capillaries is observed [45]. CAA can be associated with cerebral hemorrhages [46].

**Consensus regarding the neuropathological assessment of AD**

The first consensus report regarding neuropathological assessment of AD was proposed by Khachaturian in 1985 as a result of a joint workshop supported by four organizations (National Institute of Aging, the American Association of Retired Persons, the National Institute of Neurology and Communicative Disorders and Stroke, and the National Institute of Mental Health). The strategy proposed was based on the counts of silver stained Neuritic Plaques (NPs) in relation to the age of the patient [47]. Later in 1991, the Consortium to Establish Registry of Alzheimer’s Disease (CERAD) [48] proposed a strategy defining more in detail the brain regions to be assessed and the silver stains to be used. The criteria were based on the semi-quantitative scoring of the NPs, where the NP score is adjusted with the patient’s age at death to obtain an age related NP score. This score was then related to the clinical presentation (demented/non demented) to obtain a level (probable, possible, and definite) of certainty that the clinically observed dementia was caused by the AD related pathology. The major drawback with this criterion was that the NFT pathology was not taken into account [49].

In 1991, Braak and Braak also described, by applying the silver stain, the progression of the NFT pathology from stage I to VI. In 1997, the National Institute of Aging and the Regan Institute (NIA-RI) [50] launched their recommendations, where the CERAD NP count was combined with the Braak NFT stage. Thus, by applying the silver stains, three stages were defined: stage 1 meaning a high likelihood that the dementia is caused by the AD pathology (Braak V–VI and CERAD severe), stage 2 being intermediate likelihood (Braak stages III–IV and CERAD moderate), and stage 3 low likelihood (Braak stages I–II and CERAD mild). In 2012, the NIA-RI criteria were updated to the NI-AA criteria [51, 52], implementing the modern IHC techniques. An “ABC” staging protocol was proposed, incorporating the CERAD NP score, Braak NFT (HPr) stage, and Thal Aβ Phase. It was also recommended to assess comorbidities. An important point was raised, namely, that the neuropathological assessment, i.e., the staging of the observed alterations should be carried out independent of the clinical symptomatology. Thus, a stage of AD related pathology could be given both for cognitively unimpaired as well as demented subjects.

Over the last two decades, progress has been made in identifying the AD associated changes prior to the neuropathological assessment by using biomarkers such as cerebrospinal fluid (CSF) levels of Aβ42, HPr, and Total τ[53,54,55,57] . In AD patients, the CSF Aβ42 concentrations decrease by about 50%; this decrease is associated with the deposition of Aβ in the brain. The CSF total τ increases on average by two to three fold in AD, as well as
HPτ [53, 54]. Pathological values of two or more biomarkers are considered to reliably predict mild cognitive impairment conversion to AD [55, 56, 57].

α synucleinopathies

αS is the main component of Lewy Body (LB), the hallmark lesion of Parkinson’s Disease (PD), Parkinson’s Disease with Dementia (PDD), Dementia with Lewy Bodies (DLB)[58,59,60,61].

PD, PDD, and DLB

PD is clinically characterized by motor signs such as bradykinesia, rigidity, resting tremors, gait instability, and non-motor signs such as hallucinations, olfactory disturbances, and sleep disturbances. If the subject develops dementia over time, the clinical diagnosis given would be PDD [62]. Contrary to PDD, DLB dementia is clinically characterized by initial signs of progressive cognitive decline. Other features of DLB include parkinsonism, and visual hallucinations [4].

The neuropathological characteristics of PD, PDD, and DLB include loss of pigmented neurons in the substantia nigra, motor nucleus of vagus, and the LC, with widespread LBs and Lewy Neurites (LN) [3, 4, 5, 63,64,65]. LBs and LNs are visualized by implementing the IHC technique and antibodies directed to the αS.

Progression and distribution of αS Pathology

Already in 1984, Kosaka and colleagues suggested that three stages of DLB pathology are identified: brain stem, limbic, or neocortical [66]. In 1996, the existence of these three stages, namely, brain stem, limbic, and neocortical was confirmed by the Consortium on DLB International Workshop [67].

In 2003, Braak and colleagues proposed a more detailed classification scheme leading to 6 stages: stage 1- involvement of the dorsal motor nucleus of the vagus, stage 2- lower raphe nucleus and LC, stage 3- Substantia nigra, stage 4- Amygdala and basal nucleus of Meynert and hippocampal CA2 sector, stage 5- Cingulate cortex and finally, stage 6- Temporal, frontal, and Parietal cortex [3]. In 2005, McKeith and colleagues launched the current, commonly used, consensus guidelines incorporating the semi quantitative score and the anatomical distribution of the LBs, while applying the IHC technique and antibodies directed to the αS [4].

McKeith’s and Braak’s staging systems have been applied in several studies [68, 69, 70]. In 2009, a staging protocol for the αS pathology was proposed by the BNE [5]. The protocol is a modification of the original Braak and McKeith staging systems. Instead of semi quantitative scoring of the αS pathology, a dichotomized approach was recommended. This approach was chosen due to the significant variations observed by the BNE in the staining quality and outcome of the semi quantitative counts in the inter laboratory
setting [5]. When the protocol was applied based on a dichotomized assessment, the inter observer agreement was more than 80%.

*Multiple System Atrophy*

MSA is a rare sporadic disease clinically characterized by autonomic failure, cerebellar ataxia, or parkinsonism not responsive to Levodopa [71]. The macroscopic features seen in MSA are various, including greyish discoloration of the putamen, atrophy of the cerebellum and pons. The microscopic hallmark lesion of MSA is the αS IR glial cytoplasmic inclusions in the oligodendrocytes [72, 73,74].

**Transactive DNA binding protein 43 related proteinopathies**

TDP43 is a hallmark alteration in the Amyotrophic lateral sclerosis (ALS) and Frontotemporal lobar degeneration (FTLD-TDP) [6, 75, 76]. This protein is also seen in a subset of AD patients [8, 77, 78, 79, 80,81].

*Amyotrophic Lateral sclerosis*

ALS is a disorder characterized by degeneration of motor neurons of the motor cortex, brain stem, and the spinal cord. The clinical symptoms of ALS include progressive muscle weakness in upper and lower limbs and progressive bulbar palsy, the involvement of respiratory muscles results in respiratory failure and death [82, 83]. In addition, ALS may present with behavioral and cognitive symptoms. Several gene mutations have been reported to contribute to the pathogenesis of ALS including SOD1, TARDBP, FUS, and C9orf72 mutations [84, 85, 86].

The currently defined neuropathological phenotypes are: ALS-TDP, ALS-FUS, and ALS-SOD1. ALS-TDP cases are mainly sporadic cases with more bulbar involvement, rapid disease progression, and earlier age of onset. TDP43 is a component of the neuronal inclusions and oligodendroglial aggregates seen in ALS-TDP [75].

*FTLD-TDP*

FTLD-TDP clinical characteristics include behavioral and cognitive symptoms as well as progressive aphasia. Several genes have been reported to contribute to the pathogenesis of FTLD-TDP including TARDBP, VCP, GRN, and C9orf72 mutations [87, 88, 89, 90, 91]. Macroscopically, FTLD-TDP is characterized by frontotemporal lobar atrophy. Microscopically, it is characterized by neuronal loss, gliosis, and TDP43/IR neuronal inclusions (neuronal cytoplasmic inclusions, neuronal intranuclear inclusions, and dystrophic neurites), in addition to glial cytoplasmic inclusions that are mainly oligodendroglial [92].
Progression and distribution of TDP43 pathology

Progression and distribution of TDP43 in ALS
Four stages of progression of TDP43 pathology in ALS have been described [7]. In stage I, TDP43 inclusions are detected in the motor cortex, medulla oblongata, and the spinal cord; in stage II, further spread in the prefrontal neocortex, brain stem reticular formation, and the red nucleus; in stage III, lesions are seen in the precentral and postcentral neocortex and the striatum; and in stage IV, further involvement of the anteromedial portions of the temporal lobe and hippocampus [7].

Progression and distribution of TDP43 in behavioral variant (bv) FTLD-TDP
Four stages of progression TDP43/IR lesions in bvFTLD were described. Stage I is characterized by the involvement of the orbital gyri, gyrus rectus, and the amygdala. In stage II, there is sequential involvement of the cingulate gyrus, temporal lobe and the striatum, red nucleus, thalamus, and the pre-cerebellar nuclei. Stage III is characterized by the sequential involvement of the motor cortex and the anterior horn of the spinal cord. In stage IV, the sequential involvement of the visual cortex is observed [93].

Progression and distribution of TDP43 in AD
Recently, Josephs and colleagues described five stages of progression of TDP43 pathology in AD. In stage I, TDP43 inclusions are detected in the amygdala; in stage II, further spread in the entorhinal cortex and subiculum; in stage III, the dentate gyrus and occipitotemporal cortex; in stage IV, lesions are also seen in the inferior temporal cortex; and in stage V, a further involvement of the frontal cortex is seen [8].

Other Proteinopathies
Other proteins known to be associated with the pathogenesis of neurodegenerative disorders include; Fused in Sacroma (FUS) in FTLD and ALS, Superoxidase dismutase-1 (SOD1) in ALS, Huntingtin in Huntington’s disease, PrP in Creutzfeldt-Jakob disease[94,95,96,97,98].

Concomitant proteinopathies in dementia
The four altered proteins HPτ, Aβ, αS, and TDP43 are frequently seen concomitantly in demented subjects [99,100,101,102,103,104,105,106,107]. Subjects with AD related lesions display concomitant αS and TDP43IR. αS pathology was reported to be seen in 60% of the familial and sporadic AD; αS/IR is predominantly seen in the amygdala [108,109]. Moreover, TDP43 pathology is seen in up to 57% of the AD patients [8, 77, 78, 79, 80, 81].

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Several previous studies have demonstrated a wide spread of Aβ pathology in the striatum of the PDD and DLB patients [110,111,112]. In addition, the TDP43/IR lesions were reported to be seen in 31% of the DLB cases, in 7% of the PD, and in 19% of the PDD patients [113]. Furthermore, the accumulation of the abnormal TDP43 aggregates was previously demonstrated in the hippocampal sclerosis, Huntington’s disease, and PiD [114,115,116].

Neurodegenerative alterations in cognitively unimpaired subjects

HPτ in cognitively unimpaired subjects

Several previous post mortem reports have demonstrated HPτ/IR NFTs in the brain tissue of cognitively unimpaired subjects [117, 118, 119, 120, 121, 122]. Interestingly, Boyle and colleagues reported that HPτ/IR lesions were seen in 100% of a large cohort of cognitively unimpaired subjects [122]. Furthermore, post mortem reports have also demonstrated the existence of AGD and PSP related pathological alterations in clinically normal subjects [123, 124, 125]. Noteworthy, most subjects that are neuropathologically classified as PART are cognitively unimpaired or display minor cognitive changes [39].

Aβ in cognitively unimpaired subjects

Many previous studies have demonstrated the existence of the Aβ aggregates in the brain tissue of cognitively unimpaired subjects [118, 120, 121, 122, 126, 127]. Interestingly, Aβ pathology was reported to be seen in up to 82% of a large cohort of cognitively unimpaired subjects [122].

αS in cognitively unimpaired subjects

Several post mortem studies have documented the incidence of αS pathology in the cortical and subcortical regions in cognitively unimpaired subjects. Incidental LB related pathology (iLBRP) is seen in 8.3 to 31% of cognitively unimpaired subjects [128,129,130,131].

The clinical importance of iLBRP in cognitively unimpaired subjects was discussed in several previous studies [132,133,134,135]. It was suggested that cognitively unimpaired subjects with iLBRP might represent preclinical PD cases [132,133]. Interestingly, Delle Donne and colleagues [136] demonstrated nigral cell loss in subjects displaying iLBRP, a finding which supports that iLBRP might represent preclinical PD. In line with this, previous post mortem reports demonstrated an incidence of iLBRP in subjects dis-
playing non motor clinical features of PD such as olfactory dysfunction [137] and rapid eye movement sleep disorder [138,139].

TDP43 in cognitively unimpaired subjects
The incidence of TDP43 pathology in the brain of cognitively unimpaired subjects was previously reported [113,124,140]. TDP43/IR lesions were reported to be observed in the amygdala and/or the hippocampus in 36% of 110 cognitively unimpaired subjects [140]. Interestingly, Kovacs and colleagues studied 51 cognitively unimpaired subjects, and TDP43 pathology was reported to be seen in 7% of the subjects [124]. A lower incidence of TDP43 pathology in cognitively unimpaired subjects (3%) has also been reported [113].

Concomitant proteinopathies in cognitively unimpaired subjects
The existence of mixed pathologies in the brain tissue of aged non demented subjects have been demonstrated in a number of post mortem reports [121,122,123,124,140,141]; however, these reports are fewer when compared with the number of studies investigating mixed pathologies in demented subjects [99,100,101,102,103,104,105,106,107].

Several reports have shown concomitant AD related hallmark lesions (HP\(\tau\) and A\(\beta\)) in cognitively unimpaired subjects [117, 118, 119, 120, 121, 122]. Furthermore, studies have demonstrated concomitant HP\(\tau\), A\(\beta\), and \(\alpha\)S pathology in non-demented subjects [121,122,124]. Some studies have reported concomitant HP\(\tau\), A\(\beta\), and TDP43 pathology [124] and HP\(\tau\) and TDP43 or \(\alpha\)S and TDP43 [140] in the brain tissue of cognitively unimpaired subjects.

Normal Pressure Hydrocephalus
Idiopathic normal pressure hydrocephalus (iNPH) is a disorder characterized by cognitive impairment, gait disturbance, and urinary incontinence [142]. The cognitive impairment in iNPH is potentially reversible by ventriculoperitoneal shunt operation; however, shunt irresponsiveness can be attributed to the existence of comorbid neurodegenerative pathology. AD related pathology was reported to be seen in the cortical biopsies obtained from patients with iNPH [143,144,145,146].
Methodological considerations

The reported incidences of neurodegenerative proteinopathies in cognitively unimpaired subjects are quite various. This variation is probably due to issues such as various number of study subjects included, selection bias, age range of the study subjects, and the level of clinical assessment carried out. Furthermore, the methods used while assessing the pathology are of major significance; The post mortem delay, fixation time, selected antibody, pre-treatment used, and detection system implemented have all been reported to significantly influence the outcome [147,148,149,150,151,152].
The Present Investigation

Aims of the study

**General Aim**

To assess the incidence and distribution of the altered proteins common in age related neurodegenerative disorders in a well characterized post mortem cohort of cognitively unimpaired subjects.

The specific aims of the study are to investigate the following:

- The initiation site of HP\(\tau\) pathology in cognitively unimpaired middle aged subjects (Study I).
- The extent of AD related pathology seen in aged cognitively unimpaired subjects and the applicability of the recent National Institute on Aging – Alzheimer’s Association (NIA-AA) criteria (Study II).
- The incidence of A\(\beta\) and HP\(\tau\) pathology in cortical biopsies obtained from iNPH patients and to study whether the clinical parameters and available biomarkers reflect the brain pathology (Study III).
- The incidence and the extent of the most common altered proteins seen in the aging brain (HP\(\tau\), A\(\beta\), \(\alpha\)S, and TDP43) in a well characterized, large, cohort of cognitively unimpaired aged subjects (Study IV).
Subjects and Methods

Subjects
The brain tissue assessed in this study was either obtained post mortem (study I, II, and IV) or during a surgical procedure (study III). General demographics and selection criteria of the study subjects are given in Table 2 and 3, respectively. Ethical permission was obtained from the ethical committee at Kuopio University Hospital and from the regional ethical committee in Uppsala.

| Study    | Center | Year         | Samples | N   | Age range | Gender (M/F) |
|----------|--------|--------------|---------|-----|-----------|--------------|
| Study I  | 1      | 1992-2008    | 3       | 95  | 22-50     | 55/40        |
| Study II | 1      | 1992-2000    | 3       | 192 | 55-98     | 87/105       |
| Study III| 2      | 2010-2013    | 4       | 111 | 54-89     | 67/44        |
| Study IV | 2      | 2009-2014    | 3       | 296 | 50-102    | 185/111      |

Table 2. General description of the study subjects

Pathology department at 1, Kuopio University Hospital; 2, Uppsala University Hospital; F, Female; M, Male; 3, postmortem brain material; 4, surgical samples; N, number of subjects

Clinical assessment

The clinical data were obtained retrospectively from the hospital records blinded to the neuropathological findings (study I, II, and IV). Information regarding the cognitive status was also obtained retrospectively, but there was no neuropsychological tests carried out. Some of the subjects might thus have displayed mild cognitive impairment, but the presence of dementia was not registered in the medical records (study I, II, and IV).

iNPH cases were pre and post operatively assessed by a multidisciplinary iNPH team including a neurologist, a neurosurgeon, a trained physiotherapist, and an occupational therapist. The cognitive status was assessed by applying Mini Mental State Examination (MMSE). Subjects with a MMSE score $\geq 24$ were considered as being cognitively unimpaired, and subjects with a MMSE score $\leq 23$ were considered as being demented (study III).
Table 3. Selection criteria of included subjects

| Study  | Inclusion criteria                                                                 |
|--------|-----------------------------------------------------------------------------------|
| Study I| Age at death ≤50 years, cognitively unimpaired subjects, no concomitant brain disease (infections, primary or secondary brain tumor). |
| Study II| Age at death >50 years, cognitively unimpaired subjects, presence of neuritic plaques in modified Bielschowsky staining in the neocortex. |
| Study III| Clinical diagnosis of idiopathic normal pressure hydrocephalus, availability of cortical brain biopsy and lab and clinical data. |
| Study IV| Age at death ≥50 years, cognitively unimpaired subjects. |

Neuropathological assessment

The brains were weighed, evaluated for grossly detectable lesions and vessel abnormalities, and immersed in 10% buffered formalin for at least one week. Thereafter, the brains were sampled in a standardized manner (Table 4) (Study I, II, IV).

In study III, a frontal cortical biopsy was obtained during the surgical operation and placed in 10% buffered formalin and then embedded in paraffin.

Immunohistochemistry

For the IHC, seven µm thick sections were used. The details regarding the antibodies used are given in Table 5. For detection, a poly HPR-IHC detection kit with Romulin-3-amino-9-ethylcarbazol (AEC) chromogen was used (study I, IV). The streptavidin-biotin complex was visualized using vector red (vector red alkaline phosphatase substrate kit I, Zymed; Cat. No.SK-5100) for Aβ and DAB (Liquid DAB substrate Kit, Zymed; Cat. No.00-2014) for ubiquitin (Ubq), and HPτ (study II). For detection, the Dako EnVision FLEX detection system was used (study III and IV). The details regarding the neuroanatomical regions assessed applying IHC are given in Table 6.

Staging of the pathological lesions

Modified Bielschowsky (mBky) silver impregnation method was used for the assessment the score of NPs (study II,IV). A CERAD NP score and the level of AD neuropathological changes were given as recommended by the NIA-RI [50] (study II) and NIA-AA criteria [51, 52] (study II and IV). HPτ Braak stage [1] (study I, II, and IV), Aβ Thal phase [2] (study II and IV), αS stage [3,4,5] (study IV), and TDP43 stage [8] (study IV) were assessed as recommended.
### Table 4. Brain regions sampled and assessed

| Brain region                          | HE | Study I | Study II | Study III | Study IV |
|---------------------------------------|----|---------|----------|-----------|----------|
| Frontal Cortex, gyrus medius          | X* | X       |          | X         |          |
| Temporal Cortex, gyrus medius         | X* | X       | X        | X         |          |
| Gyrus cinguli, anterior               | X  | X       |          | X         |          |
| Parietal Cortex, inferior             | X* | X       | X        | X         |          |
| Pre-post central cortex               | X  |         |          |           |          |
| Occipital Cortex                     | X* | X       | X        | X         |          |
| Hippocampus anterior                  | X  | X       | X        | X         |          |
| Hippocampus posterior                 | X  | X       | X        | X         |          |
| Basal forebrain, incl. amygdala       | X  | X       | X        | X         |          |
| Striatum                              | X  |         |          |           |          |
| Thalamus                              | X  |         |          |           |          |
| Mesencephalon incl. Substatia Nigra   | X  | X       |          | X         |          |
| Pons, incl locus coeruleus           | X  | X       |          | X         |          |
| Medulla, incl nucleus vagus          | X  |         |          | X         |          |
| Vermis                                | X  |         |          |           |          |
| Cerebellum                            | X  | X       |          | X         |          |

* modified Bielshowsky silver stain

### Table 5. Immunohistochemical stains

| Antibody | Clone | Source     | Dilution | Pre-treatment | Study       |
|----------|-------|------------|----------|---------------|-------------|
| HP\(\tau\) | AT8   | Thermo Scientific | 1:500    | -             | I,II,III,IV |
| A\(\beta\) | 6F/3D | Dako       | 1:100    | 80 % Formic Acid, 6 hours | I,II,III,IV |
| Ubq       | -     | Dako       | 1:200    | Citrate Buffer* | II          |
| \(\alpha\)S | KM51  | NovoCastra | 1:100    | Citrate Buffer* + 80 % Formic Acid, 5min | IV          |
| TDP43     | 11-9  | Cosmo Bio  | 1:5000   | Citrate Buffer* | IV          |

HP\(\tau\), hyperphosphorylated \(\tau\); A\(\beta\), \(\beta\)-amyloid; Ubq, Ubiquitin; \(\alpha\)S, \(\alpha\)-synuclein; TDP43, trans-active response DNA binding protein 43 kDa; *Autoclave
Table 6. Neuroanatomical regions assessed applying immunohistochemistry

| Brain region                                    | Immunoistochemical staining |
|-------------------------------------------------|-----------------------------|
|                                                  | HP\(\tau\) | A\(\beta\) | \(\alpha\)S | TDP43 |
| Frontal Cortex, gyrus medius                     |             | X          |             |      |
| Temporal Cortex, gyrus medius                    | X          | X          | X           |      |
| Gyrus cinguli, anterior                          |             |            | X           |      |
| Parietal Cortex, inferior                        | X          | X          | X           |      |
| Pre-post central cortex                          |             |            | X           |      |
| Occipital Cortex                                | X          |            | X           |      |
| Hippocampus anterior                             | X          |            | X           |      |
| Hippocampus posterior                            | X          | X          | X           | X    |
| Basal forebrain, incl. amygdala                  | X          | X          | X           |      |
| Striatum                                         |             |            | X           |      |
| Thalamus                                         |             |            | X           |      |
| Mesencephalon incl. Substatia Nigra              |             |            | X           |      |
| Pons, incl locus coerules                        | X          |            |             |      |
| Medulla, incl nucleus vagus                      | X          |            | X           |      |
| Vermis                                           |             |            | X           |      |
| Cerebellum                                       | X          |            |             |      |

HP\(\tau\), hyperphosphorylated \(\tau\); A\(\beta\), \(\beta\)-amyloid; \(\alpha\)S, \(\alpha\)-synuclein; TDP43, transactive response DNA binding protein 43 kDa

Semi quantitative analysis of the pathological alterations

Semi quantitative assessments of NPs in the HP\(\tau\), Ubq, and the mBky stain were carried out for study II. For each stain, the section was first scanned at magnification x40 to select the grey matter area with the most severe involvement. Then, in each sample within the selected area, three microscopic fields were randomly chosen at 100 magnifications. The number of lesions was counted within each field and scored as 0- none, 1- 1 to 5 lesions, 2- 6 to 20 lesions, and 3- \(\geq\) 21 lesions (Study II).

In study III, semi quantitative assessment of A\(\beta\) and HP\(\tau\) /IR lesions was performed. Results were reported semi quantitatively in three levels: absent, sparse or extensive/IR.

Digital analysis

The digital quantification of the A\(\beta\)/IR and HP\(\tau\)/IR load was carried out as follows; all slides with IR were scanned using the Aperio slide scanner. The Aperio image analysis positive pixel count (PCC) version 9.1 was used. The PCC was applied in the grey matter region. The percentage of grey matter
area covered with the Aβ/IR and HPτ/IR was calculated as the stained area fraction (study III).

CSF analysis
According to the protocol from the manufacturer and using a commercial ELIZA kit, the levels of Aβ42 and HPτ and the total τ in the CSF were measured (Study III).

Statistical analysis
IBM SPSS statistics was used. The correlation between the studied variables was assessed using Spearman correlation (study I, II, III, and IV). For assessment of the statistical difference between the studied groups, Mann-Whitney-U test and Kruskal-Walis H test were applied (study III, IV). Logistic regression analysis was used to assess the relation between the variables (study III). Receiver operating characteristic analysis was used to determine the HPτ/Aβ42 cut off for identifying subjects with the IR lesions (study III).
RESULTS

HP\(\tau\) in the cognitively unimpaired subjects

Thirty-three percent of the 95 cognitively unimpaired subjects with age at death ranging from 22 to 50 years displayed HP\(\tau\)/IR lesions in the cortical and the subcortical structures (study I). HP\(\tau\) pathology was seen in the LC in 28 out of the 95 cognitively unimpaired subjects. Three out of these 28 subjects displayed concomitant HP\(\tau\) pathology in the hippocampus (study I). HP\(\tau\)/IR lesions were visualized in the cortical and the subcortical structures in 98% of the 296 subjects with age at death ranging from 50 to 102 years (study IV). HP\(\tau\) pathology was seen in the LC in 95% of the total cohort (study IV). The incidence of HP\(\tau\)/IR lesions increased with age (study I, II, and IV).

PART

Fifty two percent of the subjects fulfilled the criteria for definite PART (study IV). These subjects lacked A\(\beta\) aggregates and displayed HP\(\tau\) pathology with the Braak stages ranging from (a–IV).

HP\(\tau\) in the cortical biopsies

HP\(\tau\) pathology was observed in 25% of the 111 cortical biopsies obtained from the iNPH patients (study III) (Figure 1, 2). Lower preoperative MMSE scores corresponded with higher stained area fraction of HP\(\tau\) in the cortical biopsies. HP\(\tau\) in the CSF correlated significantly with the HP\(\tau\) stained area fraction in the biopsies.

HP\(\tau\)/IR NPs in cognitively unimpaired subjects

One hundred and ninety-two cognitively unimpaired subjects were included in study II. Sixty two percent of these subjects displayed HP\(\tau\)/IR NPs in the temporal cortex.
Figure 1. Sparse hyperphosphorylated τ Immunoreactive lesions seen in a frontal cortical biopsy obtained from a subject with normal pressure hydrocephalus diagnosis (scale bar =6mm); inset magnification x20 (scale bar=100µm), note the solitary tangle with few surrounding neurites.

Figure 2. Extensive hyperphosphorylated τ immunoreactive lesions seen in a frontal cortical biopsy obtained from a subject with normal pressure hydrocephalus diagnosis (scale bar =4mm); inset magnification x20 (scale bar=100µm), note the numerous tangles and neurites.
Aβ in the cognitively unimpaired subjects

In study I, 7 out of the 95 cognitively unimpaired subjects (7%) displayed Aβ aggregates in the cortex. In study IV, the cortical Aβ aggregates were seen in 47% of the total cohort. CAA was observed in the parietal cortex in 15% of the subjects. Six percent displayed type I, and 9% displayed type II CAA (study IV).

Aβ in the cortical biopsies

Aβ aggregates were observed in 44% of the assessed 111 biopsies obtained from the iNPH patients (study III) (Figure 3, 4). Lower preoperative MMSE scores corresponded with higher stained area fraction of Aβ in the cortical biopsies. Moreover, Aβ42 in the CSF correlated significantly with the Aβ stained area fraction in the cortical biopsies.

Figure 3. Sparse β-amyloid Immunoreactive lesions seen in a frontal cortical biopsy obtained from a subject with normal pressure hydrocephalus diagnosis (scale bar =3mm); inset magnification x20 (scale bar=100µm), note the solitary dense plaque
αS in the cognitively unimpaired subjects
αS/IR lesions were visualized in 19% of the 296 cognitively unimpaired subjects in study IV.

TDP43 in the cognitively unimpaired subjects
TDP43/IR was observed in 36% of the total cohort in study IV. The most frequently affected region was the medulla.

Concomitant pathologies in the cognitively unimpaired subjects
Thirty-one out of the 95 cognitively unimpaired subjects, with age ranging from 22 to 50 years, displayed HPτ/IR, but concomitant Aβ/IR was observed only in one subject that was in Braak stage II (study I). Concomitant HPτ and Aβ/IR were seen in 46% of the 296 cognitively unimpaired subjects, with age ranging from 50–102 (study IV). Concomitant HPτ and TDP43/IR was observed in 35% of the cognitively unimpaired subjects and 19% of displayed concomitant HPτ and αS/IR. Out of the 296 subjects, 11% displayed concomitant Aβ/HPτ and αS/IR, and 15% displayed concomitant Aβ/HPτ and TDP43/IR. All four altered proteins (HPτ/Aβ/ αS /TDP43) were detected simultaneously in 5% of the total cohort.
Concomitant pathologies in the cortical biopsies
Concomitant $\alpha$S and HP$\tau$/IR were seen in 22% of the 111 biopsies obtained from the iNPH patients (study III).

Applicability of the neuropathological criteria

Braak stages of HP$\tau$ pathology
Thirty one out of the 95 cognitively unimpaired subjects, with age at death ranging from 22–50 years, displayed HP$\tau$/IR lesions in cortical and subcortical structures. Eighty percent of these subjects were in Braak stages a – b; twenty percent of the subjects were in Braak stage I–II (study I). Seventy three percent of the 192 cognitively unimpaired subjects included in study II, with age at death ranging from 55–98, were in Braak stage I – II, 13% were in Braak stage III- IV, and 1% in Braak stage V. In study IV, Ninety-five percent of the 296 cognitively unimpaired subjects, with age at death ranging from 50 to 102 years were given a Braak stage. Forty six percent were in Braak stages a – b, 34% were in Braak stage I – II, 14% were in Braak stage III- IV, and 1% in Braak stage V. A significant correlation was noted between the Braak stage of HP$\tau$ pathology and the age at death ($r = 0.34$, $p = 0.001$) (study IV).

Thal Phases of A$\beta$ pathology
Thirty Four percent of the cognitively unimpaired subjects with A$\beta$/IR lesions were in Thal phase 1, 6% were in Thal phase 2-3 and 7% were in Thal phase 4-5. A significant correlation was noted between the Thal phase and the age at death ($r = 0.32$, $p = 0.001$) (study IV).

BNE stages of $\alpha$S pathology
In study IV, Most of the subjects with $\alpha$S/IR lesions were in McKeith mid-brain/limbic stage of $\alpha$S pathology [4] i.e. (BNE stage 1 to 5) [5]. A significant correlation was noted between the age at death and the $\alpha$S/BNE stage ($r = 0.2$, $p = 0.001$).

Joseph stages of TDP43 Pathology
Twenty three percent of the subjects with AD related pathology fulfilled the criteria for Joseph stages of TDP43 pathology from stage 1 to 5. A significant correlation was noted between the age at death and the TDP43 Joseph stage ($r = 0.3$, $p = 0.001$) (study IV).

NIAA criteria
Fifteen percent of the cognitively unimpaired subjects were classified as having an intermediate level of AD pathology based on NIAA criteria, and 85% were classified as having a low level of AD pathology. Ninety four
subjects displayed HPτ/IR NPs in the temporal cortex but were classified as having a low level of AD related pathology based on NIAA criteria (study II). In study IV, 12% of the subjects were classified as having an intermediate level of AD related pathology based on the NIA-AA criteria and only one case was classified as having a high level of AD pathology.
DISCUSSION

Our results indicate that the altered proteins (HP\(\tau\), A\(\beta\), \(\alpha\)S, and TDP43) are frequently seen in the brain tissue of cognitively unimpaired aged subjects, and the incidence increases with age. This suggests that even if neurodegeneration is common, it has to be extensive enough to cause a functional disturbance. Assessment of only one type of pathology is certainly not enough, as a substantial number of subjects seem to display mixed pathologies.

HP\(\tau\) in the cognitively unimpaired subjects

\(\tau\) Protein is a microtubule associated protein which has a fundamental role in axoplasmic transport. HP\(\tau\) is the hallmark lesion of tauopathies [11, 12, 13,14]; however, HP\(\tau\)/IR lesions can also be seen in aged non-demented subjects[117,118,119,120,121,122]. The transentorhinal cortex was presumed to be the initiation site for the HP\(\tau\) pathology. The HP\(\tau\) alteration is presumed to progress in an orderly manner as was described by Braak and Braak in 1991[36], reaching the occipital cortex in the end stage. In stages V and VI, the occipital cortex is involved and this stage is usually seen in subjects with symptoms of dementia, whereas stages I to III tend to be associated with the cognitively unimpaired subjects. Braak stage IV has been reported to be associated both with demented and with cognitively unimpaired subjects.

We observed HP\(\tau\)/IR solely in LC in 28% of the cognitively unimpaired subjects lacking HP\(\tau\)/IR in transentorhinal/entorhinal cortex (study I). This observation supports the notion that the LC might be the initiation site for the HP\(\tau\) pathology, a hypothesis proposed by Braak and colleagues in 2011[37, 38]. There are a few studies that have shed light on this issue relating to the initiation site in the LC, as most neuropathologists assess the hippocampal formation for the HP\(\tau\) pathology. One study opposing the LC as being the initiation site looked at the extent of the pathology in the LC in relation to HP\(\tau\) Braak stages [153,154]. The observation of the LC being involved early on suggests that the clinical assessment strategies and the biomarkers detecting the LC malfunction should be investigated. This is particularly of importance, as any therapeutic intervention should be initiated before the development of significant cognitive impairment.

HP\(\tau\)/IR lesions were seen in the cortical and the subcortical structures in 33% of the cognitively unimpaired subjects with age at death ranging from 22–50 years (study I) and in 98% of the subjects with age at death ranging from 50 to 102 years (study IV). The methods used in both studies are similar as well as the brain regions assessed. The difference in the obtained results is explained by the difference in the age at death of the included subjects in studies I and IV and emphasizes the significant influence of age on the incidence of HP\(\tau\)/IR. The results are in line with previous reports show-
ing that the HPτ/IR was seen in 30% of cognitively unimpaired subjects with mean age at death of 53 ± 2 years [121] and in 99% of the aged subjects [37]. This observation highlights the significance of age on the incidence of the HPτ pathology in the aging brain. In line with this, in 2013, Boyle and colleagues reported an incidence of 100% HPτ pathology while studying cognitively unimpaired subjects with mean age at death of 87 ± 7 [122].

A major factor influencing the incidence of HPτ pathology is the selection bias. In study I, all subjects with trauma or brain tumors or pharmaceutical treatments that are reported to be associated with tau hyperphosphorylation were excluded [155,156,157,158]. This probably explains the significant difference in the incidence of HPτ pathology in the LC, reported by us (29%) when compared with (90%) reported by Braak and colleagues [38]. Another aspect that should always be taken into consideration is the section thickness. Braak and colleagues used 100µm thick sections; Boyle and colleagues used 20µm thick sections when compared to our routine section thickness of 7µm (study I, IV).

Fifty two percent of the subjects fulfilled the criteria for definite PART, i.e., displayed HPτ pathology (Braak stage a–IV) but lacked the Aβ aggregates (study IV). PART was defined in 2014 when the question was raised if all subjects with AD related pathology indeed develop AD, or maybe there are those who do not progress to full blown dementia [39]. Noteworthy, definite PART subjects were significantly younger when compared to subjects with AD related pathology (study IV). Thus, PART subjects might have developed AD related pathology if they had lived longer.

HPτ/IR lesions were seen in 25% of the 111 cortical biopsies obtained from the iNPH patients (study III). This percentage is in line with the percentage of 20% reported by Pyykkö and colleagues in their cohort of iNPH patients [159]. Lower preoperative MMSE scores corresponded with higher HPτ stained area fraction in the cortical biopsies (study III). These findings are in line with a previous report [160]. In iNPH patients, HPτ in the CSF correlated significantly with the HPτ stained area fraction in the cortical biopsies, indicating that the biomarkers reflect the pathology seen in the brain (study III).

Aβ in the cognitively unimpaired subjects

Aβ protein is a product of the proteolytic cleavage of the Amyloid Precursor Protein (APP) by β&γ –secretase [161]. Based on the post mortem studies [2] Aβ aggregates are initially observed in the cortex in aged subjects. Sequentially, other brain regions are involved, and five stages of the progression of the Aβ pathology are defined, i.e., involvement of the neocortex, allocortex, the diencephalic nucleus, basal forebrain, subcortical nuclei, and finally, the cerebellum. Furthermore, the Aβ deposits in the walls of the vessels in the brain, i.e., CAA. Two types have been described, Type: I in-
volvement of all the vessel types including capillaries and type II: involvement of all the vessel types except the capillaries [45].

Aβ aggregates were seen in 7% of the 95 cognitively unimpaired subjects with age at death ranging from 22–50 years (study I). In study IV, the incidence of the Aβ aggregates in the cognitively unimpaired subjects was 47%. The difference in the obtained results in study I vs. study IV is attributed to the difference in the age range of the included subjects, i.e., the age at death of the subject in study IV was ≥ 50 years. Interestingly, Braak and colleagues [37] studied a large clinically unselected cohort with age at death ranging from 0–100 years. They reported that the Aβ aggregates were seen in 44% of their study subjects; a finding in line with our results (study IV). Noteworthy, the majority of subjects in the study by Braak and colleagues had age at death > 50 years, and quite many of them displayed advanced HPt Braak stages. However, our study in principle is not fully comparable with the study carried out by Braak and colleagues.

The significance of the methods used in the assessment of the Aβ pathology is an important factor that should be taken into account. Issues that are of interest are the brain regions assessed, tissue processing, section thickness (range from 3µm to 100µm), pretreatment -both compound and time, and certainly the antibody used [150,151]. In line with this, while applying 20µm thick sections, Boyle and colleagues [122] reported that Aβ aggregates were seen in 82% of their study subjects, with mean age at death 87 ± 7. This percentage is certainly higher than 47% observed by us when assessing a cohort with mean age at death of 76 ± 1 year (study IV). Interestingly, however, the incidence of Aβ was 62% in subjects within the age range of 80–89 and 80% in subjects older than 90 years at death (study IV). Thus, the age of the cohort and not only the thickness of the sections (7µm vs. 20µm) are of significance and explain the different percentages obtained in these two studies. Furthermore, the pretreatments used for the assessment of the Aβ/IR are of importance. Already in 1998, Kraszpulski and colleagues reported that a 6 hour formic acid pretreatment significantly increases the extent of the detected Aβ/IR aggregates in the post mortem brain tissue [151]. The various pretreatments implemented explain the difference in the results obtained by Kovacs and colleagues [124]. Specifically, Aβ/IR was seen in 39% of the subjects with age at death ranging from 77 to 87 years [124], when compared to our 59% incidence of Aβ/IR in subjects with the same age range (77 to 87 years) in study IV.

Aβ/IR was observed in 44% of the cortical biopsies obtained from the 111 iNPH patients (study III), a finding in line with previous reports [146,159]. Interestingly, this observation is close to the 47% Aβ/IR observed in our post mortem study of subjects with mean age at death of 75 ± 1 year (study IV). Similar percentages have also been previously reported [37]. Noteworthy, the relatively high incidence of Aβ aggregates in the cortical biopsies obtained from the iNPH patients emphasizes the importance of follow up of the
iNPH patients to identify cases that might develop AD with time. Lower preoperative MMSE scores corresponded with a high Aβ stained area fraction in the biopsies (study III). These results are in line with a previous report [160]. Thus, assessment of the brain tissue obtained from an iNPH patient has a diagnostic value. These findings also emphasize the importance of long-term follow-up studies of the iNPH patients. Moreover, Aβ in the CSF correlated significantly with the Aβ stained area fraction in the cortical biopsies (study III). This finding shows that the CSF biomarkers reflect the brain pathology.

Fifteen percent of the 296 cognitively unimpaired subjects with age at death ranging from 50–102 years displayed CAA. Our results are in line with previous reports, as Kovacs and colleagues [124] reported 25% incidence of CAA within the age range of 77–87 years compared with 28% in our study within the same age group.

αS in the cognitively unimpaired subject

αS is a 140 amino acid protein that is present predominantly in the presynaptic nerve terminals. The protein plays a significant role in dopamine metabolism, proteasome function and has chaperon activity; however, it may form pathological insoluble fibrils [162,163]. αS is the main component in LBs, the hallmark lesion of α-synucleinopathies: PD, PDD, and DLB [58,59,60,61]. αS/IR lesions have been described to be first seen in the brain stem structures, from where it progresses to the limbic structures and finally, the neocortex [3,4]. Interestingly, recent observations indicate that αS is also seen in the peripheral organs, suggesting that this neurodegeneration is a systemic disorder [164].

αS/IR was seen in the brain in 19% of the 296 cognitively unimpaired subjects in study IV. It has been suggested that incidental αS pathology represents preclinical PD or DLB [132,133]. Thus, this group of subjects (19%) might represent preclinical stages of α synucleinopathy. αS/IR has previously been reported to be seen in 8%–31% of the cognitively unimpaired subjects [128,129,130,131]. This variability in the incidence of αS pathology can be attributed to the case selection, brain regions assessed, and most importantly, the methods used for the detection of αS/IR.

TDP43 in the cognitively unimpaired subjects

TDP43 is a protein of 414 amino acids length and is predominantly a nuclear protein. This protein plays a role in the transcriptional repression, RNA metabolism, and gene splicing [165].

TDP43/IR lesions are seen in ALS, FTLD[6,75,76], and in AD patients [8,77,78,79,80,81]. Currently, four stages of progression of TDP43 pathology have been described in ALS, involving initially the medulla and sequen-
tially involving the red nucleus, prefrontal neocortex, pre and post central neocortex and striatum, and finally involving the temporal cortex and hippocampus [7]. Furthermore, four stages of progression TDP43/IR lesions in bvFTLD were described. Stage I is characterized by the involvement of the orbital gyri, gyrus rectus, and the amygdala. In stage II, the involvement of the cingulate gyrus, temporal lobe, and the striatum, red nucleus, thalamus, and the pre-cerebellar nuclei is seen. Stage III is characterized by the sequential involvement of the motor cortex and the anterior horn of the spinal cord. In stage IV, the involvement of the visual cortex is observed [93]. Moreover, five stages of progression of TDP43 pathology were described in AD patients, initially starting in the amygdala and sequentially involving the entorhinal cortex, subiculum, occipitotemporal cortex, inferior temporal cortex, and finally reaching the frontal cortex [8].

There are few studies regarding the incidence of TDP43 pathology in cognitively unimpaired subjects. TDP43/IR was seen in 36% of the 296 cognitively unimpaired subjects with age at death ranging from 50 to 102 when the medulla, amygdala and hippocampal formation were screened. The most frequently affected region was the medulla (study IV). In line with our results, Arnold and colleagues reported an incidence of 34% for TDP43/IR in cognitively unimpaired subjects [140]. Noteworthy, they applied 40µm thick sections when compared with our 7µm sections, and they assessed the amygdala, hippocampus, and the medulla (study IV). Thin neurites that could have been seen in the 40µm thick sections can easily be missed in a section of 7µm thickness. Thus, this might indicate that the TDP43 pathology is even more common than reported by us and by Arnold and colleagues. Thus, the incidence of TDP43 is influenced by both the brain regions assessed and the methods applied.

Our findings regarding the incidence of TDP43 pathology in cognitively unimpaired subjects are also in line with the findings reported in a recent study conducted in aging research center in Japan by Uchino and colleagues [166], in this study the medulla, amygdala, and the hippocampus were assessed and the most frequently affected region was the hippocampus. Interestingly, in study IV the most frequently affected region was the medulla. This finding may suggest that the neuroanatomical distribution of TDP43/IR lesions might be genetically i.e., racially determined.

Concomitant pathologies in the cognitively unimpaired subjects

Mixed pathologies in the brain tissue in aged cognitively unimpaired subjects have been previously reported [121,122,123,124,140]. The most frequent combination observed is Aβ + HPτ. This combination is also frequently referred to as the AD related pathology. In the large cognitively unimpaired cohort, Aβ + HPτ were seen in 46% of the subjects, with age at death
ranging from 50 to 102 years (study IV). Noteworthy, only 1% of the younger subjects with age at death ranging from 22 to 50 years displayed concomitant Aβ and HP τ pathology (study I). Aβ + HP τ pathology was seen in 22% of the 111 cortical biopsy specimens (study III). This is a finding in line with what was previously reported by Pyykkö and colleagues [159]. Remarkably, a clinical follow-up study of 63 iNPH patients revealed that as many as 16% had displayed HP τ +Aβ pathology in cortical biopsies, and half of them developed AD during the follow-up time. Thus, the findings warrant a clinical follow-up of subjects with concomitant Aβ and HP τ pathology.

Nineteen percent of the cognitively unimpaired subjects displayed concomitant HP τ and αS/IR (study IV), in line to what was reported by Kovacs and colleagues [124]. Concomitant HP τ and TDP43/IR was observed in 35% of the cognitively unimpaired subjects (study IV). Arnold and colleagues [140] reported HP τ + TDP43 to be seen in only 15% of their study material. This difference is probably primarily due to the difference in the brain regions assessed by Arnold and colleagues.

Applicability of the neuropathological criteria

Braak stages of HP τ pathology
In study I, 33% of cognitively unimpaired subjects with age at death ranging from 22–50 years displayed HP τ/IR lesions, 80% of these subjects were in Braak stages a – b and 20% were in Braak stage I–II. In study IV, Forty six percent of the cognitively unimpaired subjects with age at death ranging from 50-102 years were in Braak stages a – b, and 34% were in Braak stage I – II. The difference in the obtained results might be explained by the difference in the age at death of the included subjects in study I and IV.

Thal Phases of Aβ pathology
Most of the cognitively unimpaired subjects displaying Aβ/IR were in Thal phase 1. As expected, only 7% of the total cohorts were in advanced Thal phases 4 – 5 (study IV).

BNE stages of αS pathology
Most of the subjects with αS/IR lesions were in McKeith midbrain/limbic stage of αS pathology [4] i.e. (BNE stage 1 to 5) [5]. This observation is in line with a previous study on cognitively unimpaired subjects [131].

Joseph stages of TDP43 pathology
Recently, Joseph and colleagues identified five stages of progression of TDP43 pathology in AD cases [8]. However, Joseph staging of TDP43/IR has not been reported for cognitively unimpaired subjects previously. Interestingly, twenty three percent of the cognitively unimpaired subjects with
AD related pathology in study IV were given a Joseph stage of TDP43 pathology from stage 1 to 5.

**NIAA Criteria**

In study II, fifteen percent of the cognitively unimpaired subjects were classified as having an intermediate level of AD related pathology based on NIA-AA criteria [51, 52]. In line with this, 12% of the subjects were classified as having an intermediate level of AD related pathology based on the NIA-AA criteria and only one case was classified as having a high level of AD related pathology (study IV).
Methodological Considerations

Clinical assessment

Normal cognitive function is one of the main selection criteria in our studies (I, II, and IV). Noteworthy, detailed neuropsychological tests were not carried out, and the information regarding the cognitive status of the included subjects was obtained retrospectively from the medical records. Thus, some of the included subjects might have displayed a mild degree of cognitive impairment during life; however, none of the study subjects displayed clinical symptoms of dementia based on the medical records. Furthermore, some of the cases with more severe HPτ pathology, i.e., Braak stages ≥ 4, were hospitalized for some time prior to death; thus, clinical symptoms of dementia should certainly have been reported.

Case Selection

Our selection of cases was as unbiased as possible. In all subjects that were referred for post mortem examination due to various diseases, a neuropathological assessment was also carried out. The cohort includes subjects with risk factors known to cause protein alterations in the brain i.e., confounding variables other than aging. Subjects with disorders such as cerebrovascular diseases, diabetes, certain pharmaceutical treatments, and brain trauma reported to cause deposition of altered proteins associated with neurodegeneration were not excluded [155,156,157,158,167,168]. In order to reliably assess the influence of the above-mentioned factors on the neurodegenerative protein alteration, multi-center studies on large cohorts are required.

Methods

Anatomical Regions assessed

Regarding the incidence of HPτ pathology, we assessed the LC (study I, IV). The assessment of this region was recommended in 2011. Thus, this region has not been frequently assessed. Furthermore, we assessed TDP43/IR in the medulla, also a region that has not been frequently assessed in cognitively unimpaired subjects. A significant influence of the region assessed regarding incidence was noted.

Thickness of the sections

In the current study, we used 7µm thick sections, whereas the assessment of protein alteration has also been carried out on sections with a thickness of 20, 40, and even 100 µm. The section thickness certainly influences the obtained results.
Staining methods and tissue processing
We applied the IHC technique on formalin fixed paraffin embedded tissue. It has previously been reported that many factors should be taken into consideration, as they are known to alter the staining outcome. Issues of major importance include post mortem delay, fixation time, storage time of sections to be stained, pretreatments, and the choice of antibody used [147,148,149,150,151,152]. All methods that were applied have been shown by the Brain Net Europe to lead to reliable results, even in an inter laboratory setting.
Conclusion

- We demonstrated the early involvement of LC with the HP\(\tau\) pathology in a relatively large cohort of cognitively unimpaired mid aged subjects (study I). The early involvement of the LC region with the HP\(\tau\)/IR might be reflected in the clinical presentation of the affected subjects. Development of clinical assessment techniques and radiological investigations that reflect early functional alterations in the LC might assist in identifying subjects with early stages of neurodegeneration; thus, early therapeutic intervention could be applied prior to the occurrence of significant cognitive impairment.

- The recently updated guidelines by the National Institute on Aging – Alzheimer’s Association (NIA-AA) [51, 52] for the assessment of AD related neuropathological changes were indeed applicable in the cognitively unimpaired subjects (study II). Noteworthy, however a substantial number of subjects with HP\(\tau\)/IR NPs in the temporal cortex were classified as subjects with a low level of AD related changes and were not identified as a risk group (study II). Our recommendation is to assess the HP\(\tau\)/IR NPs in the temporal cortex in order to identify all subjects at risk.

- We showed that the AD CSF biomarkers and the MMSE scores reflect AD related pathology seen in the cortical biopsies obtained from the iNPH patients (study III). Twenty-two percent of the 111 iNPH patients included in study III displayed AD related pathology in the cortical biopsies. Thus, long-term follow-up of subjects with A\(\beta\) and HP\(\tau\) pathology in the cortical biopsies is warranted. Furthermore, subjects displaying A\(\beta\) and/or HP\(\tau\)/IR in the biopsies could be selected for inclusion in future pharmaceutical intervention trials toward these protein alterations.

- We demonstrated a relatively high incidence of neurodegenerative proteinopathies in the aged cognitively unimpaired subjects (IV). Interestingly, a substantial number of subjects fulfilled the criteria for definite PART. Another large group of subjects displayed AD related pathology. Whether PART subjects represent a different entity when compared to subjects with AD related pathology should be further investigated. The high incidence of TDP43 was a surprise, particularly, the frequent involvement of the medulla. The involvement of the medulla should be further studied in cognitively unimpaired subjects.
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