Alzheimer's disease, Aging and Centenarians

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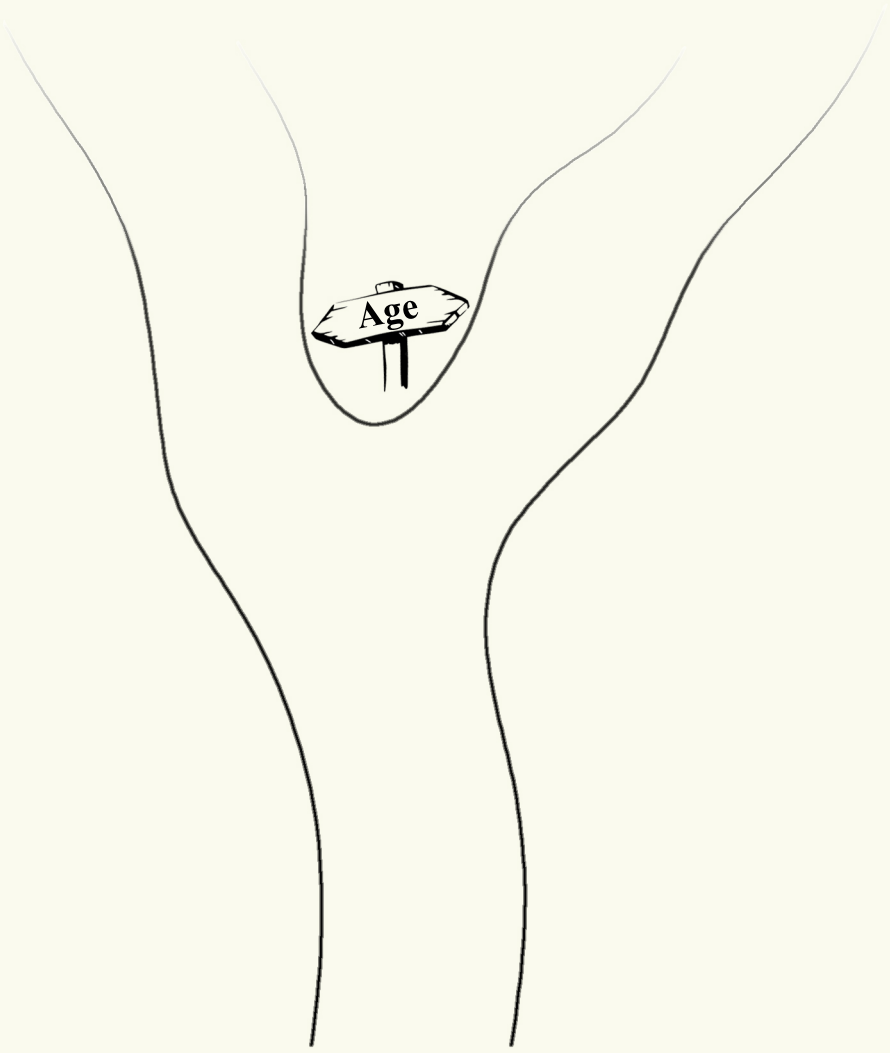

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Meng Zhang

ALZHEIMER'S DISEASE, AGING AND CENTENARIANS

ALZHEIMER'S DISEASE, AGING AND CENTENARIANS

Dissertation

for the purpose of obtaining the degree of doctor
at Delft University of Technology
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chair of the Board for Doctorates
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To the very best time.

CONTENTS

1

INTRODUCTION

有笔头千字，胸中万卷；致君尧舜，此事何难?

– 苏轼

1

Aging changes the brain structurally and functionally, promoting and accelerating cognitive impairments and susceptibility to neurodegenerative disorders even in healthy adults.[1–3] Indeed, aging is the major risk factor for most common neurodegenerative diseases, including Alzheimer's disease (AD).[4, 5] Given that the populations around the world reach higher ages (Figure 1.1A), it becomes imperative to identify approaches to halt or even reverse the cognitive decline by protecting against, or even counteracting, the effects of brain aging. In fact, brain aging and AD have similarities in many aspects, such as genomic instability, epigenetic alterations, loss of proteostasis, mitochondrial dysfunction, altered intercellular communication and neuroinflammation.[6] Therefore, we believe there is a continuum from brain aging to AD, susceptible to genetic and environmental influences.[7] With age, the average level of AD neuropathology in the post-mortem brain increases even in healthy populations[8–11] leading to an enormous number of individuals in old age who show signs of AD neuropathology and cognitive decline. However, there are exceptions, centenarians who have accumulated no/low levels of AD neuropathology or high levels of AD neuropathology but are still cognitively healthy,[12–14] indicating that AD caused cognitive decline is not inevitable. Such that, studying these centenarians should be a high priority.

1.1. EPIDEMIOLOGY, HYPOTHESES AND RISK FACTORS FOR ALZHEIMER'S DISEASE

AD is a progressive neurodegenerative disease most often associated with memory deficits and cognitive decline, ultimately leading to death.[15] In the aged population, AD is currently one of the most prevalent diseases, bringing a huge burden to patients, their relatives and society.[16, 17] The incidence of AD increases exponentially with age (Figure 1.1B): while the disease is rare before age 65 (early-onset AD), the more common type of the disease, late-onset AD, reaches an incidence of 40% per year at 100 years old.[5]

Currently, there are no successful treatments that are capable of preventing, slowing or reversing the course of AD due to our poor understanding of the disease.[18] Several competing hypotheses attempt to explain the underlying cause, but for most AD cases, the cause is still unknown.[19] The oldest hypothesis, reported in the 1970s, is the cholinergic hypothesis (Figure 1.2A), which proposed the cause of AD to be the reduced synthesis of the neurotransmitter acetylcholine.[19] The loss of cholinergic neurons was found in AD to cause alteration in cognitive function and memory loss.[20] In 1991, the amyloid cascade hypothesis postulated that extracellular amyloid beta (Aβ) deposits are the fundamental cause of the disease.[21] The amyloid cascade hypothesis (Figure 1.2B) argues that a dysregulated proteolytic cleavage of amyloid precursor protein (APP) processing occurs early in the disease process, resulting in increased deposition of the longer amyloid peptide $A\beta_{1-42}$, which forms the core of a plaque. These $A\beta_{1-42}$ plaques accumulate and then induce all the subsequent pathology, including tau aggregation, phosphorylation, neuronal attrition, and clinical dementia.[21] Contrary to the amyloid cascade hypothesis, the tau hypothesis (Figure 1.2C) proposed that abnormal tau proteins initiate the disease cascade.[22] In this model, hyperphosphorylated tau begins to pair with other fibers of tau as paired helical filaments. Eventually, they form neurofibrillary tan-

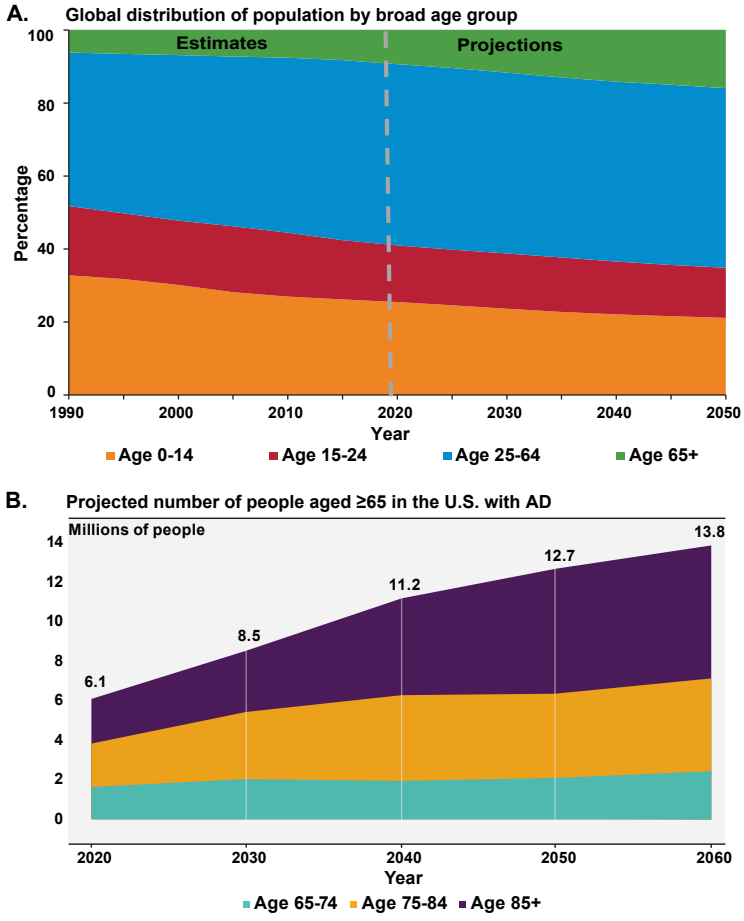

Figure 1.1: Aging society and the prevalence of Alzheimer's disease (AD). A. Global distribution of population by broad age group (1990-2050). **B.** Projected number of people age 65 and older (total and by Age) in the U.S. population with AD (2020-2060). The prevalence of AD increases exponentially due to the aging population and poses a huge burden on patients, their relatives, and society. Figure sources: World Population Ageing 2019; Alzheimer's Disease Facts and Figures 2022.

gles (NFTs) inside nerve cells.[23] During this process, the microtubules disintegrate, destroying the structure of the cell cytoskeleton, which collapses the neuron's transport system, eventually leading to apoptosis.[24] A relatively recent hypothesis is about neuroinflammation (Figure 1.2D), which argues that all aggregated misfolded proteins that are involved in neurodegenerative diseases induce oxidative stress.[25, 26] This oxidative stress leads to neuroinflammation and, in conjunction with amyloid and hyperphosphorylated tau proteins, launches and exacerbates AD.

Despite the uncertainty in the cause, there are some known risk factors for AD (Figure

1.2E). Chronological age stands out as by far the riskiest. About one-third of individuals aged 85 years and older suffer from AD dementia; the vast majority of patients are 75 years or older.[7] Accumulation of DNA damages is a well-established aging factor.[27] In this regard, increased studies reveal DNA damage as a critical risk factor for AD.[28–30] Reactive oxygen species (ROS) may be the major source of this DNA damage.[31] Next to aging, genetics and family history play an important role. Twin studies suggested that the heritability of the common form of AD ranges between 60-80%.[32] Of the many genes that influence overall risk for late-onset sporadic AD, the *APOE* genotype, which was identified in the early 1990s through linkage studies, has a greater effect than all of the others.[33–35] The *APOE* gene encodes the apolipoprotein E protein, which facilitates the transfer of cholesterol and phospholipids between cells in the brain. It is produced predominantly by astrocytes and to some extent microglia.[36] There are three *APOE* allelic isoforms: ε3, ε4, and ε2, in decreasing order of population prevalence. Carriers of the ε4 allele exhibit an increased AD risk: heterozygotes and homozygotes have 2–3 fold and 12 fold increased risk relative to noncarriers, respectively.[37] Carriers of the ε2 allele have a reduced risk relative to the most common ε3 homozygotes.[38] Apart from these uncontrollable genetic factors, some lifestyle-associated factors can also increase the risk of AD. They include the presence of vascular risk factors and insulin-resistant diabetes,[39] repeated concussions, or other forms of head trauma. A sedentary lifestyle may increase the incidence of AD,[40] whereas regular physical exercise is protective.[41] Notably, high education could also be protective. Individuals who are highly educated, or who may have experienced greater cognitive stimulation in life, appear to be protected against cognitive decline, even though they may show accumulation of AD-associated neuropathological substrates at autopsy.[14] Such findings have given room to the hypothesis of cognitive reserve or the buildup of resilience against the cognitively harmful effects of brain damage.[42–44]

1.2. HEALTHY AND UNHEALTHY BRAIN AGING

Cognitive changes can manifest with age in the absence of any disease.[45] While many researchers have focused on aging-related diseases, there are few targeted studies on the molecular biology of the aging brain in the absence of neurodegenerative diseases, neuropsychological dysfunctions or cerebral tumors of healthy older individuals. The age-associated decline in some cognitive functions can be mild, such as verbal ability, orientation, and general knowledge,[46–48] but other cognitive capabilities decline from middle ages or even earlier.[49, 50] In the middle ages, cognitive processing speed, fluid intelligence, and episodic memory decline gradually. Older individuals exhibit changes in attention, working memory, and distractibility compare to their younger counterparts.[51] However, cognitive changes attributed to age alone are rarely severe enough to impact daily life or be clinically diagnosed as mild cognitive impairment (MCI) or dementia. Also, purely age-associated cognitive decline is very slow, with changes that are perceptible only after decades.[52]

While it may seem like healthy brain aging is a slow version of AD, there are distinct processes that differentiate them. Altered gene expression regulates neural signaling during aging; for example, NMDA receptors, which are calcium-permeable ion channels that

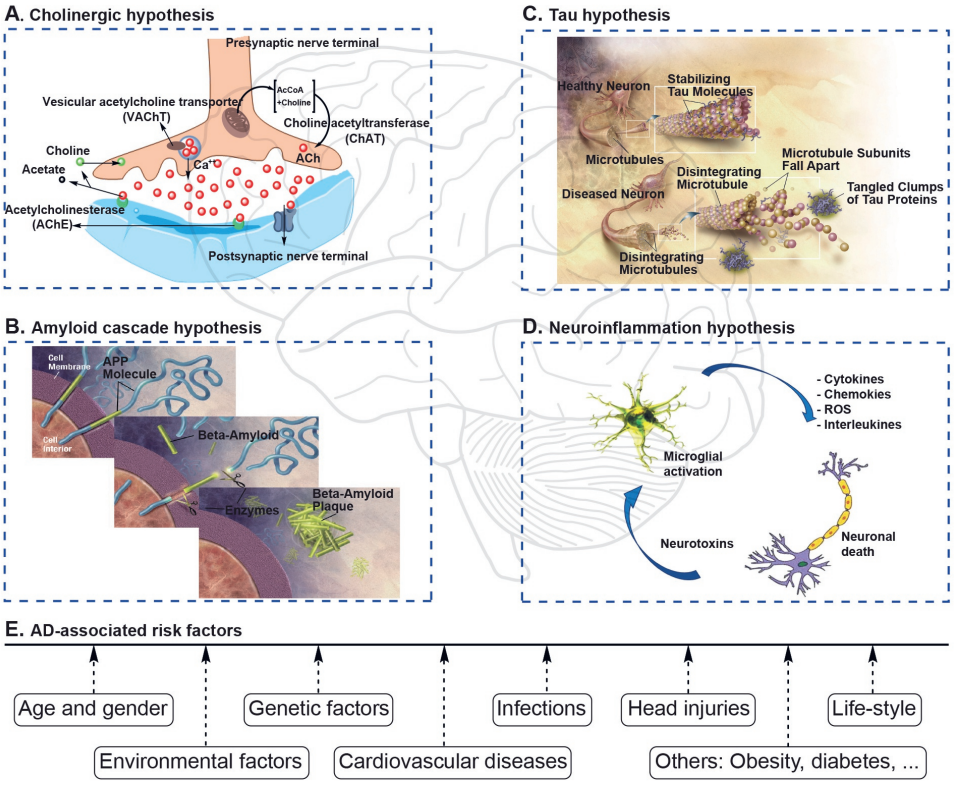

Figure 1.2: Hypotheses and risk factors for Alzheimer's disease (AD). Hypotheses have been raised to explain the cause of AD decades ago, including **A.** the cholinergic hypothesis, **B.** the amyloid cascade hypothesis, **C.** the tau hypothesis, and **D.** the neuroinflammation hypothesis. **E.** Although the cause of most AD cases is still mostly unknown, many factors have been statistically proven to increase the AD risk, which includes age and gender, genetic factors, cardiovascular diseases, lifestyle, etc. Figure sources: Zeinab Breijyeh, et al., Comprehensive Review on Alzheimer's Disease: Causes and Treatment; National institute on aging; ADEAR: "Alzheimer's Disease Education and Referral Center, a service of the National Institute on Aging."; and Wei-Wei Chen, et al., Role of neuroinflammation in neurodegenerative diseases (Review).

play an important role in the induction of synaptic plasticity, exhibit age-related changes in subunit complexes that affect how new experiences shape synaptic strength.[53, 54] However, those distinct processes between nonpathological and pathological cognitive decline are not immediately apparent. A major challenge for this field is that molecular changes associated with AD, as well as other neurodegenerative diseases begin years, even decades before their clinical symptoms appear.[55, 56] When symptoms do occur, they can also be subtle and not easily distinguished from the typical manifestations of healthy aging. Even at a molecular level, distinguishing healthy aging from disease is not straightforward. Ten to thirty percent of cognitively normal elderly (≥ 60) exhibit signs of Aβ deposition in their brain on amyloid PET imaging.[57] In addition, an analysis of

post-mortem brains indicated that 30-40% of elderly individuals aged 79 or above accumulated significant levels of AD-related neuropathology in their brain while only a small subset of individuals (15%) was clinically diagnosed with AD.[58, 59] Similarly, as many as 60% of older adults in their 60s exhibit at least the first stage of tau protein accumulation in their brains at autopsy,[60] far exceeding the population who experience actual cognitive decline. However, Aβ and tau are not the only substrates that can self-aggregate in the brains of older individuals. Lewy bodies, the intracytoplasmic neuronal accumulations of α-synuclein protein associated with Parkinson's disease, frequently accumulate in the brains of cognitively healthy elderly.[61] The same story is also observed for Tar-DNA binding protein 43 (TDP-43) in combination with hippocampal sclerosis, a recently recognized disease entity called LATE.[62, 63] What confuses researchers, even more, is that mixed-type proteinopathies are observed to be a frequent event in the brains of symptomatic and asymptomatic individuals, particularly in aged people.[64–66]

It is hard to know among a group of individuals manifesting the sorts of similar, age-associated cognitive changes who are going on to a healthy course and who are actually experiencing the first symptoms of neurodegenerative disease, like AD (Figure 1.3). It is also very difficult to know to what extent abnormal protein accumulation increases the risk of future cognitive decline. In fact, the definition of normality is also open to debate. However, the extremely rare exceptions, cognitively healthy centenarians, may give an answer.

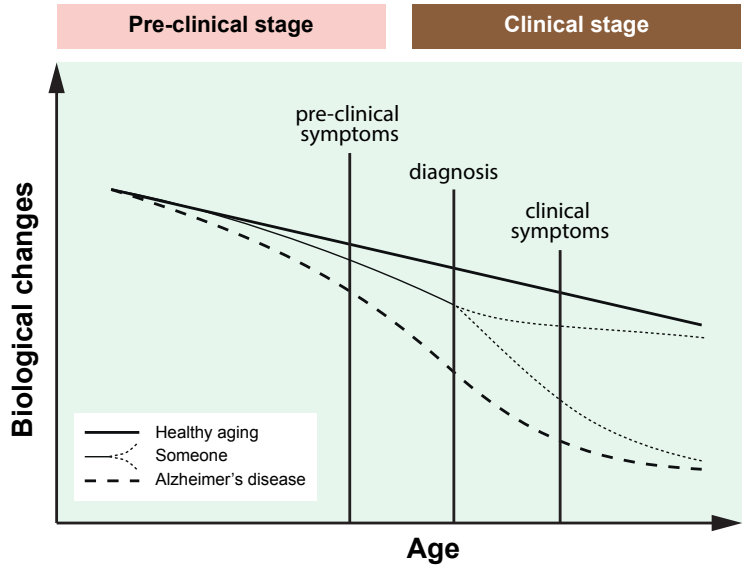

Figure 1.3: Schematic representation of the biological changes during the progressions of brain healthy aging versus AD. An individual with age-associated cognitive decline can have an uncertain cognitive fate, a benign course, or neurodegenerative dementia.

1.3. MULTIPLE-LAYER COMPARISON BETWEEN HEALTHY BRAIN AGING AND AD

Given the subtle mechanistic differences between benign aging-associated and disease-associated processes, disentangling the two is a stiflingly complex task. Comparisons between healthy brain aging and AD from multiple molecular biological layers, including but not limited to neuropathological substrates, brain proteomics, and brain somatic mutations, may be necessary. The observed differences in the aging and AD brain will also provide additional insight into the mechanisms underlying AD.

1.3.1. NEUROPATHOLOGICAL SUBSTRATES

As an archetype neurodegenerative disease, only the combined presence and accumulation of Aβ plaques and NFTs allows for a post-mortem clinical diagnosis of AD. However, as we have learned, these neuropathological substrates can also accumulate with age in the brains of cognitively healthy individuals. As such, the association between the accumulation of neuropathological substrates and cognitive decline attenuates.[8, 9] In line with this, we and others previously observed that the levels of AD-associated neuropathological substrates are highly variable in nonagenarians and centenarians with diverse cognitive performance.[10, 13, 67, 68] Trying to give an explanation, studies have indicated that a considerable fraction of amyloid deposits observed in the elderly are diffuse plaques (DPs)[13, 59], which may be primarily a benign consequence of aging. Investigating the inter-correlation between different neuropathological substrates and the correlation between each of the neuropathological substrates with cognitive performance in the context of age-continuum can help us understand which neuropathological substrates are more likely to contribute to cognitive decline in an age-independent manner. Focusing on these neuropathological substrates and investigating potential resilience and resistance to the buildup of these neuropathological substrates in centenarians will benefit the design of diagnostic strategies and treatments.

1.3.2. BRAIN PROTEOMICS

In addition to cell type- and tissue-specific regulation of protein expression, the equilibrium between synthesis and degradation determines the levels of a given protein, but many other regulatory pathways contribute to this process, including post translational modifications, protein folding, polymerization/aggregation, tagging for degradation, etc.[69] Changes in any of these aspects have been implicated in aging- and disease-related loss of protein homeostasis and would be expected to have particularly drastic consequences in tissue with largely post-mitotic cells. In the aged and diseased brain, as these homeostatic mechanisms fail, damaged proteins accumulate, leading to both loss-of-function and gain-of-function toxicity, and contribute to neuropathology.[70] Indeed, AD is characterized by the accumulation of pathogenic proteins, including amyloid precursor protein (APP), whose 40–42 amino acid fragments deposit as Aβ plaques,[71] and hyperphosphorylated microtubule-associated protein tau in NFTs. Besides, altered intercellular communication between cells of the same type, the same tissue, or across tissues at the protein level is another biological hallmark of brain aging and AD. Large-scaled brain proteomic analysis will help distinguish the proteins regulated over the

courses of aging or/and AD, and characterize these changed communications, which will further contribute to understanding common/unique molecular mechanisms. Next, investigating the specificities of these proteins in centenarians may point to therapies that can slow down or even reverse the processes of aging and AD.

1.3.3. BRAIN SOMATIC MUTATIONS

The accumulation of brain somatic mutations is another aspect of aging and AD.[30, 70] Aging results in the accumulation of damage in nuclear and mitochondrial DNA, and interference with DNA repair processes can accelerate aging.[72, 73] While glial and endothelial cells in the brain are able to divide, the vast majority of neurons do not renew, and some may live for more than a century.[74] Consequently, they need to be able to tolerate stressors and employ repair mechanisms to fix damages. With age, reactive oxygen and nitrogen species, replication errors, and other mechanisms can lead to single base mutation and single or double-strand DNA breaks in neurons.[75] Pathways which increase mutation or decrease repair could conceivably contribute to both brain aging and AD.[28, 76] Also, a recent study identified several putative pathogenic brain somatic mutations enriched in genes that are involved in hyperphosphorylation of tau.[77] These results indicate that the aggregation of these neuropathological substrates can be partly explained by the accumulation of brain somatic mutations, which raises a new hypothesis for investigating the pathogenic mechanism of AD. Investigating brain somatic mutations from a single cell level can further disentangle the processes that are involved in brain aging and AD separately or commonly.

Biological hallmarks that are involved in both brain aging and AD are not exclusively in these three layers; others like stem cell exhaustion, epigenetic alterations, mitochondrial dysfunction, and transcriptomic and genomic abnormalities are also of interest, but we will not include them in this thesis.

1.4. COGNITIVELY HEALTHY CENTENARIANS

During the last century, human life expectancy, on average, has shown an extraordinarily linear increase,[78] and by 2050 there will be 3.2 million centenarians in the world.[79] However, a consequence of an aged population is the increased prevalence of age-related diseases: an increasing fraction of older individuals who will spend part of their old age in disability or dependence on others.[80] Under this pressure, policy and research are increasingly focused on the potential of increasing healthy life or health span instead of life expectancy in recent decades.[81] In fact, there is an extremely rare population (<0.1%) reached at least 100 years of age while maintaining a high level of cognitive and physical performance, so-called cognitively healthy centenarians.[12, 82] Neurodegenerative diseases represented by AD are mostly age-dependent, leading to a blurring of the line between them and healthy aging. Healthy aging itself, as an entity, is also hard to define.[83] However, cognitively healthy centenarians are examples of healthy aging. By using these extreme controls, we can maximize the difference between neurodegenerative diseases and healthy aging processes to help us find the molecular nuances between them. Observing centenarians in the context of aging can also help us answer

when plaques, tangles, Lewy bodies, and TDP-43 accumulations are simply benign processes in an aging brain, and at what point do they become significant enough to be responsible for cognitive declines. Not surprisingly, cognitively healthy centenarians are themselves a treasure trove of research on protective genetic factors.

Thanks to the 100-plus Study: a prospective cohort study of Dutch centenarians who self-reported being cognitively healthy, as confirmed by a proxy,[12] we have a powerful weapon against age-related neurodegenerative diseases.

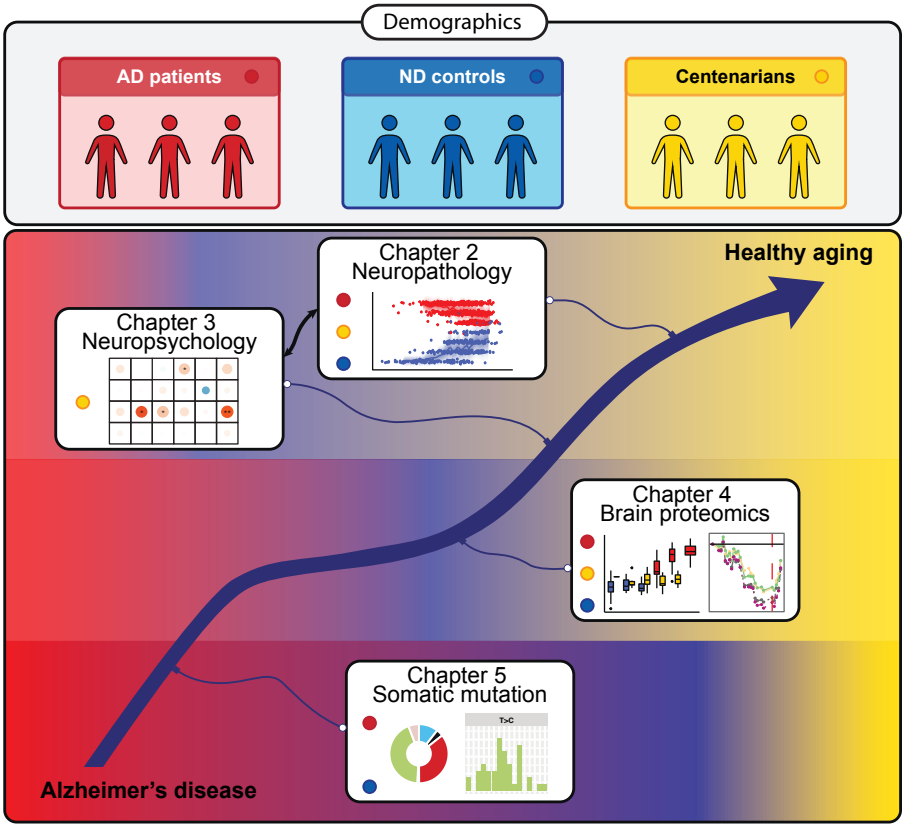

Figure 1.4: Multiple layer understanding of the interactions between brain healthy aging and AD. All chapters in this thesis are based on the analyses of different combinations of three distinct populations: (i) a sample of AD patients from the Netherlands Brain Bank (NBB) and Religious Orders Study/Memory and Aging Project (ROSMAP) cohorts, (ii) a sample of non-demented individuals from the NBB and ROSMAP cohorts, and (iii) a sample of cognitively healthy centenarians from the 100-plus Study cohort. This thesis will focus on neuropathology (chapter 2) and its correlation with neuropsychology (chapter 3), brain proteomics (chapter 4), and brain somatic mutations (chapter 5).

1.5. AIMS OF THIS THESIS AND OUTLINE

The overall objective of this thesis is to investigate centenarians as extreme controls in the context of aging and AD, and to explore the role of aging behind AD (Figure 1.4). Centered around the interaction between aging and AD, we set up our study from the perspectives of neuropathology (chapter 2) and its correlation with neuropsychology (chapter 3), brain proteomics (chapter 4), and brain somatic mutations (chapter 5). The structure of the thesis and the contributions of each chapter are as follows:

In chapter 2, we investigated AD-associated neuropathological substrates in the context of AD and aging by constructing an age-continuum from 16 to 100+ years including AD cases, non-demented controls, and non-AD demented individuals. Then, we correlated the levels of neuropathological substrates observed in the brains of centenarians with the Mini Mental State Examination (MMSE), and compared the distributions of each neuropathological substrate in centenarians and the age-continuum. We observed brains from non-demented individuals reached NIA Amyloid stage and Braak NFT stages as observed in AD patients, while CERAD NP scores remained lower. In centenarians, we observed no correlation between each neuropathological substrate and MMSE, and NIA Amyloid stages varied, Braak NFT stages and CERAD NP scores rarely exceeded certain levels. In chapter 3, we investigated the correlation between neuropathology and neuropsychology in centenarian brains using the imputed neuropathological and neuropsychological levels, composite domain and global scores, and MMSE scores. By performing factor analysis, we also assessed the neuropathology and neuropsychology correlation based on the latent factors. From the mixed pathologies, we only observed tau-associated and LATE-associated pathologies correlated with certain neuropsychological tests and cognitive domains.

In chapter 4, we turned our attention to the proteomic level. In this chapter, we investigated the proteins that regulated over Braak stages and/or ages in the post-mortem brains of AD and ND individuals. Based on the Braak stage- and/or age-correlated proteins, we identified the proteins that showed specific abundance in centenarians in terms of Braak stage and age. We identified 472 proteins that regulated with Braak stages and involved in multiple biological processes including mitochondrial, synapse, extracellular matrix, intermediate filament, epithelial cell differentiation, and glucose catabolic process. Next, we identified 174 age-correlated proteins that involved in intermediate filament, ensheathment, oligodendrocyte, and ribosome assembly biological processes. Based on these proteins, we observed that the abundance of 64 proteins was significantly different in the centenarian group compared to the AD group at Braak stage IV, and 108 proteins from diverse functions that primarily resembled the protein-levels observed at younger ages.

In chapter 5, we compared the AD and aging processes at the level of somatic mutations. By building a somatic mutation detection pipeline that contrasts genotypes derived from whole genome sequencing (WGS) data with genotypes derived from single cell RNA sequencing (scRNA-seq) data, we identified 196 putative neuronal somatic mutations and showed that the single base substitution (SBS) pattern of these putative somatic mutations is clock-like. By investigating how these somatic mutations are associated with age and AD, we identified, among others, that the RBFOX1 and KCNIP4 genes are enriched for more somatic mutations with increasing age, and the KCNQ5 and DCLK1 genes have

a higher odds ratio to accumulate somatic mutations in AD patients. Furthermore, we observed both age- and AD-specific somatic mutations presented in the K_+ channels-associated genes.
Finally, we conclude the thesis with a discussion of our contributions, limitations and future perspectives.

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2

RESILIENCE AND RESISTANCE TO THE ACCUMULATION OF AMYLOID PLAQUES AND NEUROFIBRILLARY TANGLES IN CENTENARIANS: AN AGE-CONTINUOUS PERSPECTIVE

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With increasing age, neuropathology associated with Alzheimer's disease (AD) accumulates in brains of cognitively healthy individuals: are they resilient or resistant against AD-associated neuropathologies? In 85 centenarian brains, we correlated NIA Amyloid stages, Braak-NFT stages and CERAD-NP scores with cognitive performance close to death as determined by MMSE. We assessed centenarian brains in context of 2,131 brains from AD patients, non-AD demented and non-demented individuals in an age continuum ranging 16-100+ years. With age, brains from non-demented individuals reached NIA-Amyloid and Braak-NFT stages as observed in AD patients, while CERAD-NP scores remained lower. In centenarians, NIA-Amyloid stages varied (22.4% had the highest stage 3), Braak-NFT stages rarely exceeded IV (5.9% had stage V), and CERAD-NP scores rarely exceeded 2 (4.7% had score 3); within these distributions, we observed no correlation with MMSE (NIA-Amyloid: P=.60; Braak-NFT: P=.08; CERAD-NP: P=.16). Cognitive health can be maintained despite the accumulation of high levels of AD-related neuropathological substrates.

2.1. BACKGROUND

Cognitive decline due to Alzheimer's disease (AD) is associated with the loss of neuronal synapses and dendrites which coincides with the extracellular accumulation of amyloid beta (Aβ) plaques and the intracellular aggregation of phosphorylated tau protein into neurofibrillary tangles (NFTs).[1] Whether the accumulation of these neuropathological hallmarks of AD is causative for the loss of neuronal synapses and dendrites is a matter of strong debate[2], especially since in vivo clearance of plaques and tangles by immunotherapies does not, at current, attenuate the progression of cognitive decline as hoped for[3–5]. Furthermore, the levels of these neuropathological substrates increase with age in the post-mortem brains of cognitively healthy individuals.[6–9] In fact, a large autopsy study found that 30-40% of the brains from 79-year-olds harbor significant AD-associated neuropathological changes, while only 15% of these elderly were clinically diagnosed with AD.[10, 11] In line with this, we and others previously observed that the levels of these neuropathological substrates are highly variable in nonagenarians and centenarians with diverse cognitive performance.[8, 12–16] This variability in the level of cognitive performance and the level of neuropathological substrates represents a window of opportunity to investigate whether maintaining cognitive performance during aging depends on being tolerant to the effects of accumulated AD-associated neuropathological substrates (resilience) or whether it depends on avoiding the build-up of AD-associated neuropathological substrates (resistance).

Here, we correlated the levels of neuropathological substrates observed in the brains of 85 centenarians with cognitive performance determined close to death by the Mini Mental State Examination (MMSE). Next, we compared these levels of AD-associated neuropathological substrates with those observed in the brains of 2,131 individuals, representing an age-continuum from 16 to 100+ years (851 AD cases, 654 non-demented controls, and 626 non-AD demented individuals). This allowed us to determine (1) to what extent the levels of neuropathological substrates change with age; (2) the effect of age on the potential of each AD-associated pathological substrate to distinguish between AD and cognitive health; and (3) how the inter-correlation between the levels of different

pathological substrates changes with increasing age.

2.2. METHODS

2.2.1. 100-PLUS STUDY COHORT

We included brains donated by 85 centenarians (ages at death: 100-111) who died between 2013 and 2021 and self-reported to be cognitively healthy at inclusion in the 100-plus Study cohort[17], confirmed by a proxy. For each participant, cognitive performance was assessed during a baseline visit and yearly follow-up visits. In this study, the MMSE score, an 11-item cognitive screen test with a maximum score of 30 points, from the last available visit was used to indicate the cognitive performance of each donor before death.[12, 17] Scores were imputed for missing values when <6 of the 30 points could not be scored due to sensory deficits such as hearing and vision impairment and/or general fatigue[12], otherwise MMSE was set to "missing".

2.2.2. NETHERLANDS BRAIN BANK (NBB) COHORT

Neuropathology data was obtained from 2,131 individuals, including AD cases (AD), non-demented individuals (ND) or non-AD-demented individuals (non-AD), who agreed to brain donation to the Netherlands Brain Bank (NBB, www.brainbank.nl) between 1979 and 2018. These brains formed an age-continuum from 16 to 103 years old. The diagnosis of AD is based on combination of clinical criteria of probable AD[18, 19] and histopathological confirmation by autopsy.

2.2.3. NEUROPATHOLOGICAL ASSESSMENT

Autopsies and neuropathological assessments for the NBB cohort and the 100-plus Study cohort were performed by the NBB, as described in the Supplementary material. We evaluated all donated brains according to the following criteria: (1) Aβ plaque level using the National Institute on Aging [NIA] amyloid stages;[1] (2) NFT level using Braak stages;[20–22] (3) the level of neuritic plaques [NPs], a subtype of plaque surrounded by dystrophic neurites, using the Consortium to Establish a Registry for Alzheimer's Disease [CERAD] scores;[23] and (4) the brain weight, corrected for sex.[24] Regarding the rationale for using the NIA amyloid stages, see the Supplementary material. The centenarian brains and majority of the brains in the age-continuum were evaluated by a single neuropathologist, such that interrater variability was kept to a minimum.

2.2.4. AD VS ND COMPARISON ACROSS AGE-CONTINUUM

To assess the age-related changes in the levels of AD-associated neuropathological substrates, we applied a dynamic 25-point sliding window across the ages of AD cases and ND individuals from the NBB separately. Neuropathology levels in the AD and ND brains were sorted according to age-at-death. For each neuropathological substrate, the mean level of each 25-point window was calculated. Each window encompassed a set of 12 cases with ages lower and 12 cases with ages higher than the age of the central case. Across each window-set, we calculated a confidence interval (CI) with 5% increments to

indicate the distribution of pathology levels (i.e. 5%, 10%, ..., 90%, 95% CI). Next, for each sliding window position, we calculated the difference in the average neuropathological levels between AD cases and ND individuals. The CI of the difference with 5% increments was determined by bootstrapping (n=1000).

2.2.5. DISTRIBUTION OF NEUROPATHOLOGY LEVELS BY AGE INTERVAL

The distributions of the level of each neuropathological substrate in AD cases, ND controls, and non-AD individuals were estimated separately for each age interval (i.e., <60, 60 to 69, 70 to 79, 80 to 89, ≥ 90) and visualized by generating density plots using a Gaussian kernel. Next, the overall distribution of the level of each neuropathological substrate in the NBB cohort for each age interval was estimated by summation of the densities of AD cases, ND controls, and non-AD individuals across neuropathological levels.

2.2.6. PAIRWISE CORRELATION BETWEEN DIFFERENT NEUROPATHOLOGY LEVELS

To evaluate the pairwise correlations between (1) NIA Amyloid stage and Braak-NFT stage, (2) NIA Amyloid stage and CERAD-NP score, and (3) Braak-NFT stage and CERAD-NP score with age, we merged the AD cases, non-demented controls and non-AD demented individuals as one cohort. We used a 51-point sliding window, which was constructed in the same way as the 25-point window, but using 25 cases with ages lower and 25 cases with ages higher than the age of the central brain sample. For each neuropathology pair and sliding window position, we calculated the Pearson correlation coefficient and corresponding CIs (5% increments).

2.2.7. STATISTICAL ANALYSES

We applied a linear regression model to test the association between each neuropathological substrate and MMSE in the centenarian cohort. All regressions were corrected for sex, education, and time between last acquired MMSE and death. All calculations were performed using R (version 3.6.3). Pearson correlation, and linear regression were performed using the "stats" R package.[25]

2.3. RESULTS

2.3.1. SAMPLE CHARACTERISTICS

For the 85 centenarian brain donors (74% female) distributions of age, sex, educational attainment, cognitive performance, *APOE* genotype and neuropathological assessments are shown in Table 2.1. At last available study visit, a median of 9 months (IQR: 4-13) before brain donation, the median MMSE score across all centenarians was 25 (IQR: 22-27). Of the 83 centenarians with *APOE* genotype available, seven carried one copy of the *APOE* ε4 allele, which did not correlate with the level of neuropathology (Figure 2.1, Table S2.1). Education correlated significantly with MMSE (Table S2). The levels of each neuropathological substrate did not correlate with age-at-death while corrected

for sex and education (Table S3). Based on clinical data that was provided upon autopsy and observed post-mortem neuropathology, the 2,131 NBB brain donors (56% female) were diagnosed as AD cases (AD; n=851, aged 37-102), non-demented controls (ND; n=654, aged 16-103), and individuals with non-AD dementia (non-AD; n=626, aged 16-103) (Table 2.2). Patients with non-AD dementia died with or from diverse dementia subtypes and age-related pathology: Frontotemporal dementia (FTD, 35.3%), NFT-predominant dementia (26.0%), Parkinson's disease (18.4%), Vascular dementia (15.2%), or other (5.1%), see Table S4.

Table 2.1: Characteristics of the centenarians in the 100-plus Study cohort

	100-plus Study cohort	
	n	minimum, median (IQR), maximum
Clinical demographics		
Age [y]	85	100.4, 103.2 (102.3-104.6), 111.8
Female/male	63/22	—
APOE genotype	83	E2/E2: 2, E2/E3: 15, E2/E4: 2, E3/E3: 59, E3/E4: 5
Education	85	0, 3 (1-4), 6
MMSE	85	9.4, 25 (22-27), 30
Neuropathological substrates		
NIA amyloid stage	85	2 (1-2); 0: 9.4%, 1: 35.3%, 2: 32.9%, 3: 22.4%
Braak-NFT stage	85	3 (3-4); I: 2.4%, II: 14.1%, III: 42.4%, IV: 35.3%, V: 5.9%
CERAD-NP score	85	1 (0-2); 0: 43.5%, 1: 29.4%, 2: 22.4%, 3: 4.7%
Brain weight [gr]	F: 63; M: 22	F: 820, 1,067 (1,005-1,125), 1,255 M: 990, 1,165 (1,091-1,220), 1,290 Sex-corrected: 820, 1,068 (1,005-1,125), 1,255

Abbreviations: IQR, interquartile range.

First, we investigated, for each neuropathological substrate, to what extent levels changed with increasing age in cognitively healthy individuals and in individuals diagnosed with AD (Figure 2.1 A, E, I, M). The difference between the mean levels of AD and ND is shown in figures (Figure 2.1 B, F, J, N). Then, we investigated the neuropathology levels observed in centenarians as a function of MMSE (Figure 2.1 C, G, K, O). Last we investigated, the potential of each neuropathological substrate to separate between AD and cognitively normal performance as a function of age. For this, we compared the distribution of neuropathology levels for each group across age intervals <60, 60 to 69, 70 to 79, 80 to 89, ≥ 90 (Figure 2.1 D, H, L, P). For this, we realized that post-mortem diagnosis of AD is based not only on cognitive decline, but also on having high levels of AD-associated neuropathology leading to a possible overestimation of the potential for each neuropathological substrate to separate between overall decline and cognitively normal performance. To avoid this possible bias, we additionally investigated the distribution of neuropathology levels in brains from individuals with non-AD dementia. The centenarian cohort includes all individuals with diverse cognitive performance at last study visit, regardless of levels of AD neuropathology. Thus, inclusion of non-AD dementia in the age-continuums allowed us to examine to what extent neuropathology levels in centenarians were as expected according to their age.

Table 2.2: Characteristics of the NBB donors according to age at death

Characteristic	<60 Yr	60 to 69 Yr	70 to 79 Yr	80 to 89 Yr	≥ 90 Yr	Total
Female/Male (N)	103/168	158/202	268/284	450/231	210/57	1,189/942
Neuropathological diagnosis						
	N, median (IQR)	N, median (IQR)	N, median (IQR)	N, median (IQR)	N, median (IQR)	N, median (IQR)
Alzheimer's disease (N)	42	132	204	323	150	851
NIA Amyloid stage	29, 3 (3-3)	75, 3 (3-3)	120, 3 (3-3)	159, 3 (3-3)	77, 3 (3-3)	460, 3 (3-3)
Braak-NFT stage	36, VI (VI-VI)	110, VI (V-VI)	178, V (V-VI)	292, V (V-VI)	137, V (IV-V)	753, V (V-VI)
CERAD-NP score	5, 3 (3-3)	16, 3 (3-3)	29, 3 (3-3)	28, 3 (2-3)	17, 3 (2-3)	95, 3 (3-3)
Brain weight [gr]	42, 1,017 (950-1,103)	132, 985 (890-1,065)	204, 995 (913-1,088)	323, 1,010 (941-1,084)	150, 959 (917-1,052)	851, 998 (920-1,078)
Non-dementia (N)	180	90	161	158	65	654
NIA Amyloid stage	18, 0 (0-0)	17, 0 (0-1)	65, 1 (1-2)	90, 1 (0-2)	47, 2 (1-2)	237, 1 (0-2)
Braak-NFT stage	52, 0 (0-0)	45, I (0-I)	113, I (I-II)	129, II (I-II)	55, II (I-III)	394, I (0-II)
CERAD-NP score	16, 0 (0-0)	11, 0 (0-0)	27, 0 (0-0)	36, 0 (0-0)	23, 0 (0-1)	113, 0 (0-0)
Brain weight [gr]	180, 1,267 (1,190-1,360)	90, 1,225 (1,130-1,274)	161, 1,160 (1,077-1,232)	158, 1,124 (1,050-1,199)	65, 1,060 (1,025-1,116)	654, 1,179 (1,080-1,265)
Non-AD dementia (N)	49	138	187	200	52	626
NIA Amyloid stage	14, 0 (0-0)	58, 1 (0-2)	116, 2 (1-2)	117, 2 (1-3)	35, 2 (1-2)	340, 2 (1-2)
Braak-NFT stage	15, 0 (0-I)	66, I (I-II)	145, II (I-III)	165, II (I-III)	48, III (II-III)	439, II (I-III)
CERAD-NP score	13, 0 (0-0)	35, 0 (0-0)	56, 0 (0-0)	37, 0 (0-1)	12, 0 (0-1)	153, 0 (0-0)
Brain weight [gr]	49, 926 (812-1,012)	138, 978 (837-1,100)	187, 1,036 (946-1,147)	200, 1,059 (983-1,121)	52, 1,029 (977-1,144)	626, 1,029 (935-1,125)
Total (N)	271	360	552	681	267	2,131

Figure 2.1

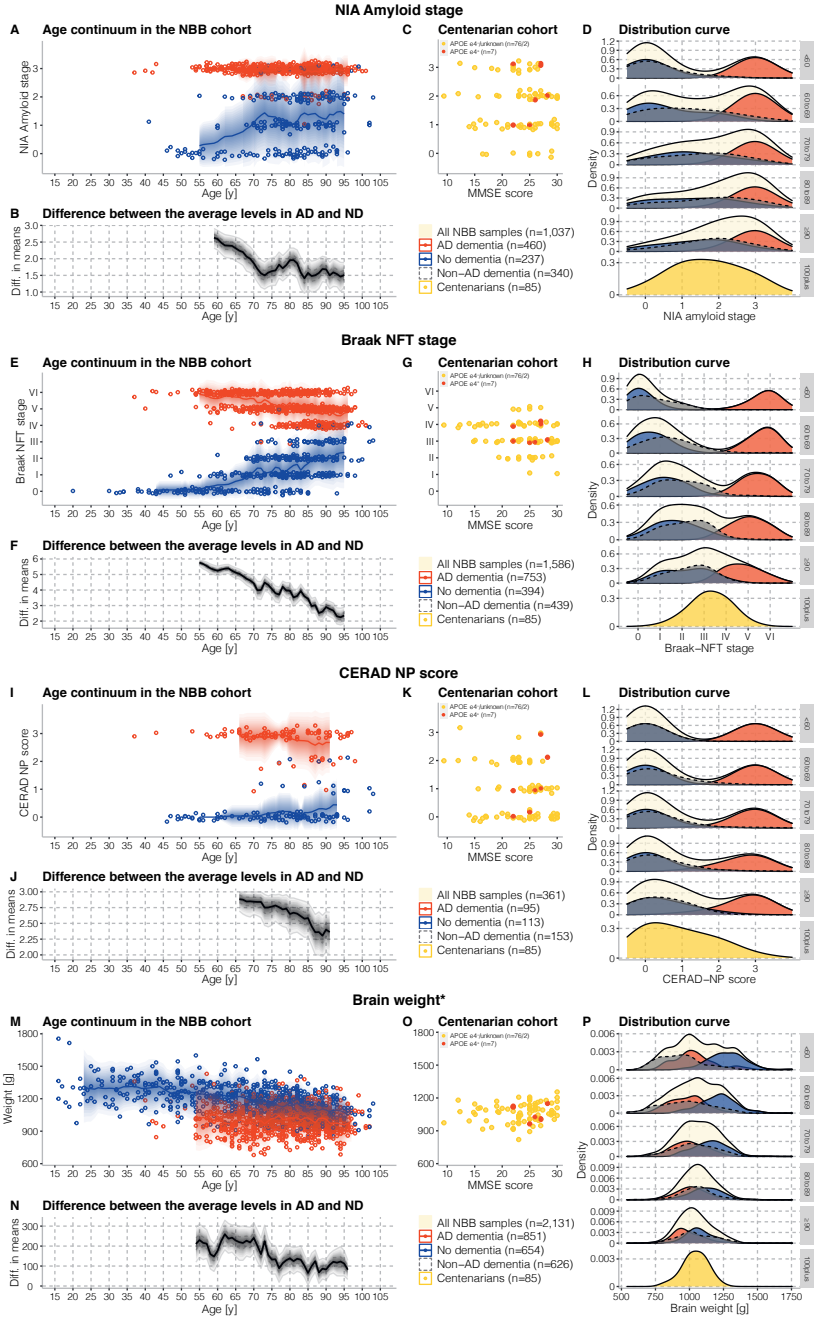
NIA Amyloid stage
A Age continuum in the NBB cohort
NIA Amyloid stage
Age [y]
B Difference between the average levels in AD and ND
Diff. in means
Age [y]
C Centenarian cohort
MMSE score
All NBB samples (n=1,037)
AD dementia (n=460)
No dementia (n=237)
Non–AD dementia (n=340)
Centenarians (n=85)
D Distribution curve
Density
NIA amyloid stage
Braak NFT stage
E Age continuum in the NBB cohort
Braak NFT stage
Age [y]
F Difference between the average levels in AD and ND
Diff. in means
Age [y]
G Centenarian cohort
MMSE score
All NBB samples (n=1,586)
AD dementia (n=753)
No dementia (n=394)
Non–AD dementia (n=439)
Centenarians (n=85)
H Distribution curve
Density
Braak–NFT stage
CERAD NP score
I Age continuum in the NBB cohort
CERAD NP score
Age [y]
J Difference between the average levels in AD and ND
Diff. in means
Age [y]
K Centenarian cohort
MMSE score
All NBB samples (n=361)
AD dementia (n=95)
No dementia (n=113)
Non–AD dementia (n=153)
Centenarians (n=85)
L Distribution curve
Density
CERAD–NP score
Brain weight*
M Age continuum in the NBB cohort
Weight [g]
Age [y]
N Difference between the average levels in AD and ND
Diff. in means
Age [y]
O Centenarian cohort
MMSE score
All NBB samples (n=2,131)
AD dementia (n=851)
No dementia (n=654)
Non–AD dementia (n=626)
Centenarians (n=85)
P Distribution curve
Density
Brain weight [g]

Figure 2.1: AD-associated neuropathological substrates (NIA Amyloid stage, Braak-NFT stage, and CERAD-NP score) and brain weight in the NBB and 100-plus Study cohort. A, E, I, M. The mean levels ±95% confidence interval (CI) of each neuropathological substrate and brain weight in an AD- and ND-age-continuum (red and blue separately) in the NBB cohort. **B, F, J, N.** The difference in the average levels of each neuropathological substrate and brain weight between AD cases and non-demented controls in the NBB age-continuum ±95% CI. **C, G, K, O.** The levels of each neuropathological substrate and brain weight across MMSE scores in centenarian cohort. Red points: the centenarian carriers of one *APOE* ε4 allele; yellow: no *APOE* ε4 allele. **D, H, L, P.** The distribution of the levels of each neuropathological substrate and brain weight for age intervals, i.e., <60, 60 to 69, 70 to 79, 80 to 89, ≥ 90, in the NBB AD (red), non-AD-dementia (light grey) and ND (blue) cohorts, and in the centenarian cohort (yellow). Beige indicates the overall distribution of the level of each neuropathological substrate in the NBB cohort (Methods). *Brain weight was corrected for sex.

2.3.2. NIA AMYLOID STAGE

The average NIA Amyloid stage was high across the AD-age-continuum, while it increased with age in the non-demented individuals (Figure 2.1A). We found an age-related decrease in the difference between the average NIA Amyloid stages between AD cases and non-demented individuals, from 2.5 at age 60 to 1.5 at age 95 (B). Of all centenarians, 9.4% had NIA Amyloid stage 0, 35.3% had stage 1, 32.9% had stage 2, and 22.4% had stage 3 (Figure 2.1C, Table 2.1), and we found no evidence for an association between NIA Amyloid stage and MMSE score ($\beta = -0.30, P = .60$; Table 2.3), nor between Thal Aβ phase and MMSE ($\beta = -0.21, P = .57$; Table S2). We observed an age-related convergence from a bimodal distribution of amyloid stages at younger ages to a unimodal distribution at older ages in the NBB cohort which was extended in the centenarian cohort (Figure 2.1D).

Table 2.3: Associations between neuropathological substrates and MMSE score

Neuropathology	Estimate β (95% CI)	P value
NIA Amyloid stage	-0.30 (-1.40, 0.81)	.60
Braak-NFT stage	-1.03 (-2.21, 0.14)	.08
CERAD-NP score	-0.78 (-1.87, 0.32)	.16
Brain weight [gr]	0.00 (-0.01, 0.02)	.54

NOTE: Using linear regression, we tested the association between the levels of each neuropathological substrate and MMSE score. The β reflects the change in MMSE score associated with one unit increase in the level of neuropathology. The associations for AD-associated neuropathological substrates and brain weight were corrected for sex, education, and time between the last available MMSE and death. Detail statistics of each regression model are provided in Table S2.

2.3.3. BRAAK-NFT STAGE

Along the age-continuum, Braak-NFT stages ranged between 0-VI. Braak-NFT stages increased with age in non-demented individuals and decreased with age in AD patients (Figure 2.1E). Accordingly, the average difference between Braak-NFT stages observed in AD cases and non-demented individuals, decreased from 6 at age 55 to 2 at age 95 (Figure 2.1F). Braak-NFT stages in centenarians ranged between I-V (Figure 2.1G): none had Braak-NFT stage 0; 2.4% had stage I; 14.1% had stage II; 42.4% had stage III; 35.3% had stage IV; and 5.9% had stage V (Table 2.1). Braak-NFT did not significantly associate with MMSE score in centenarians (β=-1.03, P=.08; Table 2.3), however, centenarians with Braak-NFT stage I-III had significantly higher MMSE scores than centenarians with Braak-NFT stage IV-V (Wilcoxon-rank-sum test: P=.04, W=1,102). Braak-NFT stage converged from a bimodal distribution at younger ages to a unimodal distribution in the centenarians (Figure 2.1H).

2.3.4. CERAD-NP SCORE

From age 75 onwards, the average CERAD-NP score increased with age in non-demented individuals and decreased in AD patients. But the changes were limited, such that the average CERAD-NP scores stayed low in non-demented older individuals and high in older AD patients (Figure 2.1I). In line with Figure 2.1I, the difference in the average CERAD-NP scores between AD and ND remained high (>2) until ages ≥ 90 (Figure 2.1J). In the centenarian cohort, 43.5% had CERAD-NP score 0; 29.4% had score 1; 22.4% had score 2; 4.7% (n=4) had score 3 (Figure 2.1K, Table 2.1). CERAD-NP scores did not significantly associate with MMSE score (β=-0.78, P=.16; Table 2.3). We observed a stable bimodal distribution in the age-continuum across NBB samples, and a unimodal distribution was only observed in centenarian cohort, with the majority having low CERAD-NP scores (Figure 2.1L).

2.3.5. BRAIN WEIGHT

The brain weight of AD patients was relatively stable across age: the median female brain weight was 1,003 gr (IQR: 930-1079); the median male brain weight was 1,170 gr (IQR: 1086-1261), and included samples that weighed <750 gr. The mean sex-corrected brain weight of ND individuals at middle age was 200 gr higher than in AD cases, but decreased with 0.27% and 0.28% per year for males and females respectively (Figure 2.1M), until at ≥ 90 years the difference in average brain weights was 100 gr (Table 2.2, Figure 2.1N). None of the ND or centenarian brains had the extremely low brain weights (<750g) observed in some young demented patients (Figure 2.1O). A regression model indicated that brain weight was not associated with the last available MMSE score in centenarians (β=0.00, P=.54; Table 2.3). While brain weights of non-AD patients were lower than AD patients at ages <60, they united the weights of AD patients and healthy controls at higher ages (Figure 2.1P).

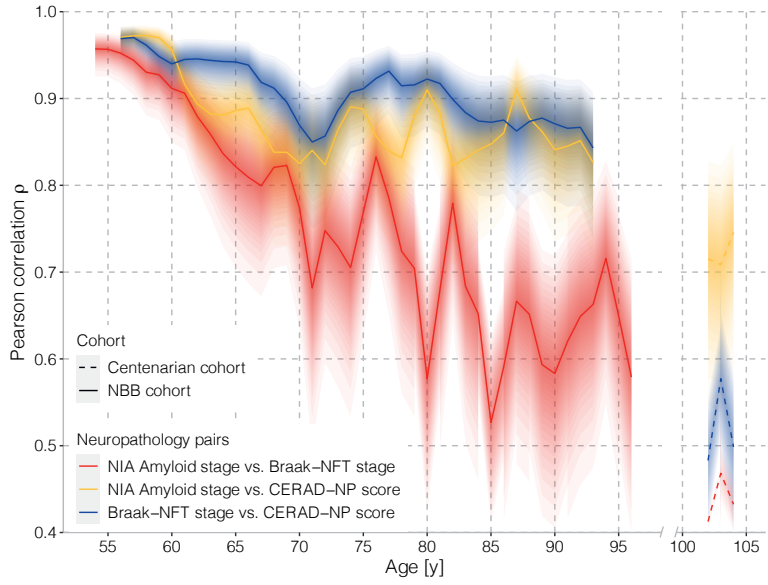

Figure 2.2: Pairwise Pearson correlation coefficient ±95% CI between AD-associated neuropathological substrates across the age-continuum in the NBB and centenarian cohorts separately. The NBB age-continuum includes AD patients, non-demented individuals, and non-AD demented individuals in a merged sample. The centenarian age-continuum includes all centenarians.

2.3.6. CORRELATIONS BETWEEN AD-ASSOCIATED PATHOLOGICAL SCORES DECREASE WITH AGE

Next, we merged all AD, non-AD and ND individuals from the NBB into one dataset and assessed the changes in pairwise correlations between the three pathology scores across an age-continuum (Figure 2). All pathologies were highly correlated at the youngest ages (r close to 1.0). Correlations decreased with age, in particular for the NIA Amyloid stage vs. Braak-NFT stage, which reached r=0.6 at >90 years. For the NIA Amyloid stage vs. CERAD-NP score and the Braak-NFT stage vs. CERAD-NP score, the correlation coefficients remained relatively high at r= 0.85, at >90 years. In the centenarian cohort, the correlation coefficients for the NIA Amyloid stage vs. CERAD-NP score remained at r= 0.75, while NIA Amyloid stage vs. Braak-NFT stage dropped to r= 0.45. Likewise, the Braak-NFT stage vs. CERAD-NP score correlation dropped to r= 0.55.

2.4. DISCUSSION

In this study we observed that, with increasing age, the levels of NIA Amyloid stage and Braak-NFT stage gradually increased in non-demented individuals. In those who reach ages of ≥ 100 years, NIA Amyloid stages, Braak-NFT stages, and CERAD-NP scores varied greatly to the extent that none of these neuropathological substrates correlated with cognitive performance as measured by MMSE. Brain weights of centenarians were ac-

cording to expectations with respect to age, and showed no correlation with cognitive performance. Our findings are in agreement with previous reports that accumulation of amyloid plaques and NFTs is a common aspect of aging[6, 7, 11, 26]. However, here we show that at extreme ages, some individuals can maintain the highest levels of cognitive performance despite accumulating levels of neuropathology equivalent to AD patients.

When focusing on amyloid plaques, we rarely observed amyloid pathology in brains <65 years, while some of the brains older than 95 years reached amyloid plaque levels similar to AD patients. Of all centenarians in this study, 9.4% resisted amyloid accumulation and had a NIA Amyloid stage of 0; cognitive performance varied widely in this group. In contrast, 22.4% of the centenarians had the highest NIA Amyloid stage 3, of whom 26% had maintained high levels of cognitive performance (MMSE ≥ 26)[27]. This is in agreement with reports showing that the correlation between amyloid plaque burden and neuronal and synaptic loss is limited.[28, 29] A first explanation for this is that a considerable fraction of Aβ deposits in the oldest old may be 'diffuse plaques' (DPs)[11, 12], depositions of aggregated non-fibrillar Aβ peptides with no organized internal architecture.[30, 31] This subtype of plaques is considered less toxic than neuritic plaques (NPs)[11], which contain a contracted central core of fibrillar Aβ peptide with neighboring dystrophic neurites and surrounded by reactive astrocytes and activated microglial cells.[31–34] In contrast to DPs, NPs are associated with the degeneration observed at the synaptic junction, i.e., the morphology of dendrites and axons with neuritic plaques was frequently abnormal.[32, 34, 35] Nevertheless, we observed that CERAD-NP scores also increased in brains of non-demented individuals across the age-continuum, but the increase remained within limits. CERAD-NP scores in centenarians were mainly within the 0-2 range, indicating that most centenarians were resistant to accumulating the highest level of NPs. Within this range, CERAD-NP scores did not correlate with MMSE scores. In fact, four centenarians (4.7%) had the highest CERAD-NP score of 3, of whom two scored ≥ 26 points on MMSE prior to death, suggesting that apparently, it is possible to be resilient to the highest NP scores[36].

Braak-NFT stages increased with age in non-demented individuals which is in agreement with previous reports[6, 7, 10]. However, we found that Braak-NFT stages decreased with age in AD cases, which suggests that at high ages, death can occur before the highest Braak-NFT stage is reached, presumably due to the competing risk of comorbidity and the effects of other co-pathologies that accumulate with age, e.g., TDP-43 and α-synuclein and vascular impairments.[3, 15–17] Most centenarians had accumulated NFTs consistent with Braak-NFT stages II-IV; only two centenarians (2.4%) resisted accumulation of NFTs beyond Braak Stage I, with variable cognitive performance. In contrast, five centenarians (5.9%) had Braak stage V, of whom three scored ≥ 25 points on the last available MMSE, indicating that resilience to high levels of accumulated tau is possible. While the association between Braak-NFT stages and MMSE did not reach significance in a regression model, centenarians with Braak stages I-III had a significantly higher MMSE than those with Braak stages IV-V. This is in line with the observation that clinical symptoms of AD often start when the Braak-NFT stage reaches stage IV[37], and the common assumption that of all AD neuropathological hallmarks, Braak-NFT stage associates strongest with cognitive performance[11, 38].

Notably, we observe a strong correlation between NIA Amyloid stage, Braak-NFT stage

and CERAD-NP score, which suggests a dependency between mechanisms supporting the accumulation of these substrates.[39] However, with age, the correlation between NIA Amyloid stage and Braak-NFT stage decreased to 0.5, indicating that the disease processes that lead to the buildup of these substrates in the elderly might be partly independent and with different etiology than at younger ages[38, 40]. For example, primary age-related tauopathy (PART) is commonly observed in aged individuals, in which NFTs occur independently of amyloid plaques.[41]

Brain weight loss starts from 40 years onwards, and amounts to 0.28% per year, which is likely due to the loss of white matter.[42] White-matter loss is associated with a decrease in processing speed, and this is characteristic for cognitive performance in the centenarian cohort.[43] The brain weight of centenarians is according to age, and showed no correlation with cognitive performance. This indicates that maintaining a high brain weight is not a prerequisite for maintaining cognitive health as measured by the MMSE.

That some centenarians were able to maintain high levels of cognitive health despite accumulating high levels of neuropathological substrates may be explained by their intrinsic resilience, i.e. a genetically defined lower vulnerability to the adverse effects of these pathologies. We previously found that, relative to a middle-aged population, the genomes of the centenarians are depleted with AD risk-alleles (including the strong risk-increasing *APOE* ε4 allele) and enriched with protective genetic variants.[44] Such a favorable genetic constellation is progressively selected for during the aging process of cognitively healthy individuals.[44, 45] This advantageous genetic constellation concerns specifically genetic variants associated with the immune response, autophagy and the endolysosomal system, mechanisms involved in the processing of many neuropathological substrates. Therefore, the resistance and resilience to accumulation of high levels of amyloid and tau may also extend to resilience to e.g. TDP-43, α-synuclein, and other neuropathological hallmarks of neurodegenerative diseases.[46] Lastly, we previously showed that the centenarians in this cohort have a relatively high educational attainment,[17, 47] which may contribute to cognitive reserve: more efficient use of existing neuron networks (i.e., neural reserve) or the ability to recruit alternate networks in response to network disruptions (i.e., neural compensation).[48–50]

One of the unique aspects of this study is that the ante mortem cognitive performance of the presented centenarian brains was tested only a few months prior to brain donation, such that correlations between neuropathology and brain function are exceptionally accurate. At study inclusion, centenarian participants self-reported to be cognitively healthy and brain donation occurred 0-6 years later. Therefore, we acknowledge that this brain cohort represents the neuropathological changes associated with the transition from cognitive health to cognitive decline, while changes associated with late-stage dementia remains unaddressed. As a measure of cognitive performance, we used the MMSE, which was the first test in our testing battery, hence despite the fatigue commonly observed at last study visit, the measure was available for almost all centenarians. However, we acknowledge that MMSE precludes the evaluation of neuropathological changes associated with different cognitive domains.[47] Likewise, we acknowledge that other neuropathological substrates may influence the observed resistance and resilience against the accumulation of amyloid and tau neuropathology. These aspects should be the focus for evaluation in future studies.

Concluding, we show that some individuals reach extreme ages with preserved cognitive health, despite accumulating levels of neuropathology similar to those observed in AD. While in vivo PET amyloid and tau imaging and CSF/plasma-based amyloid and tau levels are being implemented as biomarkers that aid clinical diagnosis of AD in memory clinics worldwide, the results of our work lead us to caution that the value of these pathologies may change with increasing age. Lastly, our results advocate for in-depth studies of these resilient brain samples to obtain a deeper understanding of the molecular mechanisms supporting the preservation of cognitive functioning until extreme ages.

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3

THE CORRELATION BETWEEN NEUROPATHOLOGY LEVELS AND COGNITIVE PERFORMANCE IN CENTENARIANS

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Neuropathological substrates associated with neurodegeneration occur in the brains of the oldest old. How does this affect cognitive performance? In 85 centenarian brains, we explored the correlations between the levels of 11 neuropathological substrates with antemortem performance on 12 neuropsychological tests. We observed levels of neuropathological substrates varied: Thal-Aβ phase up to 5, Braak-NFT stage up to V, CERAD-NP score up to 3, Thal-CAA stage up to 3, TDP-43 Stage up to 3, hippocampal sclerosis stage up to 1, Braak-LB stage up to 6, atherosclerosis stage up to 3, cerebral infarcts stage up to 1, and cerebral atrophy stage up to 2. GVD occurred in all centenarians. Some centenarians who had healthy cognitive performance also had the highest neuropathology scores. Only the Braak-NFT stage and LATE pathology (i.e., TDP-43 stage and hippocampal sclerosis) are associated significantly with performance across multiple cognitive domains. Of all cognitive tests, the clock-drawing test was particularly sensitive to levels of multiple neuropathologies.

3.1. BACKGROUND

With increasing age, the human brain commonly accumulates various proteinopathies associated with neurodegenerative diseases, which, accompanied by the concurrent loss of neuronal synapses and dendrites, is associated with the increased incidence of cognitive decline in elderly individuals.[1–6] The most common form of cognitive decline is due to Alzheimer's disease (AD), which is characterized by the accumulation of (1) amyloid beta (Aβ) plaques, (2) neuritic plaques (NPs) and (3) neurofibrillary tangles (NFTs).[7, 8] Aβ plaques are extracellular deposits of aggregated Aβ peptides. NPs are Aβ plaques that contain a contracted central core of fibrillar Aβ peptide with neighboring dystrophic neurites and are surrounded by reactive astrocytes and activated microglial cells.[9, 10] NFTs are intracellular deposits of phosphorylated tau protein aggregated into paired helical filaments. AD patients frequently co-present, to different extents, with additional neuropathological substrates associated with aging and/or other neurodegenerative disorders such as cerebral amyloid angiopathy (CAA)[11], Lewy Bodies[12, 13], atherosclerosis[14], cerebral infarcts[15], LATE pathology (characterized by Tar-DNA binding protein 43 (TDP-43) in combination with hippocampal sclerosis)[16, 17], and other cerebrovascular disorders[18]. Co-presentation of neuropathological substrates is associated with increased severity of cognitive impairment[19].

We and others previously showed that the accumulation of these neuropathological substrates not only occur in the brains of patients with AD or other dementias but that they also accumulate with age in the brains of individuals that are cognitively healthy.[1, 20–22] This leads to the question: how prevalent are these different neuropathological substrates in the oldest old, and to what extent do increased levels of each substrate associate with cognitive performance?

To investigate this, we evaluated 11 different neuropathological substrates in post-mortem brains and brain weight from well-phenotyped centenarians who participated in the 100-plus Study, an ongoing longitudinal cohort study of self-reported cognitively healthy centenarians. Previous findings in this cohort indicated that the levels of both antemortem cognitive performance and post-mortem neuropathological substrates were variable across centenarians.[20, 23] These features render this cohort ideal

for the evaluation of (1) the prevalence of and intercorrelation between the levels of different neuropathological substrates in the oldest old, and (2) the correlations between levels of neuropathological substrates and neuropsychological performance across different cognitive domains. Together, this investigation will allow for a deeper understanding of the effect of neuropathological substrates on cognitive performance at extreme ages.

3.2. MATERIALS AND METHODS

3.2.1. 100-PLUS STUDY COHORT OF CENTENARIANS

The 100-plus Study is an ongoing longitudinal cohort study of centenarians who self-report to be cognitively healthy, as confirmed by a proxy.[24] The study protocol was approved by the Medical Ethics Committee of the Amsterdam UMC. Informed consent was obtained from all participants. Brain donors consented to brain donation. The study was conducted in accordance with the Declaration of Helsinki.[25]

3.2.2. NEUROPSYCHOLOGICAL ASSESSMENT

Trained researchers visited the centenarians at their homes annually to subject them to a comprehensive neuropsychological testing battery covering five cognitive domains: memory, verbal fluency, attention/processing speed, executive functions, and visuospatial functions. A composite z-score for each of the five cognitive domians was computed, to allow associations with levels of neuropathological substrates. The Mini-Mental State Examination (MMSE) was administered[26] as a measure of global cognition, and scores on all cognitive domains were combined in a composite global cognition score. For this study, we used cognitive data collected at the last available study visit, which occurred a few months before death, to ensure a minimal time between neuropsychological measurements and neuropathological status at death.
Memory was evaluated using the immediate and delayed story recall subtest of the Rivermead Behavioral Memory Test (RBMT) and the Visual Association Test A (VAT-A)[27, 28]. Verbal fluency was measured using the Controlled Oral Word Association Test D-A-T (Letter fluency, LF) and animal fluency (AF).[29, 30] Attention/processing speed were evaluated with the digit span forward (DSF) subtest of the Wechsler Adult Intelligence Scale (WAIS-III) and the Trail Making Test (TMT) part A (scores were reversed, such that higher scores indicate better performance).[31, 32] Executive functions were evaluated using the TMT part B (scores were also reversed), key search (KS) subtest of the Behavioral Assessment of the Dysexecutive Syndrome Test Battery, and the digit span backward (DSB) subtest of the WAIS-III.[31–33] Visuospatial functions were evaluated with the number location (NL) subtest of the Visual Object and Space Perception Battery (VOSP), and the clock drawing test (CDT).[34, 35] Methods of test administration and implemented adaptations were described previously.[23]

3.2.3. NEUROPATHOLOGICAL ASSESSMENT

Autopsies were performed in collaboration with the Netherlands Brain Bank (NBB, https://www.brainbank.nl).[20] For each brain, we evaluated the level or distribution of

11 neuropathological substrates: (1) amyloid plaques (Thal-Aβ phase), (2) neurofibrillary tangles (Braak-NFT stage), (3) neuritic plaques (CERAD-NP score), (4) granulovacuolar degeneration (Thal-GVD stage), (5) cerebral amyloid angiopathy (Thal-CAA stage), (6) phosphorylated transactive response DNA-binding protein 43 (TDP-43 stage), (7) hippocampal sclerosis, (8) atherosclerosis, (9) cerebral infarcts, (10) Lewy bodies (Braak-LB stage), and (11) cerebral atrophy as well as (12) brain weight. Methods for pathology assessments and scoring strategies are described in detail in the Supplementary Material, and an overview of primary antibodies used for immunohistochemical assessments is given in Table S3.1. All centenarian brains were investigated by the same neuropathologist, keeping interrater variability to a minimum.

3.2.4. QUALITY CONTROL AND MISSING DATA IMPUTATION

Of the 395 centenarians that had been included in the 100-plus Study at the start of this analysis, 85 centenarians agreed to brain donation, allowing post-mortem neuropathological assessment (Figure 3.1). Few neuropathology staging levels were missing (Table S3.2); to make full use of the data, these were imputed across all 85 centenarians using MICE (version 3.13.0[36]) using all neuropathological substrates, sex, age-at-death, *APOE* genotype, and brain weight as variables (Supplementary Material).

At last study visit, a few months before death, fatigue, hearing and vision problems were common, which in some cases contributed to the inability to complete the cognitive testing battery[37] (Table S3.3). Missing data occurred across cognitive tests and across items within tests, and missingness became more prevalent as study visits occurred closer to the death of the centenarian. To make optimal use of available data, we used MICE to impute 1) missing MMSE items and 2) test scores across the cognitive testing battery. MMSE scores were imputed when ≤ 5 of the 30 points were missing as previously described[20], otherwise MMSE was set to "missing". Missing data for the 12 neuropsychological tests were imputed across data collected at last available visit from the 322 centenarians in the 100-plus Study cohort for whom at least half of the neuropsychological tests were available (excluding MMSE, Figure 3.1, Supplementary Material). As variables for the imputation, we included all neuropsychological test scores, imputed MMSE score, and education level (International Standard Classification of Education, ISCED). After imputation, full autopsy and full neuropsychology assessments were available for 69 centenarians, allowing the investigation of the association between neuropathology and neuropsychology (Figure 3.1).

3.2.5. STATISTICAL ANALYSES

Pairwise correlation between neuropathological substrates: The correlation between each pair of neuropathological levels, as measured in all 85 centenarian brains, was determined by calculating the Pearson correlation coefficient. Associated p-values were corrected for false discovery rates (FDR) using "Benjamini&Hochberg" method.

Factor analysis: To identify which neuropathological substrates are coregulated at extreme ages, we performed a generalized weighted least squares (GLS) factor analysis using the "oblimin" rotation method[38], with scoring based on the "tenBerge" scheme (psych R-package, version 2.1.9). The optimal number of factors was determined using

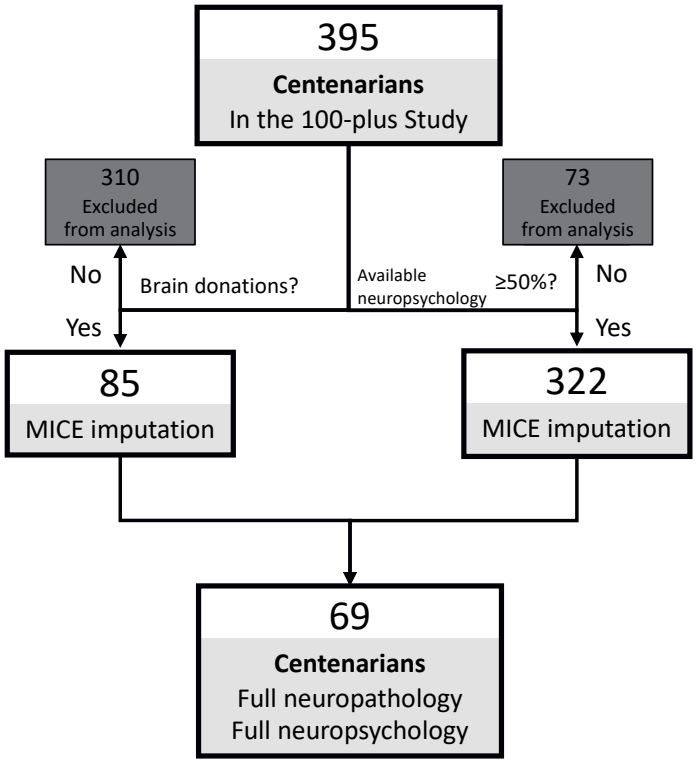

Figure 3.1: The flowchart of quality control and missing data imputation. 395 centenarians had been included in the 100-plus Study at the start of this analysis, of these, 85 centenarians had donated their brain for autopsy. The prevalence of different neuropathologies and the hidden structure was investigated in all 85 brain donors. Missing values for neuropathology were imputed across all 85 centenarian-brains using MICE. Across the 322 centenarians for whom scores of at least half of the neuropsychological tests were collected at last study visit (available neuropsychology ≥ 50%), missing scores were imputed with MICE. This resulted in 69 centenarians for whom all (imputed) neuropathology levels and all (imputed) neuropsychology test scores were available; these were included in the correlation analysis between neuropathology and neuropsychology.

the parallel analysis[39] ("nScree" function in the nFactors R-package, version 2.4.1) on neuropathological measures of all 85 centenarian brains. Paired correlations between the scores from the latent factors and brain weight were investigated using the Pearson correlation coefficient.

Regression analysis between neuropathology and neuropsychology: We applied linear regression models to investigate the correlation between neuropathological variables (explanatory variables) and neuropsychological variables (response variables). Models were corrected for age-at-death, sex, and the level of education (ISCED). *APOE* genotype does not associate with neuropathology levels or cognitive performance at these extreme ages[22] and was not corrected for. The regression coefficient was used to indicate the

strength of the correlation, and the corresponding p-value was used to indicate the significance. All response variables and explanatory variables were standardized (z-scores) to ensure the regression coefficients were comparable.
To avoid a possible outlier bias, we bootstrapped all mentioned analysis procedures (n=1,000). Pearson correlation coefficients, factor loadings and scores, and regression coefficients were calculated using the average values across bootstraps. We did not perform bootstrapping on p-values: the p-values for each analysis were determined based on the original tests, including all available centenarians. All calculations were performed using R (version 3.6.3). Pearson correlation coefficient and linear regression were performed using the "stats" R package.

3.3. RESULTS

3.3.1. SAMPLE CHARACTERISTICS

The age-at-death of the 85 centenarian brain donors ranged between 100 and 111 years, 75% was female. The last available study visit during which cognitive tests were administered occurred a median of 9 months prior to brain donation (IQR: 4-13). The median MMSE score at this last available study visit was 25 (IQR: 22-27). Of the 83 centenarians with *APOE* genotype available, seven carried one copy of the *APOE* ε4 allele, and *APOE* genotype did not associate with cognitive performance (Figure 3.2 and Table S3.4). Within this group, we observed no association between carrying the *APOE* ε4 allele and the level of neuropathological substrates (Table S3.4). The characteristics of the 69 centenarians with full autopsy and full neuropsychology assessments are available in Table 3.1.

3.3.2. THE PREVALENCE OF DIFFERENT NEUROPATHOLOGIES IN CENTENARIANS

The levels of neuropathological substrates varied widely across the 85 centenarians: none of the centenarians remained free of neuropathology, while three centenarians accumulated at least one level of all 11 neuropathological substrates (Figure S3.1). Centenarian brains had variable levels of TDP-43 stages and atherosclerosis, and high Thal-GVD stages. Further, we observed that some centenarians accumulated the high/highest level of, for example, Thal-Aβ phase up to 5 (5.9%), Braak-NFT stages up to stage V (5.9%), CERAD-NP scores up to level 3 (4.7%), Thal-CAA stages up to stage 3 (1.2%), Braak-LB stages up to stage 6 (n=1.2%), cerebral atrophy up to stage 2 (4.7%). In addition, cerebral infarcts were common in the centenarian brains (58.8%), and some had hippocampal sclerosis (22.3%). However, for the large majority of centenarians, the burden of accumulated neuropathology substrates remained with a certain limit: Braak-NFT stage $\leq IV$ (94.1%), CERAD-NP score ≤ 2 (95.3%), Thal-CAA stage ≤ 1 (91.8%), Braak-LB stage ≤ 1 (85.9%), and cerebral atrophy stage ≤ 1 (92.9%). Intriguingly, when presenting the levels of each neuropathological substrate across MMSE scores (Figure 3.2), we see that some of the centenarians with the highest neuropathology scores were among the best cognitive performers, suggesting that these individuals are resilient to the accumulation of these pathologies.

Table 3.1: Characteristics of the 69 centenarians in this analysis

	Median (Q1-Q3)
Clinical demographics	
Age [y]	103.3 (102.4-104.6)
Female/male	52/17
Education level (ISCED, 0-7)	3 (1-4)
MMSE (range: 0-30)	25 (22-26)
APOE (% with *APOE* e4 allele)	8.7% (6/69) with one *APOE* e4 allele, 0% (0/69) with two *APOE* e4 alleles
Neuropathological substrates, range of neuropathology scoring	
Thal-Aβ phase (range: 0-5)	3 (1-3)
Braak-NFT stage (range: 0-VI)	3 (3-4)
CERAD-NP score (range: 0-3)	1 (0-1)
Thal-CAA stage (range: 0-3)	1 (1-1)
TDP-43 stage (range: 0-3)	0 (0-2)
Hippocampal sclerosis (range: 0/1)	0 (0-0)
Thal-GVD stage (range: 0-5)	5 (4-5)
Atherosclerosis (range: 0-3)	2 (1-3)
Cerebral infarcts (range: 0/1)	1 (0-1)
Braak-LB stage (range: 0-6)	0 (0-0)
Cerebral atrophy (range: 0-3)	1 (0-1)
Brain weight [gr]	F: 1062 (1005-1125); M: 1175 (1150-1250)
Neuropsychological assessments, abbreviation, range of test performance	
RBMT Immediate recall Test, IR (range: 0-42)	7.0 (5.0-12.0)
RBMT Delayed recall Test, DR (range: 0-42)	4.0 (2.0-7.5)
Visual Association Test A, VAT-A (range: 0-12)	7.4 (5.0-10.0)
Letter Fluency D-A-T, LF (total count of words)	25.0 (18.0-32.0)
Animal Fluency, AF (total count of words)	10.0 (7.0-14.0)
Trail Making Test part A, TMT-A (time to finish)	87.0 (49.0-147.2.0) (not reversed)
Trail Making Test part B, TMT-B (time to finish)	232.5 (192.9-301.6) (not reversed)
Digit Span Forward, DSF (range: 0-16)	7.0 (6.0-9.0)
Digit Span Backward, DSB (range: 0-8)	5.0 (4.0-5.0)
Clock Drawing Test, CDT (range: 0-5)	3.0 (2.7-5.0)
Number Location Test, NL (range: 0-10)	8.9 (7.0-9.0)
Key Search Test, KST (range: 0-16)	5.7 (4.0-10.0)

Abbreviations: ISCED, International Standard Classification of Education.

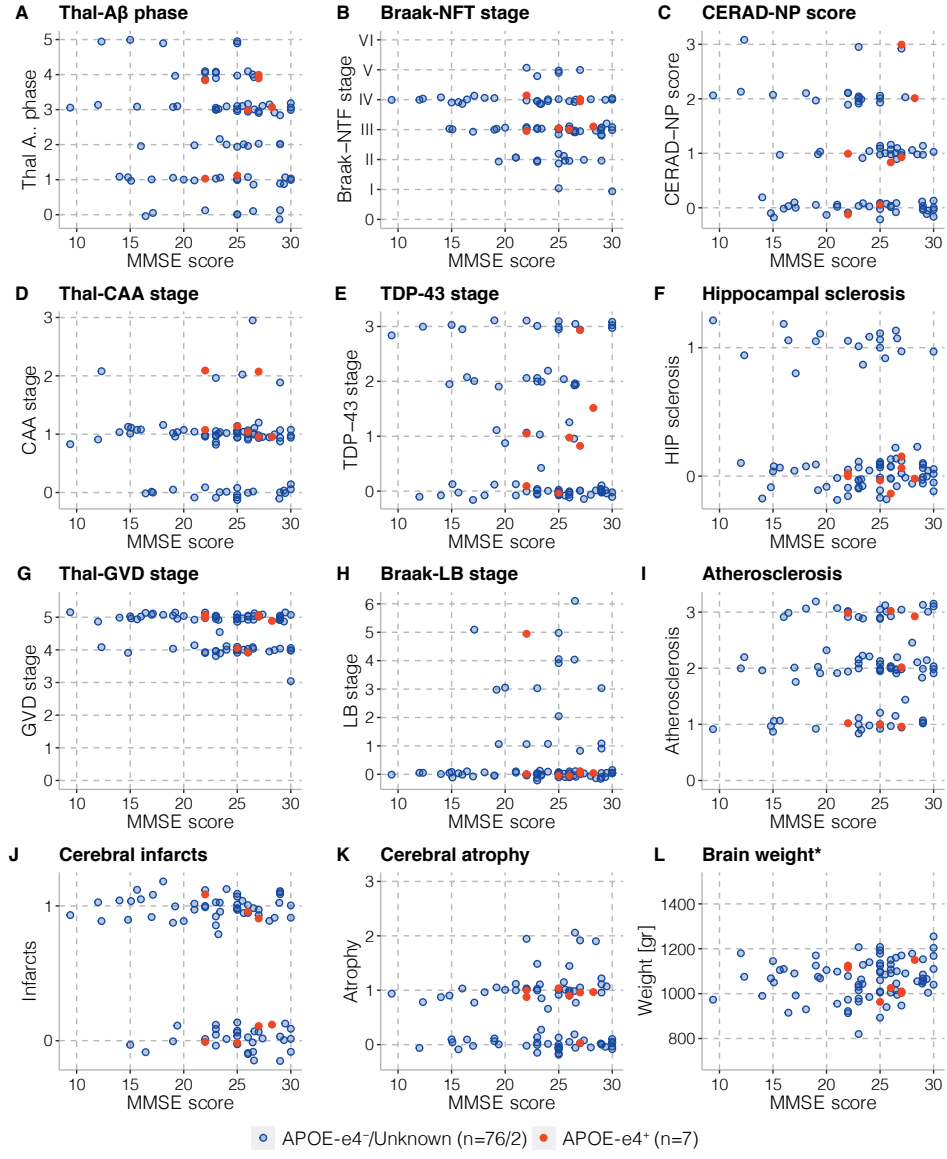

Figure 3.2: The levels of neuropathological substrates across MMSE score in 85 post-mortem brains. Red dots: centenarians with one copy of *APOE* e4 allele; blue dots: centenarians with no copy of *APOE* e4 allele or unknown *APOE* genotype. Staging scores have been given a random component to be able to distinguish the samples. *Brain weight was corrected for sex.

3.3.3. FACTOR ANALYSIS IDENTIFIES FIVE NEUROPATHOLOGY FACTORS

To explore the hidden structure of neuropathology in centenarian brains, we first evaluated the pairwise correlation between neuropathological substrates (Figure 3.3A, Ta-

ble S3.5, see Methods). We observed that CERAD-NP score correlated significantly with Thal-Aβ phase (r=0.78, FDR<.001), Braak-NFT stage (r=0.42, FDR<.001), and Thal-CAA stage (r=0.46, FDR<.001). Moreover, Thal-CAA stage correlated significantly with Thal-Aβ phase(r=0.61, FDR<.001) and cerebral atrophy(r=0.33, FDR=.02), but not with Braak-NFT stage (r=0.13, FDR=.58). Hippocampal sclerosis correlated significantly with TDP-43 stage (r=0.68, FDR<.001), and Thal-GVD stage correlated significantly with Braak-NFT stage (r=0.37, FDR=.005). Last, brain atrophy negatively correlated with brain weight (r=-0.35, FDR=.01).

Next, an Elbow and factor analysis revealed that the 11 different neuropathological substrates (i.e. excluding brain weight, as this varies between healthy individuals irrespective of age-related changes) loaded on five neuropathological factors, which we labeled as follows: 1) an amyloid factor on which the Thal-Aβ phase, CERAD-NP score, and Thal-CAA stage loaded; 2) a LATE factor on which the TDP-43 stage and hippocampal sclerosis loaded[17]; 3) a tau factor on which Braak-NFT stage, Thal-GVD stage and Braak-LB stage loaded; 4) a cerebral atrophy factor on which predominantly brain atrophy and to a lesser extent Thal-CAA stage loaded; and 5) a vascular factor onto which mainly atherosclerosis and to a lesser extent cerebral infarcts loaded (Figure 3.3B and Figure S3.2). Upon correlation of the latent factors, we observed a significant correlation between the amyloid and tau pathology factors (r=0.24, P=.01), followed by the correlation between LATE and tau pathology factors (r=0.15, P=.03). Brain weight negatively correlated with the atrophy factor (r=-0.27, P=.02) (Figure 3.3C and Table S3.6).

3.3.4. INDIVIDUAL NEUROPATHOLOGICAL SUBSTRATES VS. INDIVIDUAL NEUROPSYCHOLOGICAL TESTS

When correlating the levels of the 11 neuropathological substrates and brain weight with the performance on individual neuropsychological tests (Methods), we found that of all neuropsychological tests, the CDT showed the strongest correlation with levels of multiple neuropathological substrates (Figure 3.4, Table S3.7). Braak-NFT stage significantly correlated with immediate recall (β=-0.32, P=.008), delayed recall (β=-0.34, P=.004), VAT-A (β=-0.34, P=.005), TMT part A (β=-0.27, P=.03) and B (β=-0.26, P=.03), KS (β=-0.26, P=.02) and CDT (β=-0.35, P=.003); TDP-43 stage and hippocampal sclerosis with animal fluency (β=-0.27, P=.02; β=-0.27, P=.02) and CDT (β=-0.40, P=.001; β=-0.25, P=.04). Lastly, LB stage significantly correlated with CDT (β =-0.30, P=.03). Mentioned p-values here and below were not adjusted for multiple testing because the tests were not independent.

3.3.5. INDIVIDUAL NEUROPATHOLOGICAL SUBSTRATES VS. PREDEFINED COGNITIVE DOMAINS

A similar result was observed when investigating the correlations between neuropathology and cognitive domains (Figure 3.4 and Table S3.7). Braak-NFT stage significantly correlated with memory (β=-0.37 and P=.001), executive function (β=-0.31 and P=.004), and visuospatial function (β=-0.30 and P=.01) domains. TDP-43 stage correlated with executive function (β=-0.23 and P=.03) and visuospatial function (β=-0.27 and P=.03) domains. Hippocampal sclerosis correlated with fluency (β=-0.26 and P=.02). Braak-LB

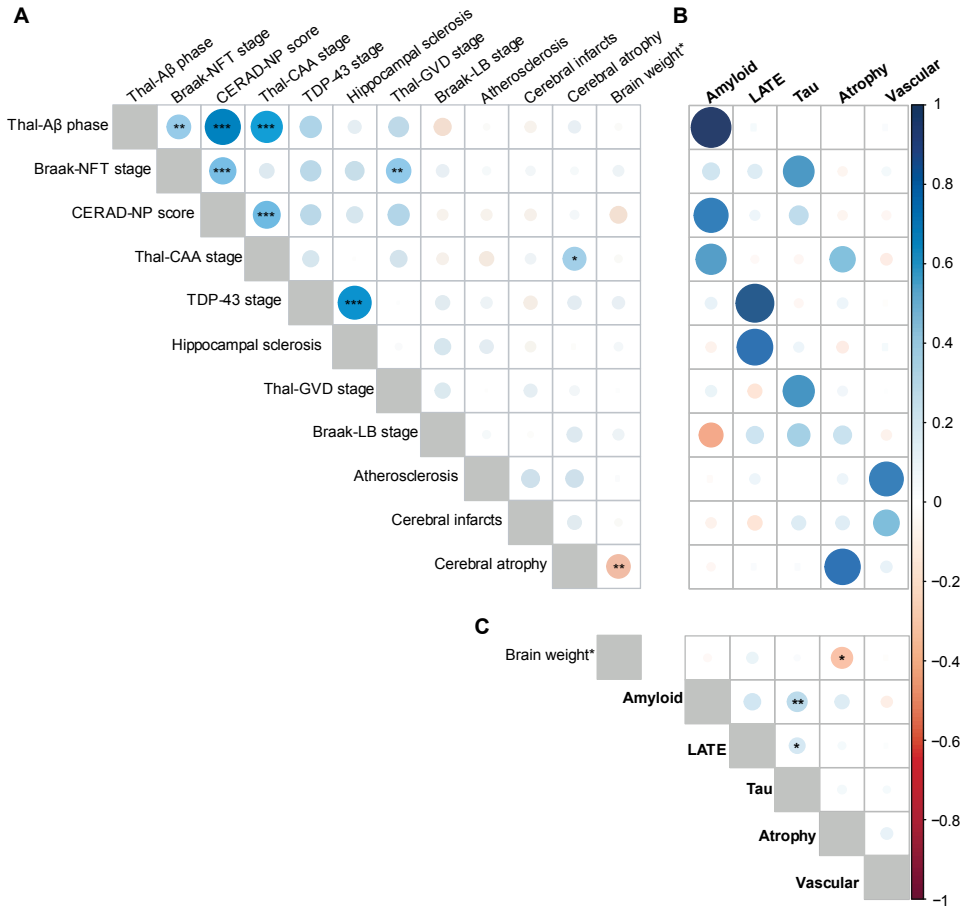

Figure 3.3: The correlation and factor analysis of neuropathological substrates. A. The pairwise correlation between neuropathological substrates and brain weight. The correlation coefficients and the p-values were calculated using Pearson correlation. All p-values were corrected for false discovery rates (FDR) using the "Benjamini&Hochberg" method. The asterisks indicate the significance of the correlation with FDR (* ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.001). **B.** An exploratory factor analysis (EFA) was performed for the 11 neuropathological substrates, and the five latent factors were determined using the Elbow method (see methods). Bold text: the factor names. The color and size of the circles indicate the loading of each neuropathological substrate on each factor, where the color blue indicates positive loads and the color red indicates negative loads. **C.** The pairwise Pearson correlation correlations between the neuropathological latent factors and brain weight. The asterisks indicate the significance of the correlation with p-value (* ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.001). The color and size of the circles indicate the strength of the Pearson correlation coefficient, where the color blue indicates a positive correlation and the color red indicates a negative correlation. *Brain weight was corrected for sex.

stage and cerebral atrophy both significantly correlated with visuospatial function (β=-

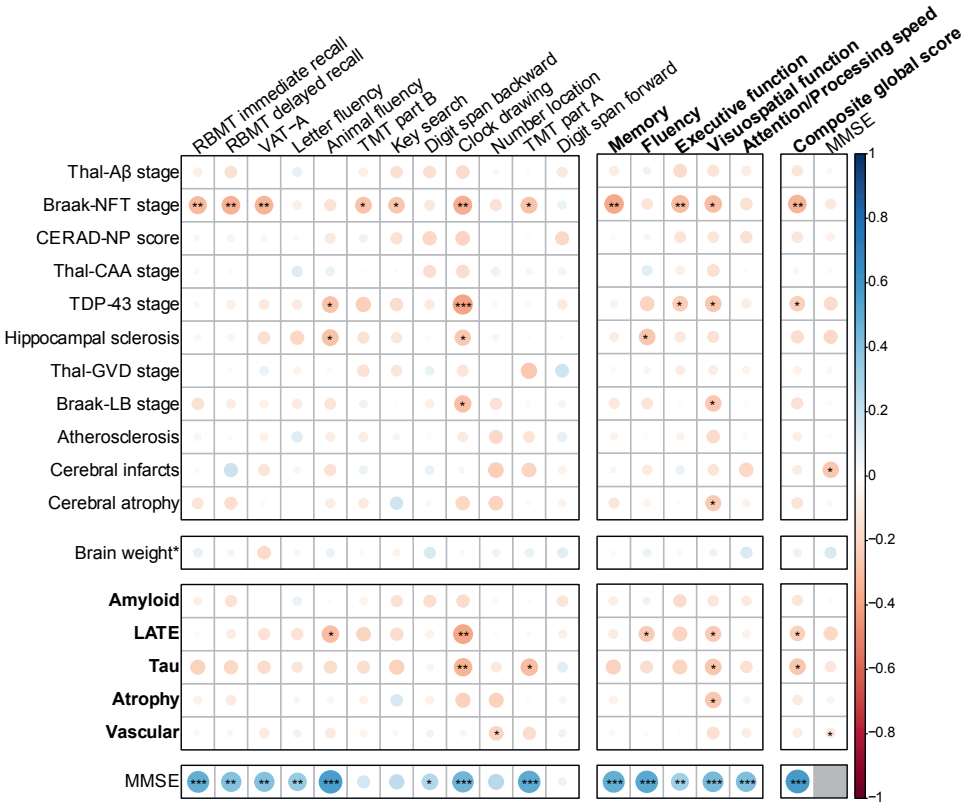

Figure 3.4: Regression analysis between neuropathology and neuropsychology (see Methods). Rows: the levels of individual neuropathological substrates, brain weight, neuropathological factors, and MMSE. Columns: performance of individual neuropsychological tests, cognitive domains, composite global cognition, and MMSE. Color and size of the circles indicate the strength of the regression coefficient, where color blue indicates positive correlation and color red indicates negative correlation. The asterisks indicate the significance of the correlation with p-value (* ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.001, uncorrected). The name of cognitive domains, composite global cognition, and neuropathology latent factors were indicated in bold text. *Brain weight was corrected for sex.

0.27, P=.04; and β=-0.25, P=.05).

3.3.6. NEUROPATHOLOGY LATENT FACTORS VS. INDIVIDUAL COGNITIVE TESTS AND COGNITIVE DOMAINS

We investigated the effect of each neuropathological factor on cognitive performance on the individual test level and domain level (Figure 3.4 and Table S3.7). We observed that the LATE factor correlated significantly with animal fluency and the CDT test scores (respectively β=-0.29, P=.01, and β=-0.37, P=.001), and also with the corresponding fluency and visuospatial function domains (respectively β=-0.25, P=.03, and β=-0.23, P=.05). The

tau factor correlated with CDT and TMT part A (respectively β=-0.29, P=.005, and β=-0.29, P=.03), and the visuospatial function domain (β=-0.24, P=.03). LATE and tau factor significantly correlated with composite global cognition (β=-0.22, P=.03; β=-0.23, P=.02). The vascular factor correlated with the NL test (β=-0.21, P=.04) and with the MMSE score (β=-0.12, P=.05) and the atrophy factor also significantly correlated with visuospatial function domain (β=-0.27, P=.03).

3.3.7. INDIVIDUAL NEUROPATHOLOGICAL SUBSTRATES VS. GLOBAL COGNITION

A significant correlation was observed between the Braak-NFT stage and the composite global cognition score (β=-0.33, P=.001), which was according to expectations given that Braak-NFT stage significantly correlated with almost all neuropsychological tests (Figure 3.4 and Table S3.7). TDP-43 stage also correlated with composited global cognition score (β=-0.23, P=.04). While the MMSE score significantly correlated with all cognitive domains as well as the composite global cognition score, neither Braak-NFT stage nor TDP-43 stage correlated with the MMSE score. MMSE, but none of the cognitive domains or composite global cognition scores, significantly correlated with cerebral infarcts (β=-0.26, P=.02).

3.4. DISCUSSION

On the individual level, neuropathological substrates varied greatly in centenarians. Overall, we observe that Braak-NFT stages and LATE pathology (i.e., TDP-43 stage and hippocampal sclerosis) significantly correlated with cognitive performance as measured only several months before brain donation. The levels of most other neuropathological substrates, including Thal-Aβ phases, Thal-GVD stages, and atherosclerosis, are associated weakly or not at all with cognitive test performance. Some centenarians maintained cognitive health despite having high burdens of neuropathological substrates, suggesting that these individuals are resilient to the associated damaging effects. Remarkably, of all neuropsychological tests, the performance on the clock-drawing-test (CDT) correlated strongest with levels of neuropathological substrates, even stronger than the composite global score or MMSE.

Overall, we observed positive correlations between Thal-Aβ phase, Braak-NFT stage, CERAD-NP score, Thal-CAA stage, TDP-43 stage, hippocampal sclerosis, and Thal-GVD stages suggesting that these substrates as a group may be functionally connected with one another. Our results further indicate that vascular changes such as atherosclerosis, cerebral infarcts, and CAA occur mostly independent from each other. Furthermore, only CAA significantly associated with cerebral atrophy in centenarians, which supports previous report that CAA can be an independent contributor to cortical atrophy[40].

Our factor analysis distinguished between an amyloid factor and a tau factor. The amyloid factor supports the established association between levels of Aβ plaques, NPs, and CAA[41]. Despite a strong correlation between Braak-NFT stage and CERAD-NP score, Braak-NFT stage loaded on the tau factors together with Braak-LB stage (which accumulated in only few centenarians) and the Thal-GVD stage (high in all centenarians). A possible explanation for this may be that both higher levels of intracellular NFTs and

α-synuclein associate with the formation of granulovacuolar bodies, which are neuronal lysosomal structures in which endocytic and specific cytosolic cargo accumulate.[42, 43] While cerebrovascular disease and amyloid accumulation previously were reported to frequently co-occur in AD[44], we observed no association between the amyloid or tau factors and the vascular factor, onto which atherosclerosis and cerebral infarcts loaded. This suggests that despite the frequent co-occurrence of these substrates, they need not in all instances be mechanistically related in centenarian brains. Indeed, while amyloid-dependent vascular factors such as CAA are prevalent in the aging human brain, there are also amyloid-independent factors that contribute to cerebral vascular disease. This includes cerebral atherosclerosis, cerebral small vessel disease (SVD, often caused by hypertensive vasculopathy) or microvascular degeneration. Also, blood-brain barrier (BBB) dysfunction are common in the ageing brain, which causes white matter lesions (WMLs) microinfarctions, lacunes or lacunar infarcts, or microbleeds.[44]

In this centenarian group, the correlation between neuropathology and cognitive performance was strongest for Braak-NFT stages, which is in line with findings in younger individuals[45, 46]. Braak-NFT stage significantly correlated with cognitive performance on neuropsychological test level, domain level and global cognition level. In contrast, Thal-Aβ phase, the other hallmark of AD, varied widely across centenarians and did not associate with performance on neuropsychological tests, despite a significant association with Braak-NFT stage. This suggests that the deposition of Aβ plaques might be a natural consequence of aging and not directly causative for functional decline in centenarians.[21, 22, 46] We previously suggested that a considerable fraction of Aβ deposits observed in elderly, including the centenarians investigated in this study (data not shown), may be diffuse plaques (DPs),[20] depositions of aggregated non-fibrillar Aβ peptides with no organized internal architecture.[9, 47] These are considered a less toxic form of Aβ plaques[46]. In contrast, NPs, as measured by CERAD-NP, include dendrites and axons with abnormal morphology, suggestive of degeneration at the synaptic junction.[10, 48, 49] CERAD-NP score increases with age in cognitively healthy individuals but the increase is limited (rarely exceeds level 2)[22]. This suggests that these centenarians resisted accumulating NPs to the highest, pathogenic levels, which may explain the lack of association with cognitive performance on any of the cognitive tests or domains. Similarly, Thal-CAA stage rarely exceeds level 2 and also did not correlate with cognitive performance, which also suggests that these centenarians resisted building up higher levels. Furthermore, GVD bodies accumulated to the highest levels in centenarian brains and the Thal-GVD stage, which also loaded onto the tau pathology factor. However, GVD levels did not correlate with any neuropsychological test. This suggests that the formation of GVD bodies may be by itself not specifically toxic.[50]

TDP-43 stage varied across centenarians and correlated strongly with hippocampal sclerosis, and as expected, these substrates both loaded onto the LATE factor. While LATE pathology is commonly observed in brains of patients with frontotemporal lobar degeneration and AD, large gaps remain in our understanding of its role in cognitive decline[17]. Moreover, TDP-43 depositions are frequently observed in the brains of the elderly[17] and we found that increased levels significantly correlated specifically with animal fluency which supports previous reports indicating that verbal fluency is more strongly affected in TDP-43 positive patients, and is less affected in those with AD pathol-

ogy.[45, 51] Next to animal fluency, TDP-43 stage also associates with a lower performance on the CDT. Altogether, this supports that TDP-43, in the context of LATE, is a neuropathological substrate that contributes to changes in cognitive performance. TDP-43 contributes to the LATE factor, which significantly correlated with the tau factor, indicating that centenarians with higher levels of LATE pathology are also more likely to have tau, which may in part contribute to the association between LATE pathology and cognitive performance.[52] We caution that the observed effects of TDP-43 and LATE pathology on cognitive performance should be replicated in larger studies.

Braak-NFT stage and TDP-43 stage significantly correlated with composite global cognition but not with MMSE. This suggests that the MMSE, as a global cognition screening tool, lacks the sensitivity to detect the specific effects of NFTs and TDP-43 on cognitive performance.[53] In contrast, Braak-NFT stage, TDP-43 stage, hippocampus sclerosis, and Braak-LB stage all significantly associate with the performance on CDT, which provides the first objective preliminary evidence that the CDT may be sensitive to critical levels of neuropathological changes. This provides further support to the reported high sensitivity and specificity of the CDT for diagnosis of AD in younger individuals[54]. However, we note that cerebral infarcts, which frequently presented in centenarians, significantly correlated with MMSE score, but not with the CDT or any other neuropsychological test. Replication in other studies will indicate whether or not MMSE is more sensitive to the cognitive deficits associated with cerebral infarcts.[55]

The availability of neuropsychological test performance measured so shortly before brain donation[22] is unique for the 100-plus Study cohort, and this greatly contributes to the reliability of correlations between neuropathology burden. While 85 centenarian brains may be considered a uniquely large sample size, it is a relatively small dataset for the identification of robust correlations. Nevertheless, we found that Braak-NFT stages and TDP-43 stages significantly associated with cognitive performance. The sample size is currently still growing, which may allow some of the observed weaker associations to reach significance in a follow-up analysis. Notably, the detected associations do not imply a (direct) causal relation. Increased sample sizes might also enable better inference of possible confounding and/or mediation effects.

Importantly, in this study, we have investigated neuropathology scores representative of the distribution of neuropathological substrates using commonly used antibodies. However, it may well be that while the distribution throughout the different brain regions may be similar loads of neuropathology per brain region may be lower in centenarians than in AD patients. Furthermore, the field is currently exploring the pathogenicity of different subtypes of neuropathological substrates[46, 56]. The accumulation of non-pathogenic neuropathological substrates might explain the observed 'resilience' to regarded toxic neuropathologies in our study subjects. Also, since the cognitive decline in human brains is mainly caused by synaptic/dendritic loss, future studies might focus on whether centenarians maintain cognitive performance due to the preserved function of synapses and dendrites during the aging process. Finally, we acknowledge that our inclusion criteria of self-reported cognitive health at age ≥ 100 years selects a unique subgroup of the population[24]. During follow-up, some of these individuals do develop dementia-related symptoms (17.4% has an MMSE <20 at the last visit), making this group ideal for correlating (preserved) cognition with observed pathological substrates at old

age. However, relative to middle-aged individuals, this group is enriched with genetic factors that associate with increased longevity[57] and depleted with genetic risk factors for AD, including the *APOE* ε4 allele[58]. Therefore, correlations observed in this group may not be representative of the entire population.
In conclusion, within the highly variable levels of neuropathological substrates in centenarian brains, Braak-NFT stages and LATE pathology significantly correlated with cognitive performance as measured shortly before brain donation. We present preliminary evidence that the performance on the clock-drawing-test CDT may be representative of higher burdens of these neuropathological substrates. To increase our understanding of the association between neuropathological burden and cognitive performance, we propose that future studies address the loads and subtypes, rather than distribution, of neuropathological substrates.

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4

NEUROPROTEOMICS OF COGNITIVELY HEALTHY CENTENARIANS IN THE CONTEXT OF AGING AND DISEASE

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Aging is often accompanied by the occurrence of neurodegenerative diseases, such as Alzheimer's disease (AD), and their associated neuropathology. Few individuals are able to maintain cognitive health throughout life, such as the centenarians from the 100-plus Study. In this single-center proteomics study on post-mortem human brain, we provide insight into the potential molecular mechanisms involved in maintaining cognitive health in old age in the abstinence of AD. A quantitative DIA/SWATH proteomics on an age-continuum of human post-mortem temporal lobe brain samples was performed (n=210) of non-demented and AD individuals, and centenarians from the 100-plus Study. We investigated the changes of these proteins with the Braak stage and age and the specificities of these proteins in centenarian brains. A total of 3,448 proteins was reliably measured over all samples. 472 proteins were found to be strongly associated with Braak stages, while 174 proteins were age-related. To identify what makes the centenarians special, we tested the abundance of proteins in centenarians by matching the Braak stage, and in comparison to AD cases, 64 proteins were differentially regulated, usually lacking or delaying the regulation seen in AD cases over the Braak stages: Ubiquitin, VGF, PSCK1, C3CD4C, MAPT, SYT12, OLFM3, PEX5L, RPH3A, KIAA1586, ACTN2, RBMX and VDAC1. Additionally, the abundance of proteins in the centenarians differed from the expected value at age 100 in 108 of the age-related proteins, interestingly corresponding to the expression level normally occurring at "younger" ages. We found a functionally diverse set of AD-related proteins that show differential expression in Braak stage matched centenarians and AD subjects. These proteins may be of relevance in cognitive resilience to the disease that characterizes the centenarians. Furthermore, we revealed several functional sets of age-related proteins (e.g. microtubular, intermediate filaments, myelin), that show a remarkably high expression in centenarians, resembling that normally observed in a median of 18-year younger non-demented individuals. These proteins may support important mechanisms related to maintaining cognitive health at extreme age.

4.1. INTRODUCTION

The incidence of aging-related diseases rises exponentially worldwide due to the increase in average life expectancy over the last century[1]. Among these, Alzheimer's disease (AD) is one of the most prevalent and devastating[2, 3]. AD is characterized by the neuropathological hallmarks of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFTs)[4, 5]. At young age, high levels of AD pathology are almost exclusively observed in those with clinical symptoms of AD, while with increasing age, these neuropathological substrates are also found in the brains of non-demented individuals[6–8]. In a sample of elderly individuals aged 79 or above, 30-40% accumulated significant levels of AD-related neuropathology, whereas only a small subset (15%) was clinically diagnosed with AD[9]. Importantly, at extreme ages, some cognitively healthy individuals accumulate levels of neuropathological substrates that can also be observed in AD patients. In fact, a subgroup of the centenarians from the 100-plus Study cohort were able to maintain the highest levels of cognitive performance, often for multiple years after reaching 100 years old, despite the accumulation of AD-related neuropathological hallmarks as observed by post-mortem brain analysis[8, 10, 11]. This suggests that those who maintain

cognitive health at high age are either resilient or resistant to the effects of accumulated AD-associated neuropathologies.

To learn how cognitive health can be maintained, it is imperative to maximally comprehend the cellular and biomolecular mechanisms of prolonged resilience and resistance against neurodegeneration-associated proteinopathies with increasing age with respect to the differential display of cognitive health. It was previously shown that specific brain proteins change levels with increasing age[12]. We therefore hypothesized that specific proteins might show different patterns in centenarians supporting cognitive health despite old age. To identify these proteins, we profiled cortical brain proteomes of 58 centenarians (aged 100-111) relative to 61 non-demented individuals and 91 AD patients covering an age range between 50 and 102 years. Using our aging cohort consisting of non-demented individuals and AD patients, we first set out to identify the proteins that changed in abundance with increasing levels of AD-associated tau levels (Braak Stages) or amyloid levels (Thal Stages). This then allowed us to identify proteins that are differentially abundant in centenarians compared to expectations based on AD-related neuropathology. Next, we investigated the proteins that change in abundance with increasing age, allowing us to identify proteins that are differentially abundant in the centenarians compared to expectations based on age. We hypothesize that these proteins are part of molecular mechanisms associated with the maintenance of cognitive health during the aging process.

4.2. MATERIAL AND METHODS

4.2.1. COHORTS

Tissues from Alzheimer's disease (AD) cases and non-demented (ND) individuals were selected from the brain cohort hosted by the Netherlands Brain Bank (NBB, https://www.brainbank.nl/). For each brain, we investigated clinical status prior to brain donation, to ascertain non-dementia and AD dementia. In total, we included 61 non-demented individuals spanning ages 50 to 96 and Braak stages 0 to III, and 91 AD cases with Braak stages from IV to VI, of which 48 AD cases had one *APOE* ε4 allele, and 43 AD cases had no *APOE* ε4 allele, spanning the ages 55 to 95 and 62 to 102, respectively. The average post-mortem delay (PMD) ranges from 2.0 to 12.9 h (mean 5.7 h).

Centenarian brains were donated to the 100-plus Study, a prospective cohort of centenarians in the Netherlands[13]. Inclusion criteria include self-reported and proxy-confirmed cognitive health and proof of age above 100 years. All participants were visited yearly at their home, where neuropsychological tests were performed. Yearly visits continued until death or until participation was no longer possible. Around 30% of 100-plus Study participants agreed to post-mortem brain donation, and tissue was collected in collaboration with NBB. At the time of tissue selection for the proteomics sample, 58 centenarians aged 100 to 111 had come to autopsy, and all were included. The average time between the last study visit and death is 9 months (±5 months), and post-mortem delay ranges from 3.4 to 12.0 h (mean 6.5 h).

Detailed information of all 210 brains analyzed in this study, their age, Amyloid stage, Braak stage and *APOE* genotype, sex, and PMD are listed in Table S4.1.

4.2.2. SAMPLE PREPARATION

Fresh frozen tissue of the middle temporal lobe (gyrus temporalis medialis, GTM2) was cut in 10 µm thick sections using a cryostat and mounted on polyethylene naphthalate-membrane slides (Leica, Herborn, DE). Sections were fixed in 100% ethanol for 1 minute and stained using 1% (wt/vol) toluidine blue in H2O (Fluka Analytical, Buchs, Switzerland) for 1 minute. Laser microdissection (LMD) was performed using a Leica AS LMD system (Leica, Wetzlar, Germany) to isolate 0.5 mm^3 of grey matter tissue and collected in 30 µl 3 M-PER lysis buffer (Thermo Scientific, Rockford, IL, USA) in 0.5 ml Eppendorf PCR tubes and stored at -80 °C until further use.
Samples were heated to 95 °C for 5 minutes and incubated in the dark with 50 mM Iodoacetamide for 30 min at room temperature. Samples were loaded on 10% Bis/Tris-polyacrylamide gels and run into the gel for 15 min at 80 V using 1.5 M Tris/Glycine SDS running buffer pH 8.3. Gels were fixed overnight and stained with colloidal Coomassie Blue G-250, before samples were cut out and small gel pieces of about 1 mm^3 were placed in 96-well Nunc filter plates (Thermo Scientific, Rockford, IL, USA). Destaining, trypsin digestion, and peptide extraction were done as described previously[14].
Collected samples were dissolved in 100 µl Mobile phase A (2% acetonitrile/0.1% formic acid) and cleaned using the OASIS filter plate (Waters Chromatography Europe BV, Etten-Leur, The Netherlands) according to the manufacturer's instruction. We used a subset of all samples to generate a peptide library comprising 5 groups: (1) a pool of 4 young AD cases, (2) a pool of 4 young ND individuals, (3) a pool of 4 old AD cases, (4) a pool 4 old ND individuals, (5) a pool of 8 centenarians. We further fractionated the sample pools using the Pierce high-pH reversed-phase fractionation spin columns (Thermo Scientific) according to manufacturer's instruction but using 0.1% acetic acid instead of 0.1% trifluoroacetic acid. The collected peptides were dried and stored at -20 °C until mass spectrometry analysis.

4.3. MASS SPECTROMETRY

4.3.1. LIBRARY GENERATION

For the spectral library generation, we performed a data-dependent acquisition (DDA) experiment using the five pooled samples (Sample preparation). Peptides were analyzed by micro LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific) coupled to the TripleTOF 5600 mass spectrometer (Sciex). Peptides were trapped on a 5 mm Pepmap 100 C18 column (300 µm i.d., 5 µm particle size, Dionex), and fractionated on a 200 mm Alltima C18 column (100 µm i.d., 3 µm particle size). The acetonitrile concentration in the mobile phase was increased from 5 to 18% in 88 min, to 25% at 98 min, 40% at 108 min and to 90% in 2 min, at a flow rate of 5 µl/min. The eluted peptides were electro-sprayed into the TripleTOF MS, with a micro-spray needle voltage of 5500 V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (350-1250 m/z, 150 msec) followed by a top 25 MS/MS (200–1800 m/z, 150 msec) at high sensitivity mode in UNIT resolution, precursor ion >150 counts/s, charge state from +2 to +5, with an exclusion time of 16 sec once the peptide was fragmented. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 5 eV. The mass spectra were searched against the human fasta database

(Uniprot_2018-05_HUMAN_201804.fasta and BIOGNOSYS_IRT.fasta) using MaxQuant software (version 1.6.3.4) with the default setting.

4.3.2. SAMPLE ANALYSIS

Next, we measured the proteome of all 210 individuals, using data independent acquisition (DIA). The same LC gradient used by DDA was employed for DIA. The DIA protocol consisted of a parent ion scan of 150 ms followed by a selection window of 8 m/z with a scan time of 80 ms and stepped through the mass range between 450 and 770 m/z. The collision energy for each window was determined based on the appropriate collision energy for a 2+ ion centered upon the window with a spread of 15 eV. The data were analyzed using Spectronaut pulsar with the default settings. Each group of eluting peptide fragments in the raw data was matched to the spectral library by Spectronaut and yielded a compound identification score for the assigned peptide. The false discovery rate (FDR) of this quality metric was provided in Spectronaut output as q-value. In total, 28,191 peptides from 4,829 unique proteins were measured in 210 proteomic profiles.

4.3.3. QUALITY CONTROL

Sample filtering: The aim of sample filtering was to remove low-quality profiles. We selected high quality samples for analyses by removing samples for which the fraction of low-quality peptides (q-value ≥ 0.01) exceeded 34%, when the fraction of low-quality peptides increased sharply (n=19). Second, we removed samples for which the distribution of peptide abundance deviated from the overall peptide abundance distribution (Kolmogorov–Smirnov distance >0.04) (n=1) (see Supplementary material and Figure S4.1 for filtering steps). After filtering, 190 proteome profiles were left for analyses.

Peptide filtering: To ensure the reliability of the measured protein abundances, we performed peptide filtering based on peptide quality measures. For each protein, if ≥ 90% of the measured samples included at least one high-quality peptide (q-value <0.01), the measurement of this protein was considered to be reliable (n=3,448); otherwise, it was considered unreliable (n=1,381). For protein measurements considered reliable, the sum of the abundance of all peptides appertaining to one protein was computed, which represented the abundance of this protein across individuals. Larger proteins are likely to have more peptides, such that the sum is likely to be larger than the sum of small proteins. This allows for the comparison of protein abundance between individuals but not between the abundance of different proteins within one individual. We log2-transformed protein abundance values for analyses (Table S4.2).

Variance explanation analysis: We used a mixed-effect linear model from the R-package "variancePartition" to assess the percentage of protein expression variance explained by age, sex, Braak stage, post-mortem delay (PMD), *APOE* genotype, and data acquisition batch. PMD, sex, and *APOE* genotype explained only minute proportions of variance in the proteomic abundance profile. However, next to Braak-stage and age (the effect of which we intended to assess in our analysis), the batch explained substantial proportions of variance (Figure S4.2). We removed the batch effect using the "Combat" R-package. After using "Combat", we reassessed protein expression variance using the mixed-effect linear model, which indicated that the proportion of variance explained by

batches was largely removed (Figure S4.2).

4.3.4. NEUROPATHOLOGICAL MEASUREMENT

Neuropathological assessments for the NBB cohort and the 100-plus Study cohort were performed by the NBB, as described in the previous study[11]. In this study, we investigated the levels of two AD hallmarks: (1) Aβ plaques using NIA Amyloid stage[4], and (2) NFTs using Braak stage[15, 16]. Brain weight was recorded during the autopsy. The centenarian brains and the majority of the brains in the age-continuum were evaluated by a single neuropathologist, such that interrater variability was kept to a minimum.

4.3.5. QUANTITATIVE IMMUNOHISTOCHEMISTRY (QIHC) OF NFTS

To assess a load of NFTs, quantitative immunohistochemistry (IHC) analysis with antibody AT8 was performed on the GTM2 tissues from a subset of post-mortem brains consisting of 76 AD cases, 30 ND individuals, and 20 centenarians. The details of IHC staining and region of interest (ROI) identification were described in the Supplementary Material. Quantitative analyses are performed with the help of ImageJ software[17]. This program is capable of processing images to a binary copy and giving quantitative results in the form of positive area percentages. A color threshold for staining images was set and maintained in all subjects.

4.3.6. CORRELATION BETWEEN PROTEIN ABUNDANCE AND BRAAK STAGE OR NIA-AMYLOID STAGE

As a first identification of proteins that correlate with Braak stages and NIA Amyloid stages, we calculated the Pearson correlation in (1) all non-demented individuals and all centenarians, and (2) all AD patients.

4.3.7. BRAAK STAGE-RELATED PROTEIN ANALYSIS: B-PROTEINS

Assuming that protein abundances change between Braak stages[18], we categorized the data from AD cases and ND individuals according to Braak stages 0/I, II, III, IV, V, and VI (Braak stages 0, and I were merged). For each protein, the significance of abundance changes across the six Braak stages was determined using a one-way ANOVA test. Next, for proteins observed to be significantly changed, we identified the Braak stage with the highest median protein abundance and the Braak stage with the lowest median protein abundance, then we calculated the log2 fold changes (LFC) between these two Braak Stages. Then, we selected the intersection across the proteins with the top 20% most significant p-values and the proteins with the top 20% absolute LFC. Up- and down-regulation were considered separately.

Second, assuming that protein abundance either increases or decreases with Braak Stage, we performed a linear regression model in which the six Braak stages were treated as continuous variables with equal numerical distances. Here, we selected the intersection of the proteins with the top 20% most significant p-values in the coefficient of linear regressions and the top 20% absolute regression coefficients. Up- and down-regulation were also considered separately.

The union of the results under each assumption was termed the “Braak stage-related proteins”, which we will refer to as ‘B-proteins’ in subsequent analysis. The proteins identified under each assumption are separately presented in two volcano plots using the ggplot2 (version 3.3.5) R-package (Figure S4.3). Also, we used the VennDiagram (version 1.6.20) package in R to indicate the intersection between the proteins identified under each assumption (Figure S4.3).

4.3.8. AGE-RELATED PROTEIN ANALYSIS: AGE-PROTEINS

Age-related proteins, to which we will refer as “Age-proteins" were identified by applying a linear regression model to ND brain donors, i.e., correlating the level of each protein with age-at-death. To remove person-specific differences we smoothed the observed protein abundances with samples of ages that are close. We first sorted the ND samples according to increasing age. Then, for each center data point of each age, we selected the left five nearest data points and right five nearest data points and then calculated the average protein abundance from these 11 data points. The extremes for which they were no five samples left or right, were excluded. For each protein, we fitted a linear regression model across smoothed abundance and age. P-values of the model coefficients were corrected for multiple testing using “Bonferroni” across all proteins tested. Changes were considered significant when corrected p-values were <0.05. Proteins that change in abundance with age are presented in volcano plots using the ggplot2 package (version 3.3.5) in R (Figure S4.4).

4.3.9. CLUSTERING OF PROTEINS

We then used hierarchical clustering to cluster the protein abundances (Pearson correlation coefficient as distance, Ward’s method as linkage[19]). For B-proteins, the clustering was performed using protein abundances observed in AD patients and ND individuals, and for Age-proteins, the clustering was performed using protein abundances observed in ND individuals. The number of clusters for proteins was defined by evaluating the height of the dendrogram.

4.3.10. CENTENARIAN-SPECIFIC BRAAK-RELATED PROTEINS: CEN-B PROTEINS

For each B-protein, we investigated whether the protein abundances between AD cases and centenarians at Braak stage IV differed using a t-test; both groups have the same level of NFT pathology according to Braak stages, but different cognitive status. The p-values were corrected for multiple testing using the “Benjamini&Hochberg” method, and centenarian-specific B-proteins, to which we will refer as “Cen-B proteins", were assessed while adhering to a 5% FDR cut-off.

4.3.11. CENTENARIAN-SPECIFIC AGE-RELATED PROTEINS: CEN-AGE PROTEINS

Next, we set out to identify protein abundances that are significantly different in centenarians than would be expected based on their age. For this, we extrapolated the abun-

dances of each Age-protein to ages >100 years (centenarians) according to the associated regression coefficient of the fitted linear model on the non-demented individuals. Then, to identify centenarian-specific age-related proteins, “Cen-Age proteins", we used a one-sided t-test (FDR<0.05) to identify significant differences between observed and expected protein abundances in centenarians.

4.3.12. PROTEIN-DEPENDENT BIOLOGICAL AGE OF CENTENARIANS

Next, to estimate the protein-specific biological brain-age of the centenarians, we calculated the difference in the average protein abundances between centenarians and younger non-demented age-continuum. For each Cen-Age protein, we grouped the non-demented individuals per 10-year age-interval (because the centenarian dataset included centenarians between 100 to 111 years old). Next, we calculated the absolute difference in the average protein abundances between centenarians and non-demented individuals from each age-interval. The age-interval resulting in the minimum absolute difference was then considered to be the biological age-interval for the centenarians based on the Cen-Age protein of interest.

Next, we assigned the mean age of non-demented individuals in this age-interval as the biological age of (all) centenarians, and the difference between this age and the mean age of the centenarians represents the presumed number of years the centenarians are biologically younger than their chronological age based on this Cen-Age protein. For each Cen-Age protein, this number of years was calculated, and the median years with interquartile range (IQR) across all Cen-Age proteins indicated how many years centenarians are biologically younger than their chronological age overall based on all Cen-Age proteins.

4.3.13. PATHWAY ENRICHMENT ANALYSIS

Pathway analysis was performed using topGO package in Bioconductor. The classic “Fisher” test was used to calculate the p-value, and the nodeSize was set to 5. The p-values were corrected using “Benjamini&Hochberg” method, and the significance of enriched gene ontology (GO) terms was assessed while adhering to a 5% FDR cutoff.

4.3.14. CELL TYPE ENRICHMENT ANALYSIS

Cell type enrichment analysis was performed as described previously[20]. Since the brain proteins were measured from the tissue of the middle temporal lobe, we used a combination of single-cell RNA-seq data of 466 cells from eight adult control donors[21] and single-nuclei RNA-seq data of 15,928 cells from eight adult control donors[22] from temporal cortical tissue to generate the normalized gene expression data and cell type label matrices, which were subsequently used for expression-weighted cell type enrichment analysis using the EWCE R-package, version 1.2.0[23]. In addition, if a protein that was measured from our samples showed values ≥ 0.5 for a certain cell type in the normalized gene expression data and cell type label matrices, it was considered a protein marker of that cell type.

4.3.15. ONLINE PROTEIN BROWSER

A protein browser is available online at "https://abc-age-3dplot.shinyapps.io/100_plus_protein_viewer/". By querying a protein symbol, the correlation with Braak stage and age, the enriched cell type, and the expression distribution in cohorts of the queried protein is displayed. It can also support multiple-proteins comparison in the "Age vs. Braak stage" tab, in this case, the average expressions across age and Braak stage for the queried proteins can be compared. In the "Centenarian specificity" tab, proteins that show centenarian specificity in terms of Braak stage and age can be inspected. The cohorts used in this analysis can be seen in the "sample characteristic" tab.

4.4. RESULTS

In this study, we aimed to identify proteins for which the abundance changed in an age- and/or pathology-dependent manner and investigate the abundances of these proteins in centenarians to identify protein abundances that make centenarians unique. We identified and quantified 4,829 unique proteins in the whole tissue proteome from the 210 individuals. Sample filtering brought the final number of samples down to 190: 88 AD patients, 53 ND controls, and 49 centenarians. Peptide filtering brought the final number of proteins down to 3,448. We used "Combat" to remove technical errors explained by MS-batch.

4.4.1. PROTEINS CHANGE WITH INCREASING BRAAK STAGE BUT NOT WITH INCREASING AMYLOID STAGE

First, we investigated the correlation between protein abundances and Braak stages in the temporal lobe of all non-demented individuals: 53 ND individuals and 49 centenarians, covering Braak stages 0-V and ages 50-111. Since the Braak stage increases with age in non-demented individuals[6, 7, 24], age correction could not be performed without losing the signal. Therefore, this analysis represents changes in protein abundance in the temporal lobe, with increasing age combined with increasing Braak stage: we identified 76 proteins after FDR correction (Figure 4.1A). For comparison, in the temporal lobe of all 88 AD patients, spanning Braak stages IV-VI, and ages 55-102), the abundance of 1,204 proteins significantly correlated with Braak stages, of which 53 overlap with the changes observed in non-demented individuals (Figure 4.1B, Table S4.3). Note that MAPT was among the 1,204 AD-associated proteins, but not among the 76 age/Braak stage related proteins.

In contrast, in the temporal lobe of the same group of non-demented individuals covering NIA Amyloid stages 0-3, we found that the abundance of only two proteins significantly correlated with NIA Amyloid stages: amyloid precursor protein (APP) and CD44 (Figure 4.1C). Interestingly, for the APP protein abundance, the signal was carried by the peptide in the Aβ fragment (LVFFAEDVGSNK), as other APP peptides including the STNLHDYGMLLPCGIDK peptide, which represents the copper binding domain (CuBD) of APP[25], remained stable across NIA Amyloid stages (Figure 4.2, Figure S4.5). Intriguingly, we observed that in the temporal lobe of AD patients (n=88), changes in protein abundance between NIA Amyloid stages 2-3 are not observable in this experiment (Fig-

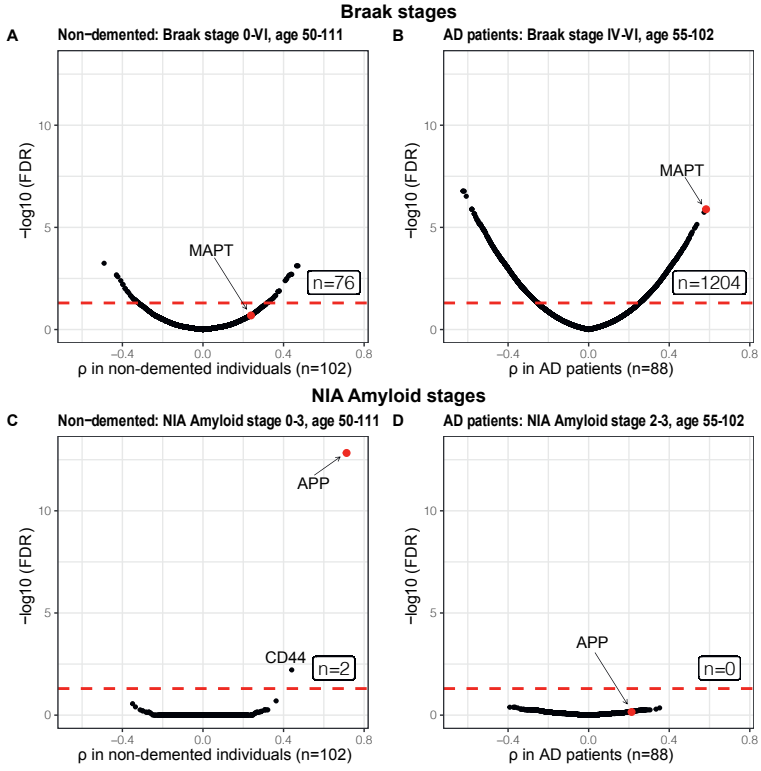

Figure 4.1: Correlation of brain proteomics with Braak and NIA Amyloid stages. Pearson correlation between protein abundances and Braak stages of each protein in non-demented individuals: 53 ND individuals and 48 centenarians (**A**) and 88 AD patients (**B**). Pearson correlation between protein abundances and NIA Amyloid stages of each protein in non-demented individuals (**C**) and AD patients (**D**). X-axis indicates the Pearson correlation coefficient (ρ) and the y-axis shows the corrected p-value (FDR) after -log10 transform.

ure 4.1D). For this reason, we applied no further focus on NIA Amyloid stage related proteins.

4.4.2. B-PROTEINS: IDENTIFICATION OF 472 PROTEINS THAT CHANGE ABUNDANCE WITH INCREASING BRAAK STAGE

To interrogate how the protein abundance in cognitively healthy centenarians with a specific Braak stage differs from 'normal' Braak stage-related protein changes, we first set out to identify Braak stage-related proteins across a pooled sample of AD cases and ND individuals aged 50-102, i.e. excluding the centenarians from the 100-plus Study. We used two approaches to detect Braak stage-related proteins (see Methods): using the ANOVA test, we selected the proteins that have both the top 20% most significant p-values and the top 20% largest log-fold-changes; using linear regression analysis, we

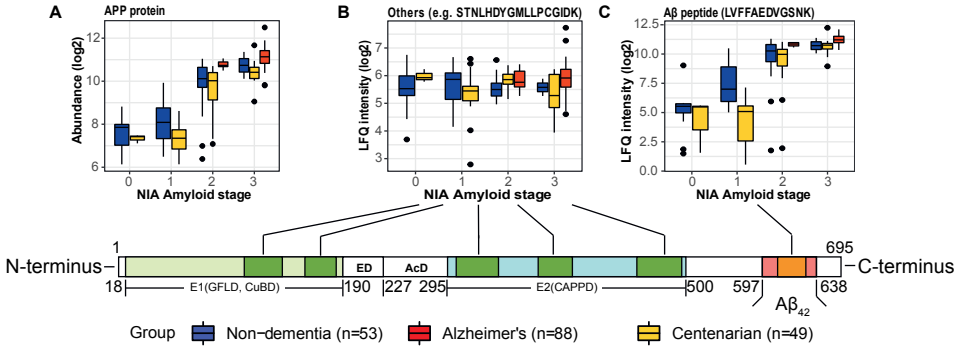

Figure 4.2: The abundance of amyloid precursor protein (APP) and APP peptides across NIA Amyloid stages. We selected the major APP isoform in the human brain as a representative, which has a length of 695 amino acids. The extracellular region of APP, much larger than the intracellular region, is divided into the E1 and E2 domains, linked by an extension domain (ED) and an acidic domain (AcD); E1 contains two subdomains including a growth factor-like domain (GFLD) and a copper-binding domain (CuBD) interacting tightly together, and E2 is the central APP domain (CAPPD). Among the measured peptides, five maintained stable abundance across the NIA Amyloid sages, including peptide STNLHDYGMLLPCGIDK; only one peptide (LVFFAEDVGSNK), which is the Aβ1-42 fragment, increased with NIA Amyloid stages. **A.** The distribution of APP abundance at each NIA Amyloid stage in ND controls, centenarians, and AD patients. **B.** Peptides from regions outside the Aβ fragment, e.g., STNLHDYGMLLPCGIDK, which represents the copper binding domain (CuBD), their label free quantification (LFQ) intensities remained stable across NIA Amyloid stages. **C.** The LFQ intensity of APP peptide segment LVFFAEDVGSNK increased with NIA Amyloid stages, which represents the Aβ1-42 fragment. All APP peptides are shown separately in Figure S4.5.

selected the proteins that have both the top 20% most significant p-values and the top 20% largest linear coefficients. Braak stage-related proteins, so-called B-proteins, comprise the union results of 472 proteins from these two approaches (Table S4.4). Of these, 246 B-proteins increased and 226 B-proteins decreased in abundance with higher Braak stages (Figure S3). Of these, 411 (87%) overlapped with the 1,204 proteins that correlated with the AD-associated increase of Braak stages (Table S4.4). Figure 4.3 illustrated the 16 proteins with the strongest correlation with Braak stages, based on the sum score of the increasing rank of p-values from ANOVA test, the increasing rank of p-values from the linear regression test, the decreasing rank of absolute log-fold-changes, and the decreasing rank of absolute linear coefficients.

To investigate the protein function of B-proteins, we first performed a hierarchical clustering, with which we identified 6 clusters: clusters C1-C2 represent B-proteins with decreased abundance with increasing Braak stage, whereas clusters C3-C6 represent B-proteins with increased abundance with increasing Braak stage (Figure 4.4A, Figure S4.6). EWCE cell type enrichment analysis indicated that C1 and C2 were both enriched with excitatory and inhibitory neuronal proteins and C3 and C6 were enriched with astrocytic and endothelial proteins (Methods and Figure 4.4B)[26, 27]. The signals in C2 and C3 were stronger than those in C1 and C6 and C4 and C5 were not enriched with

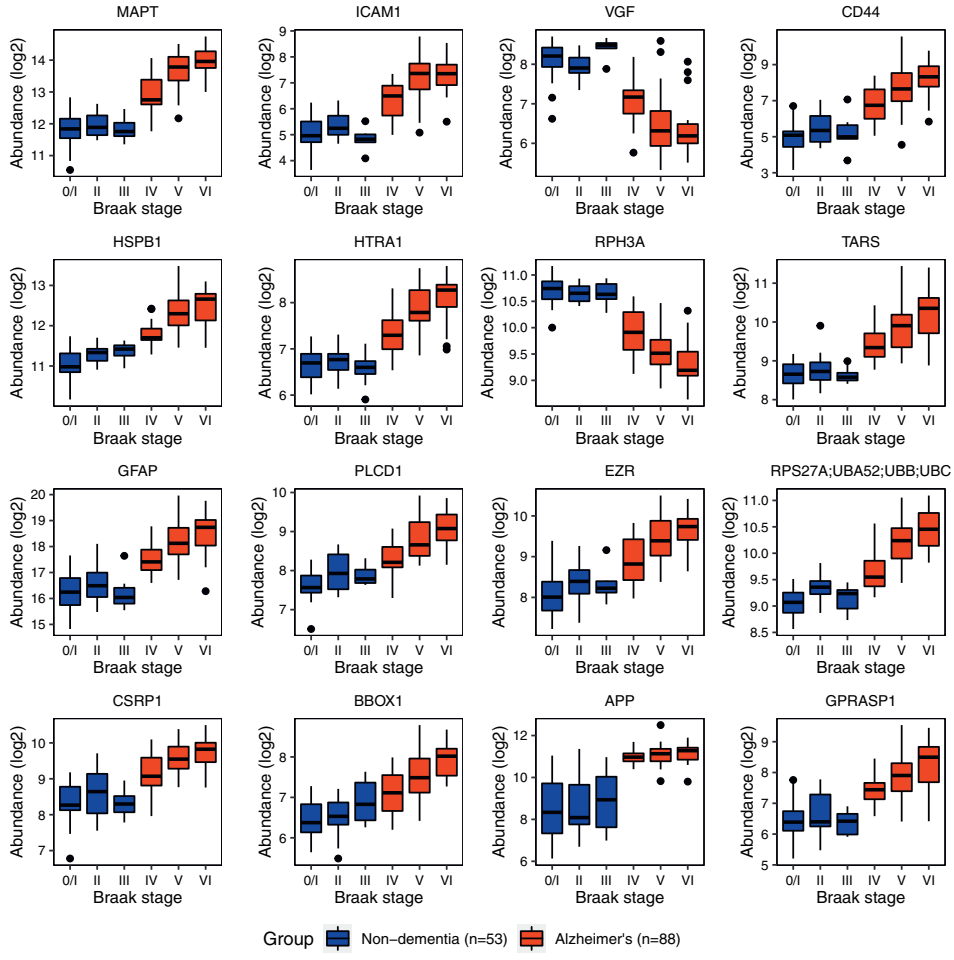

Figure 4.3: Example proteins that showed the strongest correlation with Braak stages. Y-axis indicated the protein abundances after log2 transformation; x-axis indicated the Braak stages, where Braak stage 0 and I were merged. The proteins abundances were colored based on the sample groups, i.e., blue: ND controls, red: AD cases.

proteins associated with specific cell types. A further gene ontology (GO) investigation of biological process (BP) revealed significant enrichment of proteins with ontologies for five out of the six clusters (Methods and File S5). Clusters C1, C2, and C6 were best characterized by respectively mitochondrial-, synapse-, and catabolic/metabolic-associated proteins. C3 was enriched with proteins representing multiple BP ontologies, including processes associated with epithelial cell differentiation, extracellular matrix (ECM), and intermediate filaments. C5 was enriched with proteins involved in processing pyridine-containing compounds, proteins associated with biosynthetic/metabolic processes and glucose catabolic processes.

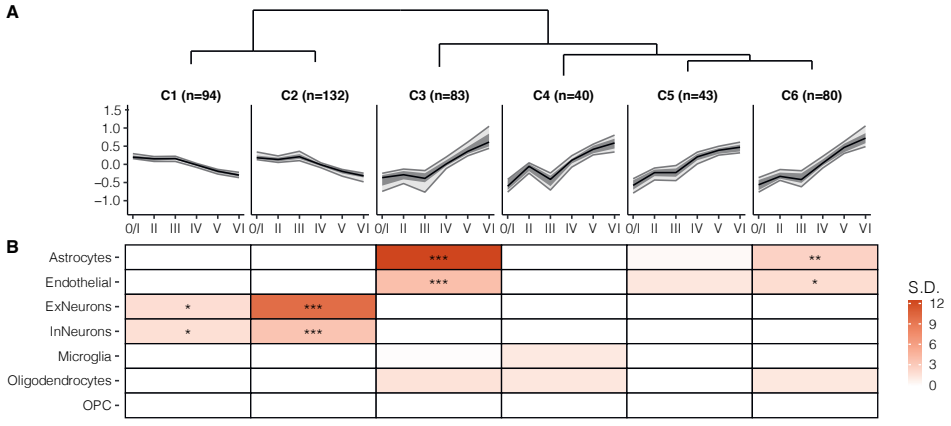

Figure 4.4: Clustering and cell type enrichment analysis for Braak stage-related proteins. A. Six intensity-correlated protein clusters were identified from the 472 Braak stage-related proteins using hierarchical clustering, of which two (C1 and C2) with median intensities increased with higher Braak stages, and four (C3 to C6) with median intensities decreased with higher Braak stages. The number n indicated the number of proteins that were assigned to the cluster. **B.** Cell type enrichment analysis for each of the six protein clusters using EWCE.

4.4.3. AGE-PROTEINS: IDENTIFICATION OF 174 PROTEINS THAT CHANGE ABUNDANCE WITH AGE IN NON-DEMENTED INDIVIDUALS

To identify protein changes associated with increasing age before 100 years, we attempted to minimize the possible confounding effects of dementia status and extreme longevity. Therefore, we used only ND controls (n=53) for this analysis. By fitting a linear regression model on smoothed protein abundance with age (see Methods), we identified 174 age-related proteins (Figure S4.7, Table S4.5). Figure 4.5 illustrated the eight proteins with the most significant correlations with age (4 positively correlated with age; 4 negatively correlated with age).

Hierarchical clustering across the 174 Age-proteins in ND controls led to the identification of 4 clusters (Figure 4.6): the abundance of proteins in cluster C1 increased with age, while the abundance of proteins in the other clusters C2, C3, and C4 decreased with age. Next, EWCW cell type enrichment analysis indicated an enriched abundance of proteins in cluster C1 associated with excitatory neurons and C3 associated with oligodendrocytes (Figure 4.6A). On the contrary, C2 and C4 were not enriched with proteins associated with specific cell types. A further GO analysis identified significant BP ontologies in clusters C3 and C4 (File S7). C3 was significantly enriched with proteins related to intermediate filament, ensheathment, and oligodendrocytes. Notably, proteins in C3 showed an accelerated decrease in abundance with age >80, indicating that oligodendrocyte-related aging may play a role after this age. The C4 cluster was significantly enriched in proteins associated with ribosome assembly, indicating interference with ribosomal biogenesis which may occur during aging.

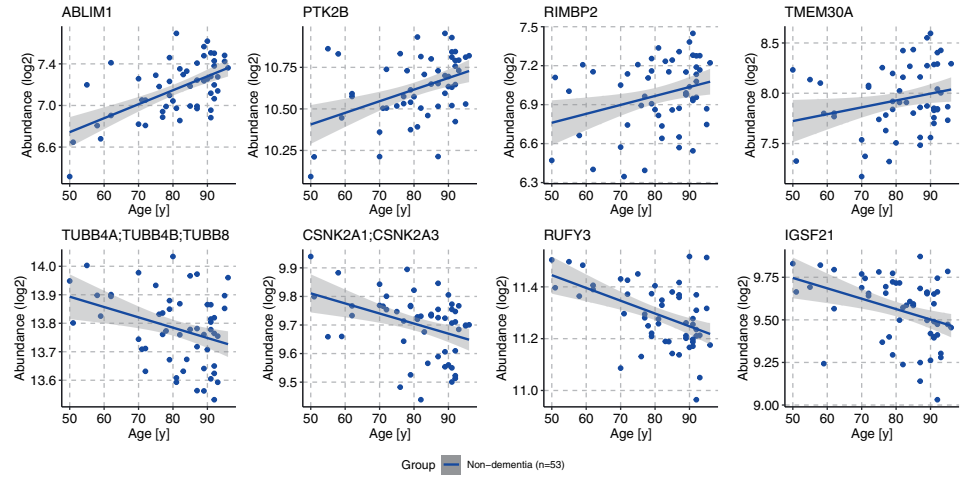

Figure 4.5: Proteins that showed the most significant correlations with ages. Y-axis indicated the protein abundances after log2 transformation; x-axis indicated the ages of ND controls.

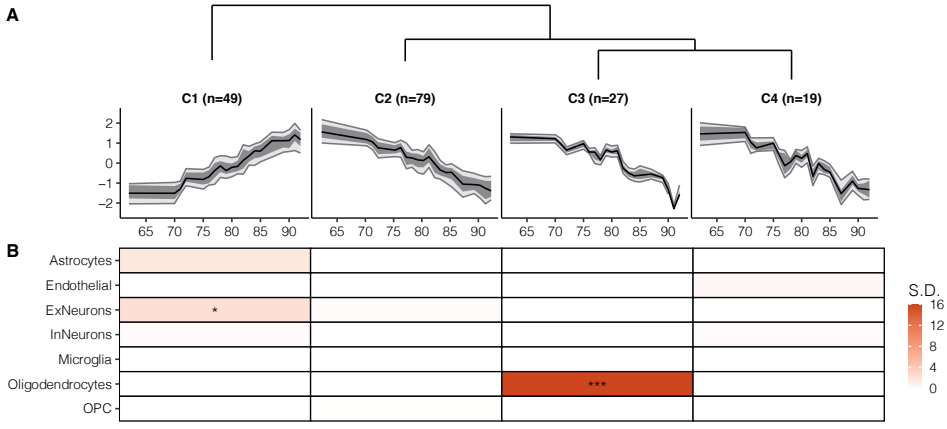

Figure 4.6: Clustering and cell type enrichment analysis for Age-related proteins. **A.** Four intensity-correlated protein clusters were identified from the 174 age-related proteins using hierarchical clustering, of which one (C1) with median intensities increased with higher ages, and three (C2 to C4) with median intensities decreased with higher ages. The number n indicated the number of proteins that were assigned to the cluster. **B.** Cell type enrichment analysis for each of the four protein clusters using EWCE.

4.4.4. CEN-B PROTEINS: THE ABUNDANCE OF 64 B-PROTEINS DIFFERENTIATES THE CENTENARIAN BRAIN FROM THE AD BRAIN

Next, we compared the protein abundance in the centenarian brain relative to what might be expected according to age or according to the level of pathology. First, we set out to identify which Braak stage-related proteins (CEN-B proteins) may explain the re-

silience to high Braak stages in centenarians. For each of the 472 B-proteins, we performed a two-sided t-test between the abundances observed in AD cases with Braak stage IV and the abundances observed in centenarians with the same Braak stage. We found that the abundance of 64 proteins was significantly different between centenarians and AD cases (FDR ≤ 0.05) (Figure S4.8, Table S4.6).

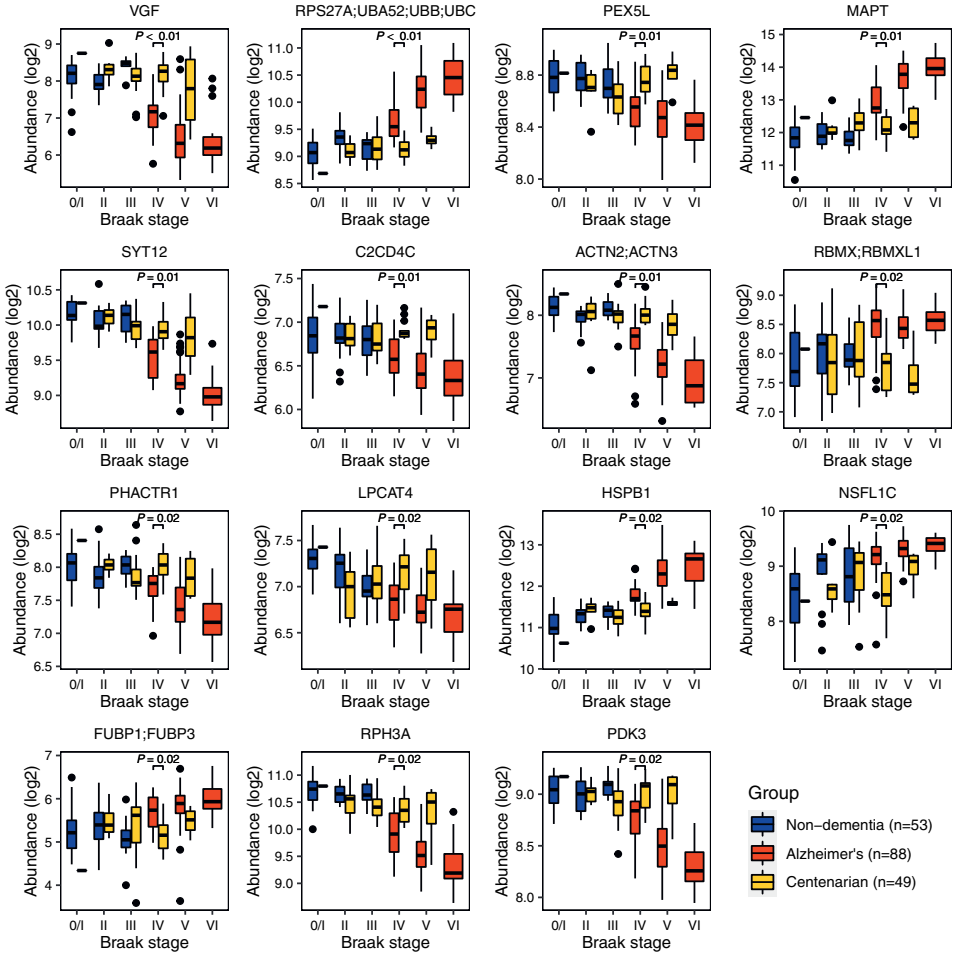

Figure 4.7: Abundance of top 15 CEN-B proteins. The abundances across Braak stages in AD, ND, and centenarian groups of the top 15 proteins with the most significant p-values. A complete list of all 64 CEN-B proteins is shown in Figure S4.8.

By mapping the 64 proteins to the six clusters of the B-proteins, we found that CEN-B proteins are significantly enriched in the protein cluster C2 (28/132) (Chi-square test, p=0.002) which is enriched in neuronal cells and involved in synapse-related BP ontologies (Figure S4.9) indicating that centenarians may specifically maintain synaptic functions compared with their counterpart Braak stage IV AD cases. The 15 most signifi-

cant CEN-B-proteins are VGF, RPS27A;UBA52;UBB;UBC, PEX5L, MAPT, SYT12, C2CD4C, ACTN2;ACTN3, RBMX;RBMXL1, PHACTR1, LPCAT4, HSPB1, NSFL1C, FUBP1;FUBP3, RPH3A, PDK3 and their abundances across Braak stages in AD, ND, and centenarian groups are shown in Figure 4.7. For a complete list of CEN-B proteins, see Figure S4.8, Table S4.6.

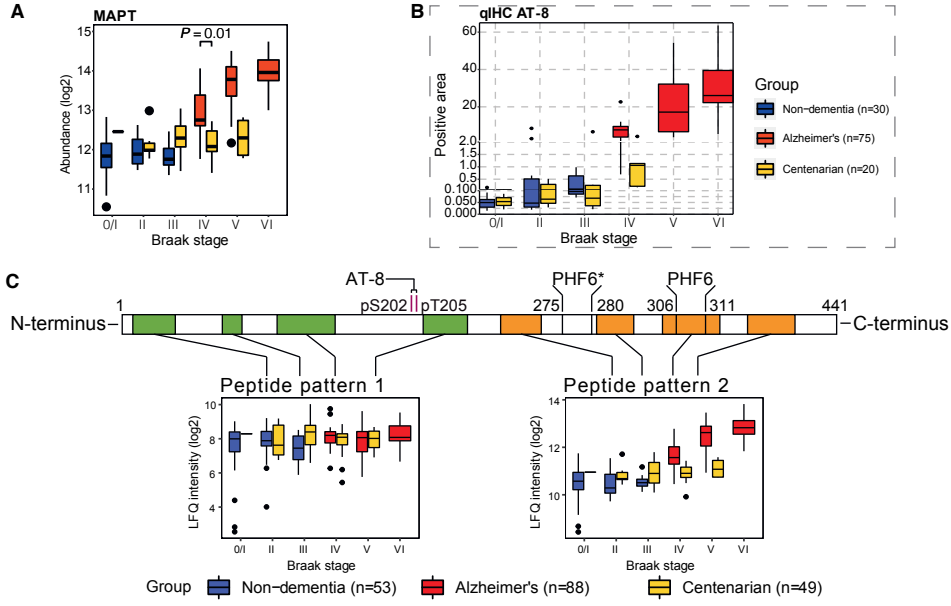

Figure 4.8: Distinguished pattern of MAPT protein in centenarian brains. A. The MAPT abundances across Braak stages in AD, ND, and centenarian groups. **B.** Quantified positive area of AT-8 staining of a subset of the cohort (20 centenarians, 75 AD cases, and 30 ND individuals) plotted against Braak stages in three different groups. **C.** Peptides from the N-terminal part of MAPT did not change with increasing Braak stages in AD nor in centenarians, while peptides from the MTBR regions increased with Braak stages in AD cases, but not in centenarians.

One of the proteins in this list deserves specific attention: the microtubule-associated protein tau (MAPT), the source protein for neurofibrillary tangles which spread to different brain regions as described by the Braak stage[15] (Figure 4.8A). To rule out that any misclassification of Braak stages could be the source of differential MAPT abundance levels, we reconfirmed the Braak stages in the five centenarians and five AD cases using gallyas and AT-8 staining. Quantitative immunohistochemistry (qIHC) for phosphorylated tau (AT-8 antibody) was available for a subset of the tissues used for this proteomics measurement (20 centenarians, 75 AD cases, and 30 ND individuals), where we also observed a deviation between AD patients and centenarians at Braak stage IV (Figure 4.8B). Upon investigation of the abundances of all measured MAPT peptides across Braak stages, we found that peptides from the N-terminus of the MAPT protein had similar abundances between AD patients and centenarians at identical Braak stages while peptides from/near the microtubule-binding region (MTBR) showed an up-regulation

with Braak stages in AD patients but not in centenarians (Figure 4.8C).

4.4.5. CEN-AGE PROTEINS: AGE-RELATED PROTEINS INDICATE THAT CENTENARIANS HAVE A YOUNGER BRAIN THAN EXPECTED FOR THEIR AGE

Next, we investigated whether the brain-aging trajectory in the centenarians deviates from non-demented individuals. We tested whether the observed abundance of each Age-protein in the centenarians matched the expected abundance, as determined when extrapolating the linear regression model, trained on all non-demented individuals between the ages of 50 and 96 to ages >100 (Methods). Among the 174 Age-proteins, 108 proteins had significant differences between observed abundance and expected abundance in centenarians (CEN-Age proteins, Table S4.7). After comprehensively investigating the 108 CA-proteins (Table 4.1), we found that several proteins showed a consistent decrease with age in ND individuals but higher levels in centenarians, or a consistent increase with age, but lower abundance in centenarians (Figure 4.9). These proteins may play an important role in against age-related decline in brain function. When focusing on the proteins for which the brains of centenarians have an abundance representative of brains from younger individuals, we identified three proteins that are part of chaperonin-containing TCP1 complex (CCT), that have an abundance corresponding to those observed in brains 26.2 years younger. The abundance of CACNA1E, a protein important for information processing by modulation of firing patterns of neurons, also corresponds to abundances observed in the brains of individuals 26.8 years younger. CACNA1E is part of several proteins associated with neuronal or synaptic activity for which centenarians have abundances representative of individuals with younger ages (RIMBP2, CACNA1E, PTK2B, NCDN, IGSF21, SYPL1, PNCK). Furthermore, we found that the abundance of several tubulin, tubulin-associated or neurofilament proteins: i.e. TUBB4A, TUBB, TUBB3, MAP1B, suggesting that the number of in-tact neurons is significantly higher in centenarians than expected for their age.
Using protein-dependent biological age estimation (Methods), we found that the average abundances of most of the 108 CEN-Age proteins in the centenarian brains showed a minimal difference between the average abundances in the brain of ND individuals aged 80-89 years old (Figure 4.10); in other words, based on these 108 proteins, the centenarian brain is, a median of 18 (IQR:13-23) years younger than their chronological age. For the 66 remaining Age-proteins, the protein abundance in the centenarian brain corresponded with the abundance in non-demented individuals with almost the highest age-interval 89-98, i.e., of a median of 11 (IQR:10-13) years younger than their chronological age (Figure 4.10).

4.4.6. ABUNDANCE CHANGES OF PREVIOUSLY AD-ASSOCIATED PROTEINS IN AD AND AGING

Lastly, from the Age-proteins or B-proteins, we manually selected proteins in 23 protein families or functional groups based on previous reports of their involvement in AD (Table 4.2), and analyzed the abundances across Braak Stages and aging (Figure 4.9, Figure S4.9). The protein abundances across Braak stages were investigated in the merged

Table 4.1: protein-abundance in centenarian-brains representative of younger brains

Mechanistic process	Proteins adhering to mechanistic process	Years younger in centenarians Median (IQR)
Neuronal/synaptic activity	RIMBP2, CACNA1E, PTK2B, NCDN, IGSF21, SYPL1, PNCK	26.8 (IQR:19.8-26.8)
TriC complex	CCT3, CCT6A, CCT6B, CCT6A;CCT6B	26.2 (IQR:25.7-27.1)
Cell cycle	RALA, CIT, CDK1, LZTS1, NAP1L1, NUMA1	24.9 (IQR:23.7-27.4)
Ubiquitin/protein degradation	CAND1;CAND2, USP7, USP11	23.5 (IQR:21.9-25.8)
Endolysosomal trafficking	SNX5, SNX9, STX5, TPD52L1, CHMP1A, RAP1A;RAP1B, ANXA11	22.5 (IQR:22.4-24.9)
Microtubules/neurofilament	TUBB, TUBB4A, MAP1B, INA, SIRT2, CNP, ABLIM1, NEFL, NEFM	20.0 (IQR:12.8-22.4)
Adhesion protein	MCAM, CNTN1, LGALS3BP	18.2 (IQR:16.8-20.2)
Immune	IRGQ, C2;CFB, PTGES2, SSB	17.9 (IQR:14.7-22.0)
Cellular homeostasis	HADHA, PON2, SHMT2, AK3, PLEKHB1, CARNS1	17.7 (IQR:15.9-22.2)
Ribosome	RPL31, RPS13, RPS3	17.3 (IQR:14.1-20.4)
Transmembrane signaling	PDE1A;PDE1C, GNA13, PDPK1;PDPK2P	14.0 (IQR:12.5-17.0)

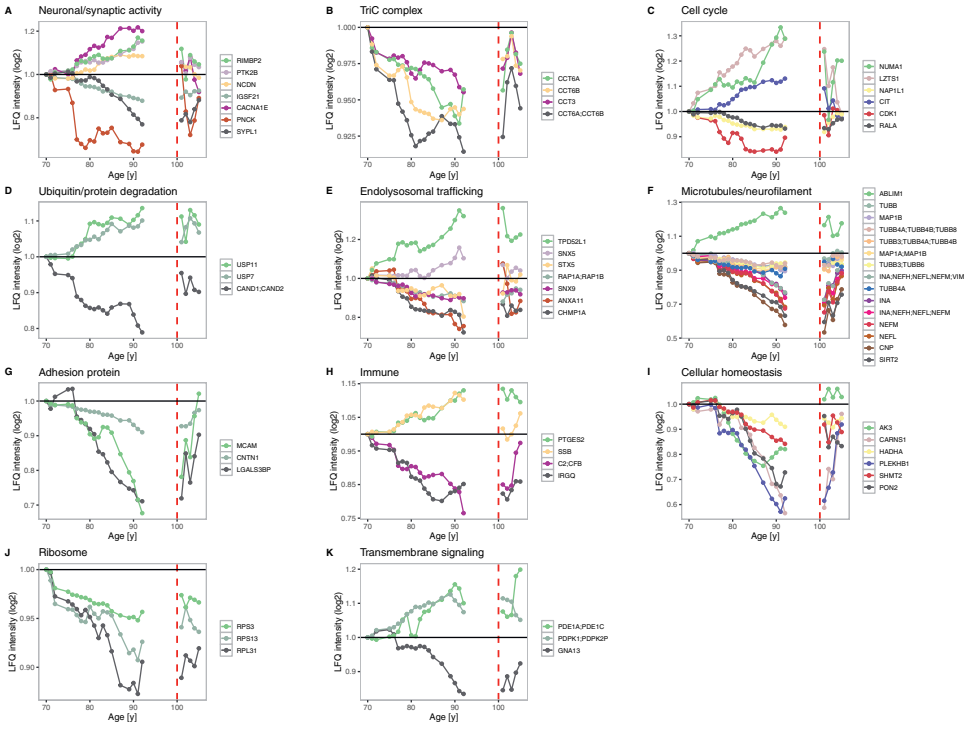

Figure 4.9: The smoothed protein abundance vs age in ND individuals and centenarians for CEN-Age proteins. For proteins belonging to each group the smoothed protein abundances (Methods) for ND individuals and centenarians is plotted versus age. The vertical red dashed line indicates the abundance in the age continuum until 100 years, and abundance levels measured in centenarians.

group of AD and ND individuals, while protein abundances across ages were investigated in the ND group only.

4.5. DISCUSSION

With this work, we were able, for the first time, to identify brain proteins associated with the molecular mechanisms that support the preservation of cognitive health until extreme ages. The centenarians of the 100-plus Study appear to defy biological aging[13, 28], which leads to the question of whether these individuals are biologically younger than their chronological age, and to what extent this is reflected in their brain proteome. Based on the abundance of 108 proteins that change with age in the temporal lobe of non-demented individuals, we found that the centenarian temporal lobe is in a median of 18 years, and up to 28 years younger than expected by age. The proteins involved point towards key proteomic changes associated with cellular processes including protein aggregation, cellular senescence/cell apoptosis, proliferation, endolysosomal trafficking, neuronal and synaptic activity, and cytoskeletal proteostasis. Further, it is known that

Table 4.2: Highlighted proteins in each protein family or functional group

Group & protein	Descriptions
Cytoskeleton: B-proteins: MAPT Age-proteins: None	From the selected 12 proteins in the cytoskeleton protein family, only the MAPT protein shows a strong increase in abundance after Braak stage III, (Figure 4.11a.1). Intriguingly, MAPT does not change with increasing age. (Figure 4.11a.2)
Intermediate filaments (IF): B-proteins: GFAP, VIM, and SYNM Age-proteins: INA, NEFL, NEFM, and NEFH	Type III (GFAP and VIM) and type IV (SYNM) IFs increase strongly after Braak stage III (Figure 4.11b.1). Neurofilaments (i.e., INA, NEFL, NEFM, and NEFH), which are abundant along the axons of vertebrate neurons, decrease consistently with age (Figure 4.11b.2).
Proteasome: B-proteins: PSME1, PSME2 Age-proteins: None	The abundance of proteasome activators PSME1 and PSME2 but not PSME3 are strongly increased after Braak stage III, whereas the abundance of most 20S and some 26S proteasomal subunit proteins increased moderately (Figure 4.11c.1). No significant correlation with age was observed for the abundance of proteasome proteins (Figure 4.11c.2).
Mitochondrial proteins: B-proteins: SLC25A46 Age-proteins: None	Mitochondrial proteins related to fission and fusion show a strong overall down-regulation after Braak stage III, for example SLC25A46 (Figure 4.11d.1). No explicit changes are observed with age (Figure 4.11d.2).
Synaptic proteins: B-proteins: STX1A, SNAP25, SYT1, SYNGR1, DLG4, SYNGAP1, GRIN1, GRIA2, GRIN2B, GRIA3 Age-proteins: SHANK2	Abundance of synaptic proteins have previously been shown to be substantially decreased in AD. We observed a strong decrease after Braak stage III for the abundances of presynaptic scaffolding-associated proteins STX1A and SNAP25, presynaptic vesicle release-associated proteins SYT1 and SYNGR1, postsynaptic scaffold-associated proteins DLG4 and SYNGAP1, and ionotropic glutamate receptor proteins GRIN1, GRIA2, GRIN2B, and GRIA3, (Figure 4.11e.1, f.1, g.1, and h.1). With the exception of the abundance of postsynaptic scaffold protein SHANK2, we observe no relationship with age for these proteins, (Figure 4.11e.2, f.2, g.2, and h.2).
Myelin proteins: B-proteins: None Age-proteins: PLP1, CNP, MAG, MOG	The abundance of the myelin proteins PLP1, CNP, MAG and MOG strongly decrease with age (Figure 4.11i.2) but not with Braak stage (Figure 4.11i.1).

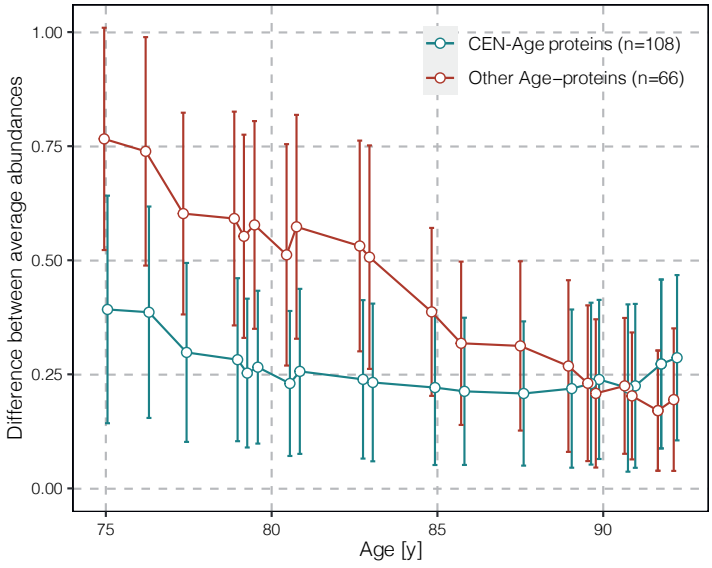

Figure 4.10: The absolute differences in the average abundances between centenarians and ND controls in age intervals. Blue line: the average absolute differences across 108 CEN-Age proteins. The minimum difference was observed at age 85-88, so the protein abundance in centenarians resembles the protein abundance observed in lower-aged ND controls. Red line: the average absolute differences across 66 other Age-proteins. The minimum difference in protein abundance between centenarians and ND controls was observed at the highest ages, so protein abundance in centenarians resembled the protein abundance observed in ND controls with the highest ages. The vertical bars indicated the standard error of the average absolute difference. Average protein abundances in ND controls were measured across samples covering a window size of 10 years. See 'protein-dependent biological age of centenarians' in Methods.

NFTs and Amyloid plaques accumulate in the brain of non-demented elderly[6, 7] including the cognitively healthy centenarians as studied in this work[24]. Here, we found that increasing Braak Stages in the temporal lobe of non-demented individuals (including the centenarians) involves a changed abundance of 76 proteins, while in individuals with AD increasing Braak stages involved a changed abundance of many more, 1204 proteins. In sharp contrast, proteins involved in increasing Amyloid stages in the temporal lobe of ND individuals and centenarians are limited to Amyloid beta and CD44. These results suggest that the Braak stage is a stronger indicator of disease-associated proteomic changes in the temporal lobe than the Amyloid stage.

With increasing age, we observe a marked decrease in the abundance of proteins such as microtubules, actin, neurofilaments and myelin that make up the cytoskeleton of brain cells, including neurons and neuronal synapses. The abundances of these proteins were all significantly higher in the centenarians of the 100-plus Study than expected for their age, suggesting that centenarians resisted the age-related loss of brain cells. One possible explanation for this may lie in the strongest signal observed in centenarians;

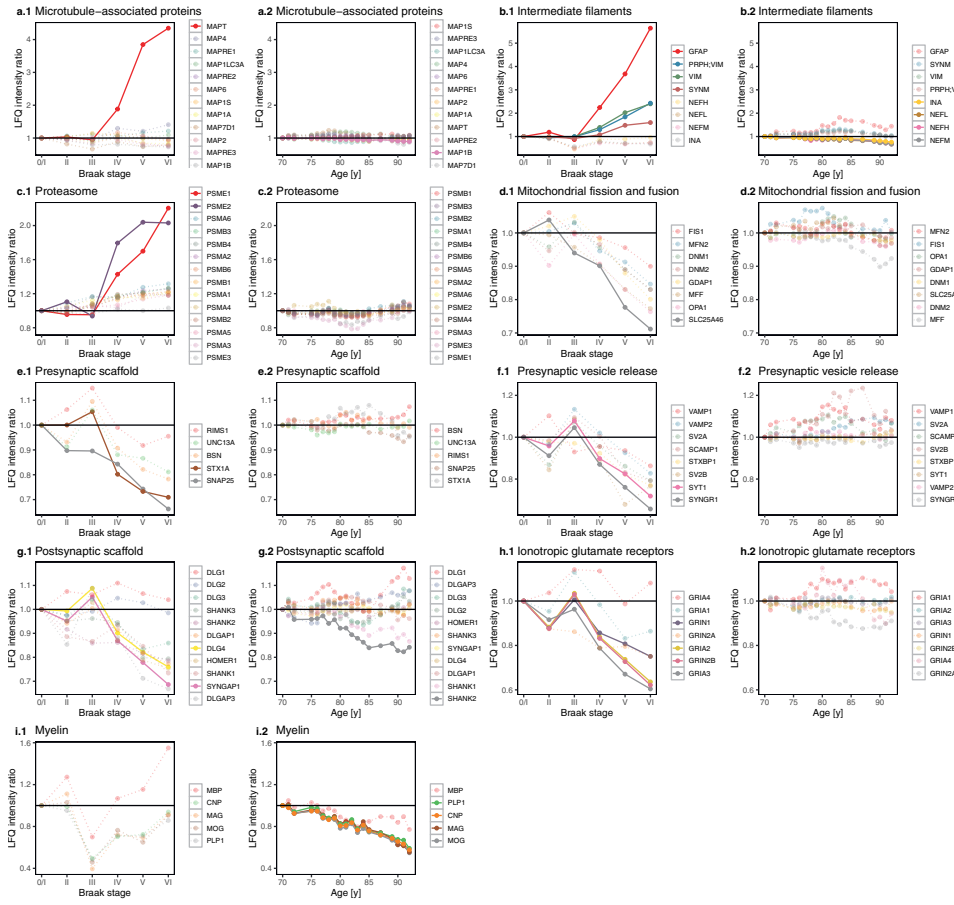

Figure 4.11: Abundance changes of selected proteins with Braak stage and age, in context of the proteins that belong to the same functional groups. Functional protein groups are displayed along Braak stages in AD and ND groups (left panel), and along age only in the ND group (right panel). Y axis: LFQ intensity ratio: the average abundance at each Braak stage (i.e., 0/I, II, III, IV, V, VI) is depicted relative to the average abundance at Braak stage 0/I. The average protein abundance of each age was calculated using a sliding window method (see Methods). Similarly, for each protein, the changes in average abundance with age are shown as the ratio relative to the average abundance at age 70. Protein names are ordered based on the abundance ratio at Braak stage VI or highest ages, respectively. Protein abundance that significantly changes with Braak stages or age (respectively B-proteins or Age-proteins) are drawn in a solid line with dark colors; non-significant changes are depicted using a dashed line with light colors. Abbreviations: LFQ, label-free quantification. (Similar plots for the other 14 additional protein families or functional groups are shown in Figure S4.9).

the maintained abundance of the CCT3, CCT6A, CCT6B subunits of the TRiC-complex, which is representative of levels observed in 26-year younger brains, the maximum observed difference between expected and observed protein abundances in centenarians.

The TRiC-mediated complex (Tailless complex polypeptide 1 (TCP-1) Ring Complex) is also known as the eukaryotic CCT (chaperonin containing TCP1) complex, which assists in the folding of up to 10% of the cellular proteome in the cytosol, including actin and tubulin, and importantly, it prevents protein aggregation[29]. The TRiC complex comprises two rings, each ring composed of eight subunits which are encoded by their own gene (CCT1 through CCT8), and with a folding cavity in the center of each ring. CTT expression data presented by Brehme et al., 2014[30], suggests that during aging, Alzheimer's and Huntington's disease, the expression of one or more CCT genes wanes, leading to loss of assembled CCT oligomer[31, 32]. In line with these previous observations, our data indicate that the abundance of CCT subunits CCT3, CCT6B, CCT6A decrease with age for most people, but that centenarians have maintained an abundance of these subunits in accordance with levels observed in 28-year younger brains. This is important, as amyloid and tau, which accumulate in AD, are client proteins of many chaperones and co-chaperones (Hsp90/CHIP and Hsp70 complexes), including CCT, that control their stabilization and degradation [29, 33]. Next to amyloid, tau, and actin, many of the proteins discussed below, which have an abundance in centenarian's representative of a much younger brain, are substrates of the TRiC-complex (i.e. CCT3, CCT6A, and CCT6B). Therefore, maintained cognitive health until extreme ages may in part depend on the prolonged TRiC-mediated proteostasis.

While Amyloid plaques accumulate in the brain of non-demented elderly[6, 7, 24], the increase of NIA-Amyloid stages from 0 to VI, which appertained to the analyzed non-demented individuals, did not correlate with changes in protein abundance in the temporal lobe, apart from changes in the abundance of the Amyloid-β peptide and CD44 protein. Thus, our results indicate that the accumulation of Aβ at a non-symptomatic, early AD stage, may have only a very limited effect on tissue protein expression. We acknowledge that we investigated only the temporal lobe cortex, but if this is at all representative of other brain regions, approaches designed to remove Aβ from the brain[34] at the pre-symptomatic stage might have a limited impact on the expression of other disease-associated proteins. Previous reports suggest that in the context of AD, Aβ deposition can affect cellular processes directly, for instance by affecting ion channel function and changing neuronal excitability[35]. However, the differential effect on protein expression between NIA-Amyloid Stages 2 and 3, appertaining to the AD patients included in this study, maybe too minor to be identified with our proteomics approach.

On the other hand, our results suggest that tau accumulation is representative of a broad spectrum of molecular changes underlying AD, even at the non-symptomatic stage. Braak stage-related proteins show linear changes in abundance after Braak stage III, i.e., in Braak stages IV-VI neurodegeneration becomes increasingly prominent in the temporal lobe[18]. Intriguingly, we observed tau spreading up to Braak Stages V in ND cases and non-demented centenarians, but MAPT levels in the temporal lobe of ND individuals and centenarians remain low with increasing Braak stages while they increase markedly in the temporal lobe of AD cases. The MAPT increase in AD cases depended fully on peptides from the microtubule binding region of tau (MTBR), which forms the aggregated core of the NFT that remains upon neuronal cell-death[36]. In line with this, AT-8 staining of the temporal lobes of AD cases and ND individuals and centenarians reveals that centenarians may have the same spatial spread of phosphorylated tau (as

measured by Braak-Stage) compared to AD patients, but with less AT-8 positive area in their brains, and with lower densities of neuropil threads than AD cases. The fact that we do not observe this increased level of MTBR-tau in ND individuals or centenarians suggests that, despite having the same Braak stages, there may be less aggregated tau and neuronal loss in centenarians than in AD cases, which may, in part, be a result of a functional TRiC complex.

Apart from MAPT, several other Braak stage-related proteins are not affected in centenarians despite having the same Braak stages. For example, synaptic proteins: VGF, STX1A, CEP170B, OLFM1, CASKIN1, and SNAP25 show a significant decrease with Braak stages in AD cases but remain high with increasing Braak stage in centenarians. These proteins were previously reported to associate with cognitive performance and neuropathology in older humans[37]: despite accumulating high levels/spreading of tau, the maintained high abundance of these proteins may have a protective effect against cognitive decline. Our proteomic analysis cannot distinguish between cause or effect regarding tau, such that it remains unclear whether removing tau would lead to a healthier brain proteomic constellation[38], or whether investments in maintenance of the abundance of other proteins may be a more successful approach.

Furthermore, for several additional proteins, the abundance in centenarians was representative of a much younger brain. For example, the age-related down-regulation of MCAM, CDK1, and WRN is associated with the acceleration of cellular senescence[39–41]. Also, the age-related decreased abundance of the PIGK, RHOG, and PNMA8B proteins might be associated with the neuronal apoptosis[42–44]. In addition, age-related down-regulation of CHMP1A and ANLN protein may associate with neuronal cell proliferation[45, 46]. For all these proteins, centenarians-maintained levels representative of a much younger brain.

Likewise, SIRT2 is an NAD+ dependent deacetylase and is well known to be involved in a multitude of age-related neurodegenerative diseases. Park and co-workers reported that SIRT2 is a tumor suppressor that connects aging, acetylome, cell cycle signaling, and carcinogenesis[47]. Increased expression of SIRT2 is a novel marker of cellular senescence dependence on wild type p53 status[48]. Furthermore, it is thought to have effects in different stages of development and aging, as well as influence myelination, which would be in line with the effect of age on myelin proteins[49–51].

Other tumor suppressors are CSDE1 and CHMP1A; Avolio and co-workers indicated that depletion of CSDE1 leads to senescence bypass, cell immortalization, and tumor formation[52] and Mochida[46] showed that CHMP1A serves as a critical link between cytoplasmic signals and BMI1-mediated chromatin modifications that regulate the proliferation of central nervous system progenitor cells. The abundances of these proteins in centenarian brains resemble the levels observed in healthy younger individuals indicating less dysregulated cell cycles. In fact, several studies have suggested that the incidence of cancer and the importance of cancer as a cause of death may decline after age 95[53, 54].

We also observed centenarian specific differential expression for four myelin proteins. MAG, which is a critical protein for the formation and maintenance of myelin sheaths[55], and MOG which has been investigated as a player in demyelination[56], which are also inhibitors of axonal sprouting needed for the formation of synapses[57,

58]. CNP is an enzyme that is essential for axonal survival[59], and SLC44A1 is a choline transporter that is involved in myelin production and membrane and phospholipid synthesis[60, 61]. Here also, the expression in centenarians resembles that observed at younger ages. These findings are of interest with respect to the known myelin sheath reduction with age[58, 62]. In particular, loss of white matter and myelin is thought to be involved in age-related cognitive decline[58, 63, 64] and myelin disruption has long been considered a feature of AD, as described already by Alois Alzheimer[58]. Our data suggest that the myelin decrease observed in AD is primarily a feature of increased age in AD cases and exemplifies that observations in AD and aging can be strongly intertwined and potentially interpreted incorrectly. As myelin proteins are markers of oligodendrocytes, the observed decrease of these proteins in abundance may indicate the consistent loss of oligodendrocytes with age in non-demented individuals, or that oligodendrocytes within the aging brain have a reduced capacity for producing and maintaining healthy myelin sheaths[65].

Similarly, the neurofilament proteins (NF), i.e., NEFL, NEFM, NEFH, and alpha-internexin (INA), show a decrease in abundance with aging, in which healthy centenarians have a substantially higher abundance of three of these four proteins (NEFL, NEFM, and INA) than healthy individuals between ages 90-100. NF proteins, especially NEFL, have recently gained a lot of attention as possible biomarkers of neurodegeneration in CSF and plasma[66] and these changes are known to be linked to changes in both grey and white matter[67]. While the exact mechanisms are not known, the increases in CSF or plasma levels are thought to be reflected by a decrease in the grey matter tissue as analyzed here. However, since NF protein levels are highly dependent on aging, caution needs to be applied when using NF as an AD biomarker. Although NFs in the brain have been traditionally viewed as axonal structural components, evidence has revealed that distinctive assemblies of NF subunits are integral components of synapses, especially at postsynaptic sites[68, 69]. Within the synaptic compartment, the individual subunits differentially modulate neurotransmission and behavior through interactions with specific neurotransmitter receptors[70]. These newly uncovered functions suggest that alterations of NFs not only underlie axonopathy in various neurological disorders but also may play vital roles in cognition. The higher abundances of these proteins in centenarian brains than in healthy individuals between ages 90-100 might explain the maintained cognition of centenarians from the 100-plus Study.

The design of our study, which includes a unique and large cohort of cognitively healthy centenarians, the availability of a large, age-distributed cohort of Alzheimer's patients and non-demented controls allowed for the first time to reveal proteins in the post-mortem brain associated with resistance and resilience against AD and proteins differentially age-expressed in centenarians. The study design also has its disadvantages. As in any cohort, neuropathology, age, and dementia status are highly entangled, such that, to investigate the correlation between protein abundances and either one of the aforementioned aspects, the other two will be confounders. To minimize the effect of confounders, different combinations of the three groups were used to answer specific questions. In our analysis, we measured the bulk proteome, such that we were not able to associate changes in protein abundance with changes in cell type. Therefore, we used single cell expression data from other datasets to assign cell types. However, we ac-

knowledge that RNA expression may in several instances not be a proxy for protein expression[71], which may affect our analysis of cell-type involvement. Furthermore, we acknowledge that we were able to observe changes only in those proteins that can currently be detected, leaving many others out of the current scope. For example, relative to other cell types, microglia are sparse in the brain, such that the detection of microglia-specific proteins may have gone unnoticed in proteomic detection. Therefore, future experiments will involve applying novel versions of proteomics, including many more proteins, on this dataset. Additionally, it may be informative to associate with tau and amyloid loads, instead of spreads, as measured by Braak stage and NIA-Amyloid scores.

4.6. CONCLUSION

Taken together, in this study we found a large group of proteins that are dysregulated related to age, AD pathology, or relevant to both of these contexts. Several distinct functional sets of proteins show coherent dysregulation. Importantly, we found a functionally diverse set of AD-related proteins that shows a non-concordant expression in Braak stage-matched centenarians and AD cases. Several synaptic proteins show specific abundances in centenarians in terms of the Braak stage, indicating that cognitive resilience potential might relate to synaptic function. Age-related proteins whose abundances in centenarians resemble the expression levels observed in a median of 18 years younger non-demented individuals might be of relevance for maintaining cognitive health at old age. Reducing cellular senescence and maintaining myelinated neuronal tracts are among the prominent features of centenarians.

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5

Identifying Aging and Alzheimer Disease–Associated Somatic Variations in Excitatory Neurons From the Human Frontal Cortex

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With age, somatic mutations accumulated in human brain cells can lead to various neurological disorders and brain tumors. Since the incidence rate of Alzheimer's disease (AD) increases exponentially with age, investigating the association between AD and the accumulation of somatic mutation can help understand the etiology of AD. We designed a somatic mutation detection workflow by contrasting genotypes derived from WGS data with genotypes derived from scRNA-seq data, and applied this workflow to 76 participants from the ROSMAP cohort. We focused only on excitatory neurons, the dominant cell type in the scRNA-seq data. We identified 196 sites that harbored at least one individual with an excitatory neuron-specific somatic mutation (ENSM), and these 196 sites were mapped to 127 genes. The single base substitution (SBS) pattern of the putative ENSMs was best explained by signature SBS5 from the COSMIC mutational signatures, a clock-like pattern correlating with the age of the individual. The count of ENSMs per individual also showed an increasing trend with age. Among the mutated sites, we found two sites to have significantly more mutations in older individuals (16:6899517 (*RBFOX1*), p=0.04; 4:21788463 (*KCNIP4*), p<0.05). Also, two sites were found to have a higher odds ratio to detect a somatic mutation in AD samples (6:73374221 (*KCNQ5*), p=0.01 and 13:36667102 (*DCLK1*), p=0.02). 32 genes that harbor somatic mutations unique to AD and the *KCNQ5* and *DCLK1* genes were used for GO-term enrichment analysis. We found the AD-specific ENSMs enriched in the GO-term "vocalization behavior" and "intraspecies interaction between organisms". Interestingly, we observed both age- and AD-specific ENSMs enriched in the K+ channels-associated genes. Our results show that combining scRNA-seq and WGS data can successfully detect putative somatic mutations. The putative somatic mutations detected from ROSMAP dataset have provided new insights into the association of AD and aging with brain somatic mutagenesis.

5.1. INTRODUCTION

Somatic mutations are post-zygotic genetic variations that can result in genetically different cells within a single organism.[1] Possible reasons for the occurrence and accumulation of somatic mutations in human brains are errors occurring during DNA replication and gradual failing of DNA repair mechanisms caused by extensive oxidative stress.[2, 3] Previous studies have shown that brain somatic mutations originating in neuronal stem/progenitor cells can lead to various neurological disorders and brain tumors.[4–6] While mutations in post-mitotic neurons have been found to play an important role in age-related and neurodegenerative diseases,[7] this association remains relatively poorly understood. The link between the accumulation of age-related mutations in neurons and neurodegenerative disease is intuitively worth exploring, considering aging is a major risk factor for many neurodegenerative diseases, like Alzheimer's disease (AD)[8].

AD is the most predominant form of dementia, and characterized by the extracellular accumulation of amyloid beta (Aβ) plaques and the intracellular aggregation of phosphorylated tau protein into neurofibrillary tangles (NFTs).[9] A recent study identified several putative pathogenic brain somatic mutations enriched in genes that are involved in hyperphosphorylation of tau.[10] These results indicate that the aggregation of these neuropathological substrates can be partly explained by the accumulation of brain so-

matic mutations, which raises a new direction for investigating the pathogenic mechanism of AD.
Most age-related somatic mutations are only present in a small group of post-mitotic neurons or even in a single neuron. For this reason, ultra-deep bulk sequencing and matched peripheral tissues are often required.[10] This type of data is often generated for one specific research question with relatively high cost and are not always available from public databases. In contrast, the availability of public single cell RNA sequencing (scRNA-seq) datasets has exploded due to continuous technological innovations, increasing throughput, and decreasing costs.[11] scRNA-seq data is most often used for expression-based analyses, such as revealing complex and rare cell populations, uncovering regulatory relationships between genes, and tracking the trajectories of distinct cell lineages in development.[12, 13] We hypothesized that scRNA-seq data can also be used to detect somatic mutations. We are not the first to realize this, in fact, other studies pioneered on different solutions to call variants in this setting. For example, Prashant et al.,[14] compares three different variant callers (GATK, Strelka2, Mutect2) and show that a two-fold higher number of SNVs can be detected from the pooled scRNA-seq as compared to bulk data. As another example, Vu et al.,[15] developed a specific variant caller (SCmut) that can identify specific cells that harbor mutations discovered in bulk-cell data by smartly controlling the false positives. Both studies applied their methodology to detect single cell somatic mutations in cancer.
In this study, we designed a workflow to detect brain-specific somatic mutation by contrasting genotypes identified with whole genome sequencing (WGS) data with genotypes identified with scRNA-seq data. To call variants in single cell data we exploit the VarTrix caller from 10x Genomics[16] and apply various filters to ensure their quality. For each putative somatic mutation, we investigated associated genes and their respective relationship with AD and age. Additionally, we investigated whether AD and age coincide with an increasing number of somatic mutations.

5.2. MATERIALS AND METHODS

5.2.1. CASE SELECTION

Single nuclei RNA sequencing (snRNA-seq) data and whole genome sequencing (WGS) data were obtained from the Religious Order Study (ROS) and the Rush Memory and Aging Project (MAP), two longitudinal cohort studies of aging and dementia.[17] Information collected as part of these studies, collectively known as ROSMAP, includes clinical data, detailed post-mortem pathological evaluations and tissue omics profiling. The snRNA-seq data used in this project were from three sources: 1) snRNAseqMFC study (n=24), 2) snRNAseqAD_TREM2 study (n=32), and 3) snRNAseqPFC_BA10 study (n=48). All specimens for these three snRNA-seq data sources were collected post-mortem from the frontal cortex, sub-regions might slightly differ between studies. The snRNA-seq data from the three studies were all sequenced according to the 10x Genomics manufacturer's protocol. Detailed information for cell partitioning, reverse transcription, library construction, and sequencing run configuration for the three studies is available on Synapse (snRNAseqMFC: syn16780177, snRNAseqAD_TREM2: syn21682120, snRNAseqPFC_BA10: syn21261143). WGS data was from a subset of the ROSMAP participants

with DNA obtained from brain tissue, whole blood or lymphocytes transformed with the EBV virus. The details for WGS library preparation and sequencing, and WGS Germline variants calling were described previously.[18] The individuals (n=90) that have both snRNA-seq data and WGS data (27 from brain tissue and 63 from whole blood) available were selected for this study. Individuals annotated with no cognitive impairment or mild cognitive impairment were defined as non-demented (ND) controls; AD patients with or without other cause of cognitive impairment were defined as AD samples.

5.2.2. STANDARD PROTOCOL APPROVALS, REGISTRATIONS, AND PATIENT CONSENTS

The parent studies and sub-studies were all approved by an Institutional Review Board of Rush University Medical Center and all participants signed an informed consent, Anatomical Gift Act, and a repository consent to share data and biospecimens.

5.2.3. CELL TYPE ANNOTATION

Each snRNA-seq dataset was separately processed for clustering and cell type annotation which was done as follows. The processed count matrix was loaded in Seurat 3.2.2.[19] The data was log-normalized and scaled before analysis. Next, with the 2,000 most variable genes (default with Seurat), principal components analysis (PCA) was performed. The number of principal components used for clustering was determined using the elbow method. Further, Seurat's FindNeighbours and FindCluster functions were used, which utilizes Louvain clustering, the resolution was set at 0.5. A UMAP plot (Supplementary Figure S5.1) was made to visualise and inspect the clusters. The following cell types were identified using known and previously used markers: excitatory neurons (*SLC17A7*, *CAMK2A*, *NRGN*), inhibitory neurons (*GAD1*, *GAD2*), astrocytes (*AQP4*, *GFAP*), oligodendrocytes (*MBP*, *MOBP*, *PLP1*), oligodendrocyte progenitor cell (*PDGFRA*, *VCAN*, *CSPG4*), microglia (*CSF1R*, *CD74*, *C3*) and endothelial cells (*FLT1*, *CLDN5*).[20] Based on the markers' expression patterns across clusters determined by Seurat's FindMarkers function, cell types were assigned to cells (Supplementary Figure S5.1). When clusters were characterized by markers of multiple cell types, they were assigned "Unknown".

5.2.4. SNRNA-SEQ SHORT VARIANTS CALLING

Single nuclei RNA reads were mapped to the reference human genome GRCh37 using STAR aligner (STAR v2.7.9a). After alignment, duplicate reads were identified using Mark-Duplicates (Picard v2.25.0) and reads with unannotated cell barcodes were removed using samtools (smatools v1.11). Reads containing Ns in their cigar string were splitted into multiple supplementary alignments using SplitNCigarReads (GATK v4.2.0.0) to match the conventions of DNA aligner. Base Quality Recalibration was performed per-sample to detect and correct for patterns of systematic errors in the base quality scores using BaseRecalibrator and ApplyBQSR (GATK v4.2.0.0). Short variant discovery was performed on chromosome 1-22 with a two-step process. HaplotypeCaller was run on each sample separately in GVCF mode (GATK v4.2.0.0) producing an intermediate file format called gVCF (for genomic VCF). gVCFs from each individual were

combined together and run through a joint genotyping step (GATK v4.2.0.0) to produce a multi-sample VCF file. Supplementary Figure S5.2 indicates the steps of snRNA-seq short variants calling in a flow chart. Variant filtration was then performed using bcftools (bcftools v1.11). A basic hard-filtering was performed using cutoffs of 1) the total read depth DP<50000; 2) the quality of calling QUAL>100; 3) the quality by depth QD>2; 4) the strand odds ratio SOR<2; and 5) the strand bias Fisher's exact test FS<10.

5.2.5. IDENTICAL INDIVIDUAL CHECK USING IBD ESTIMATION

To make sure the sequences of snRNA-seq and WGS are matching and from the same individual, we performed a pairwise identical by descent (IBD) estimation using filtered variants from snRNA-seq and WGS in a combined VCF file. The estimation was calculated using PLINK v1.9.[21] The proportion IBD value PI_HAT from the output of PLINK was used as the estimator, when the profiles are from the same individual the PI_HAT value will be close to 1, otherwise, it will be close to 0.

5.2.6. SOMATIC MUTATION DETECTION USING VARTRIX

VarTrix, a software tool for extracting single cell variant information from 10x Genomics single cell data, was used to detect somatic mutations. For single nuclei gene expression data, VarTrix requires a pre-called variant set in VCF format, an associated set of alignments in BAM or CRAM format, a genome FASTA file, and a cell barcodes file produced by Cell Ranger as input. After an exploratory phase, we observed that only cells annotated as excitatory neuron had enough read coverage for somatic mutation detection. Therefore, for each individual, a subset of the BAM file including only reads from cells annotated as excitatory neuron was used as the input of VarTrix. Correspondingly, the pre-called variant set was also detected from the subset of the BAM file which only including barcodes from cells annotated as excitatory neuron.
Human reference genome GRCh37 was used as the genome FASTA file. In this study, VarTrix was run in coverage mode generating a reference coverage matrix and an alternate coverage matrix indicating the number of reads that support the reference allele and the alternate allele. These matrices were later used for filtering variant sites and detecting somatic mutations in the excitatory neurons.
Since the snRNA-seq data were collected from three studies, the average coverage varied between different sources. To minimize the batch effect from different studies, we filtered the variant site based on the read number of each individual. Specifically, we calculated a cutoff C_i for each individual i as below:

$$C_i = \frac{n_i}{\sum_{i=1}^{N} n_i / N} C$$

where n_i is the number of reads for individual i, N is the number of individuals. The constant value C is set as 25 to guarantee that a sufficient amount of reads (>5) can support a variant site for every samples. A variant site would be used for somatic mutation detection when for all individuals the read depth at this site is higher than the cut-off C_i for that individual. Next, a somatic mutation was identified as present in one individual when: 1) the genotype of this individual at the site in WGS was ref/ref and the ratio of

reads that support the alternate allele in snRNA-seq is larger than 0.1 at the same site, or 2) the genotype of this individual at the site in WGS was alt/alt and the ratio of reads that support the reference allele in snRNA-seq is larger than 0.1 at the same site. When the genotype of an individual at a certain site was heterozygote in WGS, we ignored the site for that individual, regardless of the allele ratio in snRNA-seq, because we cannot distinguish an observed homozygous variant at a site in snRNA-seq is due to somatic mutagenesis or reads missing when there is a heterozygous variant in WGS at the same site.

5.2.7. MUTATION SIGNATURE ANALYSIS

To characterize the contribution of mutation signatures, we pooled all putative somatic single nucleotide variations (SNVs) for signature analysis. We formatted the pooled SNVs in a VCF file and used it as input for running Mutalisk[22] with the following configurations: maximum likelihood estimation (MLE) method; linear regression. The input file was compared with 30 single base substitution (SBS) signatures from the COSMIC mutational signatures database. The best model of signature combination was suggested from the tool by considering the Bayesian information criterion (BIC).

5.2.8. VARIANTS ANNOTATION AND EFFECT PREDICTION

The gene annotation and functional effect prediction for all putative variants were performed using SnpEff (SnpEff v5.0).[23] The human genome GRCh37 was used as reference genome. If there were multiple genes mapping to one variant site, the gene having higher putative effect was used for the disease and age association analyses.

5.2.9. GO-TERM ENRICHMENT ANALYSIS

The gene ontology (GO-term) enrichment analysis was performed using “topGo" package[24] in R and compressed by REVIGO[25] with semantic similarity score “Lin".[26] The genes that were annotated to the variant sites with read depths higher than the cutoffs for all samples were used as background. The p-values from the uneliminated GO-terms were corrected using “Benjamini & Hochberg" method, significant results were reported with false discovery rate (FDR) < 0.05.

5.2.10. STATISTICAL ANALYSIS

All calculations were performed using R (version 3.6.3). Wilcoxon rank sum test, linear regression, Fisher’s exact test, and logistic regression were performed using the “stats" R package.[27] By categorizing the “presence" of a somatic mutation as 1 and the “absence" of a somatic mutation as 0, the logistic function was defined as: $p = 1/(1 + exp(-(\beta_0 + \beta_1 age + \beta_2 group)))$, where age is the age of the sample at death, $group$ is the assigned group for the individual based on the cogdx category, and $\beta_{0..2}$ are the coefficients of the intercept and the explanatory variables. For this analysis, only individuals from the AD and ND group were used.

5.3. RESULTS

5.3.1. EXCITATORY NEURON-SPECIFIC SOMATIC MUTATIONS (ENSMS)

To study somatic mutations acquired over age and between demented (AD) and non-demented (ND) persons, we retrieved data from 90 participants from the ROSMAP study for which WGS data in blood or brain as well as single nuclei RNA sequencing (snRNA-seq) data of the frontal cortex was present (Methods). Since the snRNA-seq data (n=90) were collected within three different studies, the read coverage for samples varied between the studies (Figure 5.1A). To reduce the bias generated from the unbalanced read coverage, we excluded individuals (n=9) with a total read count smaller than 6×10^7, and applied a sample-specific cut-off for the required read coverage to detect a somatic mutation based on the total read count per sample (Methods). Cells from the snRNA-seq data were annotated according to seven major cell types (Methods). As the amount of cells varied for different cell types (Figure 5.1B), we first explored the feasibility of detecting somatic mutations for each cell type. This exploratory analysis showed that somatic mutations could only be detected from the excitatory neurons (when requiring a minimum number of reads (≥ 5) per sample for a putative variant site, Methods), the dominate cell type in our snRNA-seq data. This underpins that a sufficient amount of cells is needed for snRNA-seq based somatic mutation detection. As a consequence, we focus our analysis on excitatory neurons only. To further ensure data quality, we excluded individuals (n=5) which had less than 200 excitatory neurons. After filtering, 76 participants (23 from the snRNAseqMFC study, 30 from the snRNAseqPFC_BA10 study, and 23 from the snRNAseqAD_TREM2 study) had an adequate read coverage and sufficient number of excitatory neurons. The demographic data (sex, age at death, and cognitive diagnosis (cogdx) categories[28]) of these participants are given in Table 5.1. More than 72% of them were 85 years of age or older at death; 56% were women. Individuals were grouped based on their cognitive diagnosis in either being non-demented (n=42) or being an AD sample (n=33).

Table 5.1: Summary characteristics of selected sample from the ROSMAP study.

Group	Cogdx*	n	Sex	Age, mean ± SD (range)
Non-demented	1	33	23 F; 19 M	85.7 ±4.2 (76-90)
	2	8		
	3	1		
Alzheimer's disease	4	32	19 F; 14 M	87.1 ±3.9 (74-90)
	5	1		
Other dementia	6	1	1 F	83

* Cognitive diagnosis (cogdx) is defined as six categories: 1 NCI: No cognitive impairment (No impaired domains); 2 MCI: Mild cognitive impairment (One impaired domain) and NO other cause of CI; 3 MCI: Mild cognitive impairment (One impaired domain) AND another cause of CI; 4 AD: Alzheimer's dementia and NO other cause of CI (NINCDS PROB AD); 5 AD: Alzheimer's dementia AND another cause of CI (NINCDS POSS AD); 6 Other dementia: Other primary cause of dementia.

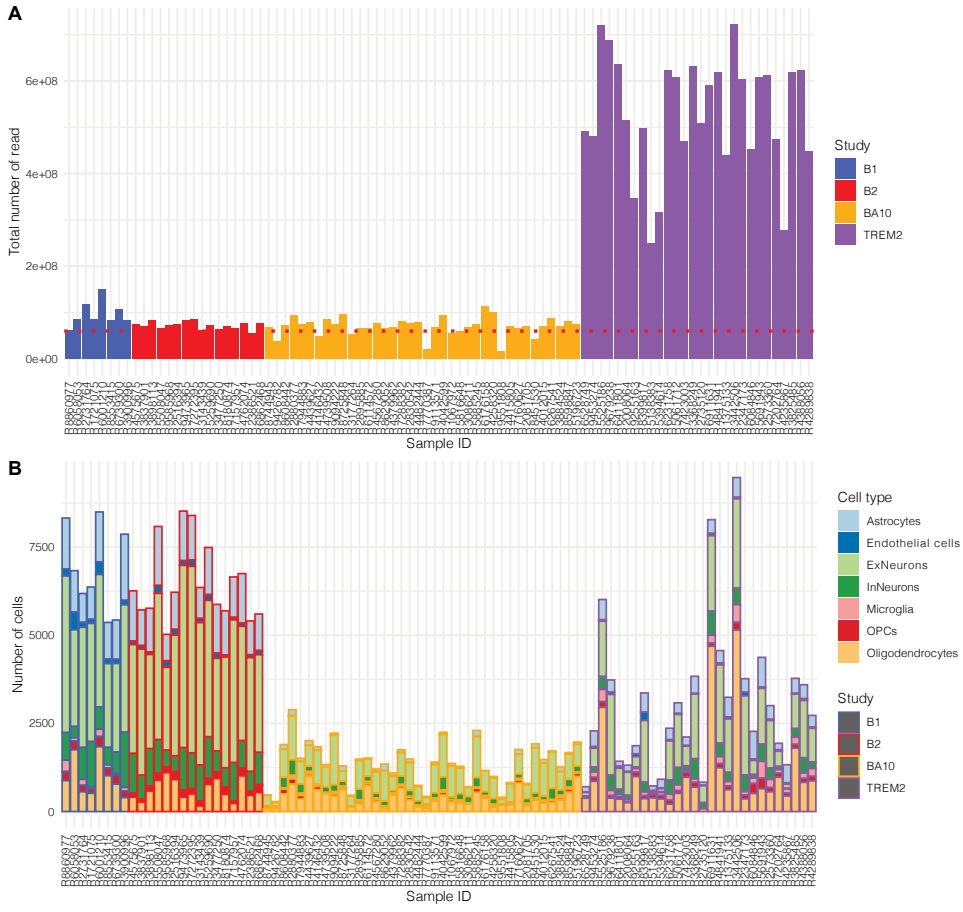

Figure 5.1: Single nuclei RNA (snRNA) reads and cell count across selected samples. Participants (n=90) from the ROSMAP project with both single nuclei RNA sequencing (snRNA-seq) data and whole genome sequencing (WGS) data available were selected for this study. **A.** The distribution of the number of snRNA reads across individuals. The dashed red line indicates the cutoff of $< 6 \times 10^7$ for the minimal read coverage, i.e. individuals below this line were excluded from the study (n=9). The colors indicated the study that included an individual. Individuals who colored either blue or red were from the two batches (B1 and B2) of the snRNAseqMFC study. Individuals colored orange were from the snRNAseqAD_BA10 study, and individuals colored purple from the snRNAseqPFC_TREM2 study. **B.** The number of cells per cell type per individual. The cell types were distinguished with seven different colors (see legend). The colors of the edges indicated different studies, as in **A**. Abbreviation: ExNeurons, excitatory neurons; InNeurons, inhibitory neurons; OPCs, oligodendrocyte progenitor cells.

5.3.2. SUMMARY OF DETECTED ENSMS

Somatic mutations in the 76 participants were detected using the workflow described in the Methods. For that the snRNA-seq data of the excitatory neurons are compared

to WGS data of blood (n=23) or brain (n=53). IBD estimation using shared variant sites confirmed the matching between the snRNA-seq and WGS samples (pair-wised PI_HAT >0.85, Supplementary Figure S5.3, Methods). From the 9,751,193 short variants called from the snRNA-seq data, we identified 196 sites that harbored excitatory neuron-specific somatic mutations (ENSMs). These genetic sites map to 127 genes (Methods), and 104 sites among them were single-nucleotide variants (SNVs). From these 196 sites, 98 were shared between multiple individuals n>2, and thus are recurrent somatic mutations (Supplementary Figure S5.4). A few sites have mutations present in almost all individual genomes, which are likely to be either RNA editing events[29]; transcription errors, which can occur in a wide variety of genetic contexts with several different patterns[30, 31]; or technical errors[32]. 53 sites have mutations uniquely present in the brains of the AD samples (Supplementary Table S5.1). Per individual genome the number of ENSMs ranged from 24 to 41. This does not seem to contradict the other observations that found an average of 12 somatic SNVs in hippocampal formation tissue using deep bulk exome sequencing[10], and an average amount of 1700 somatic mutations (substitutions 1500; indels 200) in neurons using a whole-genome duplex single-cell sequencing protocol[33]. However, this comparison might be complicated by the differences in sequencing and somatic mutation detection methods, as well as brain regions.

5.3.3. NUMBER OF ENSMS INCREASE WITH AGE

To characterize the ENSMs, a mutation signature analysis was performed on the 104 detected putative somatic SNVs (Methods). The results show that, from the 30 COSMIC mutational signatures, SBS5 best explains the observed pattern of putative somatic SNVs by Mutalisk (Figure 5.2, Supplementary Figure S5.5). SBS5 is a clock-like signature, i.e., the number of mutations correlates with the age of the individual. This suggests that the underlying mutational processes of the found ENSMs might be part of the normal aging process in excitatory neurons.[34] A previous study using bulk exome sequencing also found an abundance of the SBS5 signature in aged brain tissues.[10]

When studying the count of somatic mutation in our analyses, we found only a slight increase with age (β=0.15, Figure 5.3A) that was not statistically significant (p=0.12). Similar results were observed when performing the same analysis in AD samples and ND individuals separately (Supplementary Figure S5.6). We should note that the number of samples is relatively low and represent a relatively narrow age range (from 74 to 90 years old). Moreover, participants with an age older than 90 years were all censored by age 90, which could also influence the significance of the age trend. A significant trend is observed when we exclude individuals at age 90 from the regression (β=0.37, P=0.005; Supplementary Figure S5.7).

5.3.4. *RBFOX1* AND *KCNIP4* HARBOR AGE-ASSOCIATING ENSMS

As several detected ENSMs are being detected in multiple individual genomes (Supplementary Figure S5.4), we next tested the association of age with somatic mutation prevalence for each site individually using logistic regression (Methods). We added AD status as an explanatory term and excluded the sample with other primary causes of dementia (Methods) from this analysis. Two sites (16:6899517 (*RBFOX1*), p=0.04; 4:21788463 (*KC-*

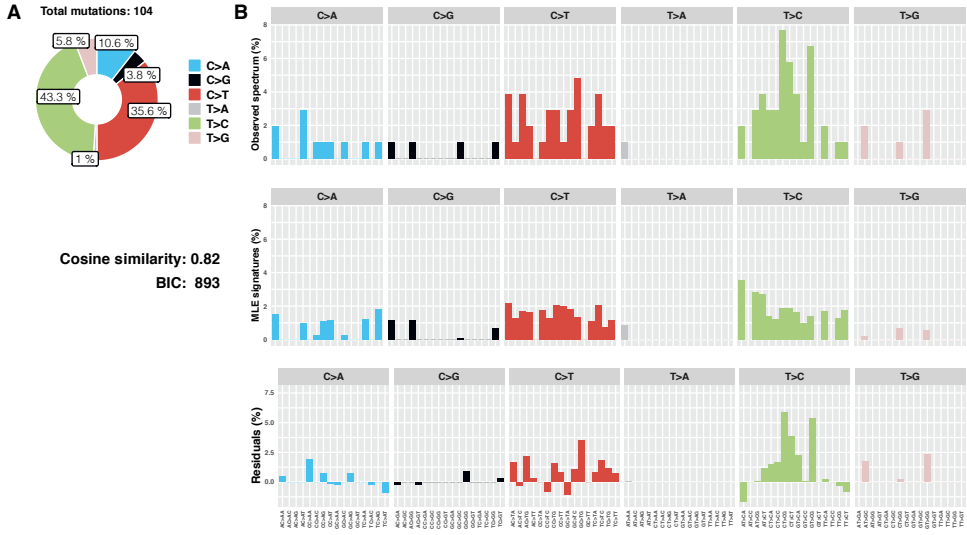

Figure 5.2: The mutation signature of 104 putative excitatory neuron-specific single nucleotide variations (SNVs) in the brain. Among the 30 COSMIC single base substitution (SBS) signatures, SBS5 was identified as the model that best explains the observed pattern of putative somatic SNVs by Mutalisk. The cosine similarity with the 104 putative excitatory neuron-specific SNVs and the corresponding Bayesian information criterion (BIC) for each COSMIC SBS signature are shown in Supplementary Figure S5.5. **A.** The percentage of each substitution subtype in the 104 putative excitatory neuron-specific SNVs. Subtype T>C and C>T are the dominate subtypes and account for 43.3% and 35.6% of the fraction separately. **B.** The top panel shows the observed distribution of 104 putative excitatory neuron-specific SNVs across the 96 possible mutation types; the middle panel shows the distribution of the identified signature (SBS5); the bottom panel shows the difference of each base substitution subtype between the top and middle panel. The same plots of the other top 5 mutational signatures in cosine similarity (i.e., signatures 25, 12, 26, and 9, except for signature 5) are shown in Supplementary Figure S5.5.

NIP4), p<0.05) are found to have significantly more mutations in older individuals. The age distributions in mutated and un-mutated samples for these two sites are shown in Figure 5.4. Some caution should be treated when interpreting this plot for individuals older than 90 years, as these are all mapped to 90 years old. Such that to assess the effect due to censored age, we performed a sensitivity analysis by removing samples with age ≥ 90. The results indicated stronger signals for these two sites (16:6899517 (*RBFOX1*), p=0.02; 4:21788463 (*KCNIP4*), p=0.03; Supplementary Figure S5.8).

5.3.5. ENSM SITES IN *KCNQ5* AND *DCLK1* ASSOCIATE WITH AD STATUS

Genes that were enriched with somatic mutations in AD samples might have a higher possibility to be associated with AD. We found 53 ENSM sites that were only detected in AD samples. This prompted the question whether the number of ENSMs associate with AD status. A Wilcoxon rank sum test indicated that there was no significant difference

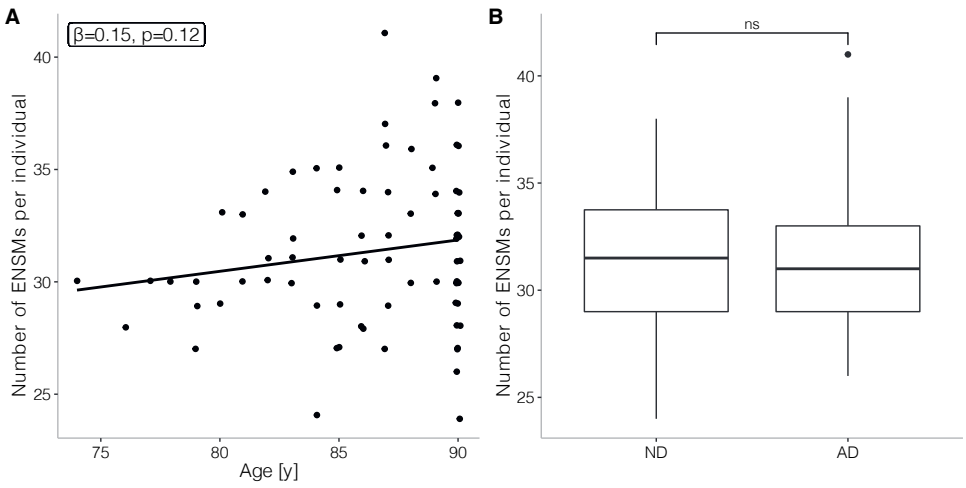

Figure 5.3: Quantitative comparison of the number of excitatory neuron-specific somatic mutations (ENSMs) in terms of AD and aging. A. The number of ENSMs per individual against the age of the individual. The line shows how this number regresses with age. The significance of the coefficient ($\beta \neq 0$) was tested using a t-test. The same analysis for AD and non-AD samples separately is shown in Supplementary File S5.6. **B.** Boxplot of the number of ENSMs in non-demented controls (ND) and AD patients (AD). The Wilcoxon rank sum test does not show a significance difference (ns).

5

(p=0.71) in the average count of ENSMs between AD samples and non-demented controls (Figure 5.3B). This finding is in line with a previous report[10, 33, 35] that indicated that somatic mutations are associated with AD in certain patterns, but not by amount.
Next, we examined whether the occurrence of an ENSM is overrepresented within AD samples. A Fisher's exact test that identifies sites that have a higher odds ratio to detect a somatic mutation in AD samples (Methods), yielded two sites with significant odds ratios. These sites are mapped to two genes (6:73374221 (*KCNQ5*), p=0.01, and 13:36667102 (*DCLK1*), p=0.02).

5.3.6. GENES HARBORING AD SPECIFIC ENSMS DO RELATE TO ALZHEIMER OR PROCESSES INVOLVED IN ALZHEIMER

The 53 AD specific ENSM sites map to 42 genes. When we exclude genes for which also an ENSM occurs in an ND individual (n=10), we end up with 32 genes that have ENSMs only seen in AD samples (Supplementary File S5.2). Among these 32 genes, there are several well-known AD-associated genes, like *SLC30A3*, *TTL*, and *CTSB*, which thus harbor somatic mutations unique for AD.
Together with the two genes for which AD samples had a higher occurrence of ENSMs (*KCNQ5* and *DCLK1*), we conducted a GO-term analysis to investigate the biological pathways that may be involved (Methods). The most enriched biological process is "vocalization behavior" (FDR<0.001); also, "intraspecies interaction between organisms" is found to be significant (FDR<0.04), and detected genes with these functions

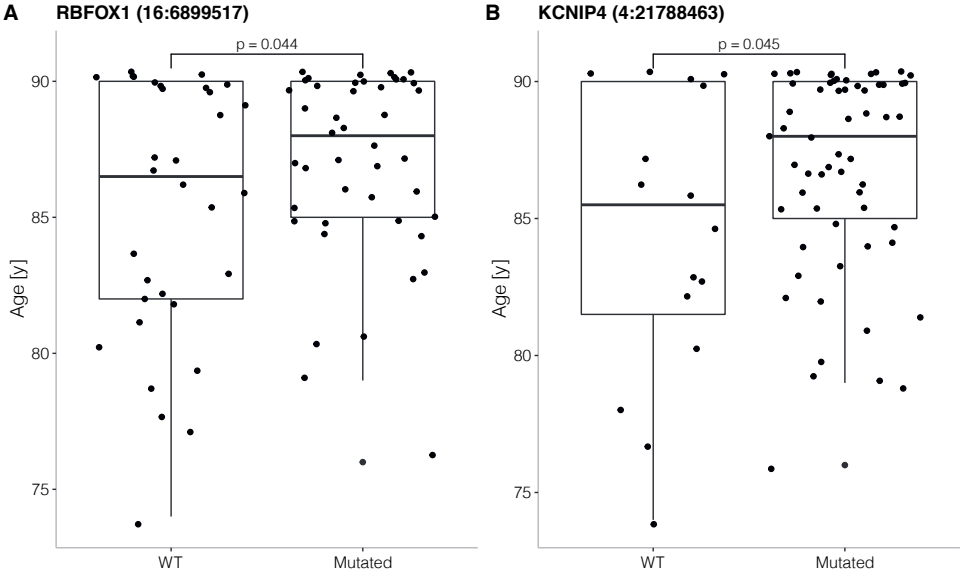

Figure 5.4: The occurrence of somatic mutation with age in (A) *RBFOX1* and (B) *KCNIP4* genes. Red dots: AD cases; blue dots: non-demented (ND) individuals. Logistic regression was used to test the prevalence of somatic mutations with increasing age.

are *DLG4*, *CNTNAP2*, and *NRXN3* (Figure 5.5). Our results also identified a group of genes (*CACNA1B*, *CNTNAP2*, *DLG4*, *KCNQ3*, and *KCNQ5*) enriched with the GO-term "ion channel complex" (FDR<0.03). *KCNQ* genes encode five members of the K_v7 family of K^+ channel subunits ($K_v7.1$–7.5). Four of these ($K_v7.2$–7.5) are expressed in the nervous system.[36] Concerning AD-related neuropathology, a link between Aβ accumulation and K_v7 channels has been reported by some studies.[37, 38]

5.4. DISCUSSION

Late-onset Alzheimer's disease, whose incidence increases with age, is often referred to as an age-related disease. Although the accumulation of Aβ peptide and phosphorylated tau protein are the neuropathological main characteristics of AD, they fail to fully explain the molecular pathogenesis. As such, a cell-level investigation might be necessary to study the underlying pathogenic mechanism. Here, we identified somatic mutations using public data collected from 76 ROSMAP donors and investigated their associations with AD and aging.

Although scRNA-seq data are normally used for expression-based analyses, our results have shown that scRNAseq data can be used for the detection of somatic mutations at a cell-type specific level. As long as RNA sequences align correctly to a reference genome, the pipeline that was used for variant calling can be used for both bulk RNA-seq and scRNAseq data.[39] However, calling variants for each cell separately is not efficient, suffers from low coverage, and each cell is likely to have a unique set of identified variants.

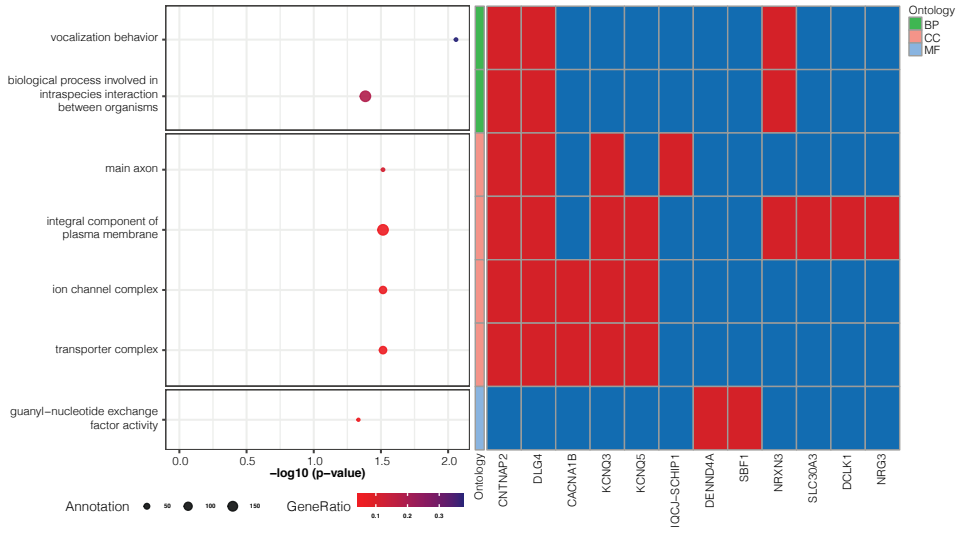

Figure 5.5: GO-terms enriched with genes having AD-specific ENSMs. 32 genes that have ENSMs only seen in AD samples, and the *KCNQ5* and *DCLK1* genes that have a higher occurrence in AD samples are used in the GO-term enrichment analysis. The left panel of the figure shows the enriched terms, their corrected p-value, the number of genes annotated with that term (size of circle), and the fraction of overlapping genes that harbor an AD-specific ENSM (color of circle). The FDR corrected significant GO-terms are grouped into three categories: Biological Process (BP), Cellular Component (CC), and Molecular Function (MF). The right panel shows the subset of genes having an AD-specific ENSM that are annotated with the enriched GO terms, red squares, while a blue square indicates that the gene does not have that annotation. Those genes that are not annotated with any of these GO-terms are not included in this panel.

For this reason, we aggregated cells per individual and per cell-type, generating cell-type specific pseudo-bulk data. An exploratory run of this workflow revealed that we were only able to confidently detect somatic mutations for excitatory neuron as this was the most abundant cell type in the snRNA-seq data and thus resulting in sufficient read coverage. Hence, it is imperative to have a sufficient amount of cells or relatively deep sequencing to reliably detect somatic mutations from scRNA-seq data.

Our analysis showed that the prevalence of somatic mutations in the *KCNIP4* and *RBFOX1* genes are associated with increasing age (when corrected for AD status). *KCNIP4* encodes a member of the family of voltage-gated potassium (K^+) channel-interacting proteins (*KCNIPs*), which suggests altered ion transports/channels may be associated with the aging process.[40] *RBFOX1* is a neuron-specific splicing factor predicted to regulate neuronal splicing networks clinically implicated in neurodevelopmental disorders.[41, 42] The increased somatic mutations in *RBFOX1* with age indicates neurodevelopmental disorders may also associate with human brain aging.

We detected the occurrence of somatic mutations within some well-known AD-associated genes, like *SLC30A3, TTL*, and *CTSB*. *SLC30A3* is known to be down-regulated in the prefrontal cortex of AD patients.[43] *SLC30A3* is assumed to play a protective role

against ER stress, which has been thought to be involved to neurodegenerative diseases such as AD.[44] *TTL* is a cytosolic enzyme involved in the post-translational modification of alpha-tubulin.[45] A previous study found that levels of *TTL* were decreased in lysates from AD brains compared to age-matched controls and that, in contrast, D2 tubulin was significantly higher in the AD brains, indicating that loss of *TTL* and accompanying accumulation of D2 tubulin are hallmarks of both sporadic and familial AD.[46] Gene *CSTB* encodes cystatin B (CSTB), an endogenous inhibitor of cystine proteases.[47] Human *CSTB* has been proposed to be a partner of Aβ and colocalises with intracellular inclusions of Aβ in cultured cells.[48] Protein levels of CSTB have been also reported to increase in the brains of AD patients.[49] Apart from these well-known AD-associated genes, we also identified the *DCLK1* gene harbored more somatic mutations in AD patients. A study reported that *DCLK1*, which has both microtubule-polymerizing activity and protein kinase activity, phosphorylates *MAP7D1* on Ser 315 to facilitate the axon elongation of cortical neurons.[50] These observations suggest that somatic mutations may initiate or are involved in the AD process in many ways.

Advance AD-related dementia is often accompanied with language problems, behavioral issues and cognitive decline.[8] Our results identified AD-associated somatic mutations in the genes *CNTNAP2*, *DLG4*, and *NRXN3*, which are involved in, among other processes, vocalization behavior and intraspecies interaction between organisms. These results may indicate that AD-related speech or language problems and withdrawal from social activities might be associated with somatic mutations in excitatory neurons. In addition, we identified AD-associated somatic mutations in *CACNA1B*, *CNTNAP2*, *DLG4*, *KCNQ3* and *KCNQ5*, which are all ion-channels or involved with ion-channels. Previous studies have reported on the possible role of altered neuronal excitability, controlled by different ion channels and their associated proteins, occurring early during AD pathogenesis.[51, 52] Specifically K^+ channels which are the most numerous and diverse channels present in the mammalian brain, may partly explain this alteration in neuronal excitability.[53] Also, a dysfunction of K^+ channels has been observed in fibroblasts[54] and platelets[49] of AD patients. Additionally, Aβ has been demonstrated to not only be involve in the AD pathogenesis, but also modulate K^+ channel activities[55] and may have a physiological role in controlling neuronal excitability[56]. Somatic mutations involved in K+ channels were detected to associate with both AD and age indicating the existence of common processes behind neurodegenerative disease and aging. It also seems that K^+ channels are naturally subjected to oxidation by reactive oxygen species (ROS) in both aging and neurodegenerative disease which are characterized by high levels of ROS.[57]

Calling variants and detecting somatic mutations from public scRNA-seq data expands the use and scope of scRNA-seq data, and may provide new insight into post-zygotic genetic change at a cell-type specific level. The use of a single cell-type (excitatory neuron) and the minimal read coverage requirement minimized biases driven by gene-specific expression. However, some limitations can also not be ignored. With the workflow that was used, the results are sensitive to the chosen settings of the parameters. RNA editing events and transcription errors that happen in RNA sequences might also be identified as somatic mutations using this workflow, which may explain the recurrent mutations that we identified. However, the association between this type of mutation and AD or aging could also be interesting.[58] Additionally, as the workflow is relatively complex,

quality control was highly critical for this study. Another limitation of this study is the relative narrow age range of the included individuals. Moreover, ages above 90 were censored to be 90. These two factors may explain that we only found a relative weak association between age and the accumulation of somatic mutations. On the other hand, the significant trend after removing individuals with an age higher than 90 might also suggest that nonagenarians and centenarians generally have a healthier individual genome. Next, heterozygous variants from the WGS data were ignored in this study (due to potential ambiguity as a result of differences in gene expression). Therefore, many potential somatic mutations were excluded from the start. Also, to reduce the effect of technical noise, we need more than 10% of the reads to support a mutational base, which may exclude the mutations present in just one or a few neurons. Finally, as 10x scRNA-seq data was used to detect somatic mutations, only variants located on the DNA that gets transcribed into mRNA were detected.
Our study has explored the feasibility of using scRNA-seq data to generate potential new insights into the association of AD and aging with brain somatic mutagenesis. It should be noted that follow-up studies with larger cohorts are required to validate our results.

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6

Discussion

用舍由时，行藏在我，袖手何妨闲处看。
身长健，但优游卒岁，且斗尊前。

– 苏轼

Aging seems destined to be accompanied by dysfunction occurrence and error accumulation, with AD being one facet of aging-induced vulnerabilities that is susceptible to genetic and environmental influences. In contrast, cognitively healthy centenarians, such as those from the 100-plus Study, point towards the possibility of healthy longevity, although very rare. About 10% of all centenarians can be considered to be in healthy cognitive and physical conditions.[1, 2] They may have specific characteristics that delay or counteract the onset of cognitive decline and other age-associated disorders, suggesting the need to explore the mechanisms underlying their maintenance of cognitive health. This chapter discusses the most important findings presented in this thesis, including the proteomic specificities in cognitively healthy centenarians. We will also address challenges, limitations, and future perspectives.

6.1. NEUROPATHOLOGY AND AGING

In addition to clinical symptoms, the accumulation of neuropathological substrates is another observable macroscopic sign of brain change. Although we do not know whether the accumulation of neuropathology causes AD, or AD causes the neuropathological accumulation, at least the levels of neuropathological substrates provide an indication for the progression of AD, to some extent.[3] Contrarily, our study (Chapter 2) and other studies have reported the increase of average neuropathological levels with age in non-demented populations,[4–7] suggesting that the accumulation of neuropathological substrates can be in part a normal consequence of aging. An accompanying cognitive decline can then distinguish whether it is a benign or disease process. In fact, in the centenarian cohort, accumulation of neuropathology and cognitive decline are not perfectly correlated, and the same is true between neuropathologies (Chapter 2). We believe that centenarians who have accumulated similar neuropathological levels as AD patients and maintained normal cognition possess some resilience potential, or, at least, the accumulated neuropathological substrates are less toxic in their brains than their AD counterparts. For the centenarians who are resilient to the effect of high levels of neuropathology, the accumulated neuropathology is driven by aging, but the brains of these centenarians are built so robust (e.g., greater intracranial volume, higher synapse density, or higher neurogenesis) that they can tolerant the adverse effects before resulting cognitive impairment.[8–10] Or, there are some compensatory mechanisms in their brains that can protect against these adverse effects, including the ability to use existing cognitive networks more efficiently (i.e., neural reserve) or to recruit alternate networks in response to network disruptions (i.e., neural compensation).[11, 12] Another explanation for the attenuated correlation can be that the accumulated neuropathological substrates in the brains of centenarians are of a less toxic form, for example, the Aβ deposition in diffuse plaques (DPs).[13, 14] In chapter 4, we tried to reveal the resilience mechanisms from the brain proteomics level; however, future studies focusing on distinguishing the different forms of neuropathological substrates may also help to explain the cause of AD.

AD is characterized by the extracellular accumulation of (1) Aβ plaques or (2) neuritic plaques (NPs) and (3) the intracellular aggregation of NFTs. In the middle-age or younger population, the three AD-associated neuropathological substrates are highly correlated

in the post-mortem brains (Chapter 2), but this does not necessarily mean that they are mutually dependent. For AD patients in the middle age, AD-associated vulnerability is the exclusive consequence driven by aging so that AD-associated neuropathological substrates can develop to the highest levels before death. While for middle-aged non-demented patients, AD-associated vulnerability is not the major consequence of aging, so all neuropathological levels stay low. This bimodal distribution of neuropathological levels leads to a high correlation (close to 1) between them for middle and younger ages. With increasing age, other competing vulnerabilities that can also cause death start to manifest, so that the accumulation of AD-associated neuropathological substrates has not yet reached the highest levels before death, leading to a confounding decrease of some neuropathological levels in AD patients with age.[6, 13, 15–17] Also, due to aging induced vulnerability, AD-associated neuropathological substrates increase with age in non-demented individuals. Based on these two reasons, AD-associated neuropathological substrates changes from a bimodal distribution in middle age to a unimodal distribution in the oldest old, and the correlations between them decrease correspondingly. Therefore, we believe that the correlation between AD-associated neuropathological substrates based on younger population may over-estimated and a cascade model might be doubtful. But there are indeed some commons between these neuropathological substrates as significant correlations are still observed in cognitive healthy centenarians. To investigate the early dependence between different neuropathological substrates, a large cohort study in non-demented individuals may help.

6.2. NEUROPATHOLOGY AND NEUROPSYCHOLOGY IN CENTENARIANS

Since the vulnerability of many neurodegenerative diseases increases with age, a mixed neuropathology is often observed in the brains of the oldest old.[18–21] In our study (Chapter 3), variable levels of mixed neuropathologies and variable cognitive performance across multiple cognitive domains[22] are observed simultaneously in the brains of centenarians. However, the vulnerabilities of the different neuropathologies are not the same, even in the oldest old. No centenarian avoids the buildup of Braak-NFT stage, GVD stage, and atherosclerosis, while only a few centenarians stay in the Thal Aβ phase or CAA stage 0, or have no cerebral infarcts. In contrast, a considerable fraction of centenarians does not develop CERAD-NP score, TDP-43 stage, hippocampal sclerosis, Lewy body stage, and cerebral atrophy. Compared with the centenarians who have accumulated certain levels of these neuropathological substrates, a resistance potential should be considered to present in those centenarians who do not develop these neuropathological substrates. Research on these centenarians may help develop strategies to prevent the accumulation of these neuropathological substrates or their combinations (i.e., CERAD-NP score, TDP-43 stage, hippocampal sclerosis, Lewy body stage, and cerebral atrophy). Among these neuropathological substrates, a factor analysis identified the tau pathology (Braak-NFT stage, LB stage, and GVD stage) and LATE pathology (TDP-43 stage and hippocampal sclerosis) showed significant correlations with specific neuropsychology factors in the centenarian brains. Thus, developing therapeutic regimens that target these neuropathological substrates should be a priority.

6.3. BRAIN PROTEOMICS IN AD AND AGING

Alterations in the brain proteome are direct reflections of AD and aging processes at the molecular level. In chapter 4, we identified proteins that changed expression over Braak stages or/and ages. If we disregard the magnitude of the effect size, more than half of the proteins we measured showed significant changes with Braak stages, suggesting that the biological changes associated with AD are systemic, at least for late-onset AD.[23] The proteins that are associated with the Braak stages are involved in mitochondrial, synapse, extracellular matrix, intermediate filament, epithelial cell differentiation, and glucose catabolic processes, suggesting that these biological processes are most affected by AD. A considerable (65.5%) part of age-related proteins also showed significance in this protein set, suggesting that those proteins are related to processes that are common to AD and aging[24]. This may also serve as evidence that AD is an aging-induced vulnerability.

Among the proteins that regulated with Braak stages or/and ages, centenarians showed specificities. For some Braak stage-related proteins, centenarians showed significantly different or delayed expression compared to AD patients at the same Braak stage (stage IV), indicating that these proteins may contribute to the resilience potential in centenarian brains. More specifically, synaptic or synapse-associated proteins are highly present in this set of proteins, suggesting that centenarians have stronger synaptic functions.[25] From this perspective, AD-related changes occur in the brains of centenarians, but they can counteract the harmful effects to maintain cognitive health. However, we also observed that the microtubule associated protein tau protein (MAPT), hallmark of AD tangle formation, showed specificity in centenarians. This could give room to the hypothesis that observed NFTs in centenarian brains are a harmless form or that the process of NFTs accumulation in centenarian brains is harmless.[26, 27] Next, we also observed that in centenarian brains, most age-related proteins resembled the protein levels observed in non-demented younger individuals. This indicates maintaining healthy cognition at extreme ages requires systemic robustness of brain function.[28, 29] This systemic robustness may, in turn, help the centenarian avoid the vulnerability of AD. Thus, a systematic perspective may be necessary when studying the processes of AD and aging.

6.4. BRAIN SOMATIC MUTATION IN AD AND AGING

Brain somatic mutation is another modifiable biological feature that will accumulate during aging. This genomic instability can also be considered an aging-induced vulnerability.[30] Using single-cell RNA sequencing (scRNA-seq) data as well as whole genome sequencing (WGS) data from the Religious Orders Study/Memory and Aging Project cohorts (ROSMAP), we detected several aging- or AD-associated excitatory neuron-specific somatic mutations (ENSMs) (Chapter 5). We showed that the number of ENSMs increased with age in human brains, and certain somatic mutations occurred more frequently in the brains of AD patients and older individuals. Somatic mutations can occur due to multiple mutational processes, including the intrinsic slight infidelity of the DNA replication machinery, exogenous or endogenous mutagen exposures, enzymatic modification of DNA, and defective DNA repair.[31] The single base substitution (SBS) type of the ENSMs that we identified in AD and non-demented individuals resembles

an age-related 'clock-like' signature (SBS5), which is observed in other normal cells as well as in essentially all cancer cells.[32] This indicates aging shows a stronger correlation with the accumulation of brain somatic mutations as compared with AD. However, certain types of sporadic AD can be related to somatic mutations in the autosomal dominant AD genes, although it's very rare[33]. Also, somatic mutations may contribute to AD progression by affecting the genes involved in the accumulation of AD-associated neuropathological substrates[34]. Somatic mutations may increase the risk of AD or are partly involved in AD progression, but they are just another aging-induced vulnerability, not the common cause of AD. Nonetheless, studies of somatic mutations and DNA repair mechanisms will contribute to a deeper understanding of aging and, to more or less, part of AD.

6.5. COGNITIVELY HEALTHY CENTENARIANS AS A DEFINITION OF HEALTHY AGING

In the previous sections, we have discussed the hypothesis that AD is an aging-induced vulnerability. In contrast, cognitively healthy centenarians define what is healthy brain aging. Their genomes, transcriptomes, proteomes, and metagenomes can be used as super-controls to enhance and help to identify the molecular distinction between different aging-induced vulnerabilities. For example, the inclusion of centenarians in genetic studies increased the effect size, which translated into higher statistical power to detect significant associations.[35] In chapter 4, we observed the expression of age-related proteins showed an opposite behavior in centenarians. However, centenarians are still rare which limits their using as super-controls as it precludes the possibility to gather very large sample sizes. Our findings, however, might have methodological implications, i.e., quantitative or qualitative, cross-sectional or longitudinal, in terms of study design and sample collection for future studies of AD, other age-related diseases, and aging. In addition, defining centenarians as the limit of human beings might be doubtable. Current aging theories are assigned to the damage concept, whereby the accumulation of damage may cause biological systems to fail, or to the programmed aging concept, whereby the inherent internal processes may cause aging.[36] Based on either concept, there must be ways to further extend the lifespan of humans by either avoiding the accumulation of errors or inhibiting the aging-promoting genes. But we propose that how to achieve a healthy end life should be the first problem to solve.

6.6. LIMITATIONS

Investigating centenarians in the context of aging and AD is not without limitations. Apart from the difficulty in collecting a large sample size,[2] the definition of cognitively healthy centenarians is not straightforward. During the study inclusion, all centenarians self-reported to be cognitively healthy, which was confirmed by a proxy. Next to that, the 100-plus Study implements a neuropsychological tests battery that is specialized for the diagnosis of cognitive decline and dementia. While these tests allow for assessing the performances of different cognitive domains, they are not developed for extreme ages. To date, no specific neuropsychological assessment protocol has been developed

to score the cognitive performance at such old ages.[37] Nonetheless, our study has been the first to identify cognitive assessment protocols that may be more appropriate for centenarians.[38] Given these protocols, any study of extreme ages anywhere in the world can identify subsets of individuals who maintain cognitive abilities at extreme ages in a standardized manner. This will eventually lead to more collaborative efforts, which will be crucial for aging and AD research. In addition to the ambiguity of definition, the terminal decline is another limitation in centenarian studies. In our previous study, we observed a higher rate of decline and mortality rate in centenarians who dropped out of the study,[39] indicating a potential terminal decline.[40, 41] Consequently, these individuals had only a short follow-up; and even the neuropsychological assessment at the last visit cannot faithfully reflect the cognitive status at death. Furthermore, at the molecular level, some proteins expression levels can also change dramatically close to death. Due to the above reasons, i.e., small sample size, ambiguous definition, and terminal decline, when studying cognitively healthy centenarians as a sample, the assumptions of independent and identically distributed random variables (IID) may not be well satisfied, leading to less robust and reproducible results.

6.7. FUTURE PERSPECTIVES

The biological features that will change during aging and AD processes are not only neuropathological substrates, brain proteome, and somatic genome. Other changes like stem cell exhaustion[42, 43], epigenetic alterations[44, 45], mitochondrial dysfunction[46, 47], transcriptomic and genomic abnormalities[48], etc., can be the directions for future studies. Given the results from our brain proteomic study, we would expect centenarians will also show some specific patterns in these biological features. Furthermore, studies that focus on the connections between these biological features will help the understanding of aging and AD in a systematic way. Based on the hypothesis that aging-induced vulnerability are susceptible to genetic and environmental influences, studies on the genetic profiles and environmental factors of centenarians may point to the causality of healthy brain aging and will, in turn, help to explain AD. Notably, the 100-plus Study is currently enrolling additional cognitively healthy centenarians and their family members, allowing the further exploration of the unique characteristics of these individuals from the perspectives mentioned above. Lastly, building a reference genome using the DNA sequences from cognitively healthy centenarians will define a healthy aging human and this effort will help the collaboration with other studies.

6.8. CONCLUSIONS

The main finding of this thesis is the characterization of centenarians' resilience and resistance potential to neuropathologies and aging, and to discover the proteins that associate with these potentials. In this thesis, we selectively explore the biological features involved in AD and aging, in which the boundaries between aging and AD are not always clear-cut, and investigate centenarians in both the context of AD and aging. We believe the findings in this thesis are instrumental for future studies dealing with AD, aging, and other age-related disorders, and should inspire more efforts in centenarian studies.

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SUMMARY

A remarkable accomplishment of human beings in recent centuries is the extension of their average lifespan, for example by improved living conditions. A downside of this achievement is that aging-related diseases, such as Alzheimer's disease (AD), have risen, and as a consequence are impacting the lives of an increasing fraction of individuals. Although many conclude that aging is the main risk factor for AD, it might be better to say AD is one facet of aging. Indeed, the boundaries between brain aging and AD are largely fluent in the elderly. Nevertheless, dementia and AD are not inevitable. In fact, a small proportion of the population (< 0.1%) reaches at least 100 years old while maintaining to have a healthy cognition. These rare but remarkable centenarians could shed light on how to escape age-related diseases.

This thesis studies cognitive healthy centenarians as extreme controls in the context of aging and AD. Based on a large cohort of data, this thesis indeed shows that some centenarians escaped the buildup of some neuropathologies, indicating resistance to these neuropathologies. Contrarily, this thesis also shows that average levels of AD-associated neuropathologies increase with age in non-demented individuals, whereas these neuropathologies decrease with age in AD cases. Most intriguingly, this thesis shows that some centenarians with the highest cognitive performance, did accumulate the highest levels of some neuropathologies, yet remained cognitive healthy. This thesis then speculates that these observations point towards a resilience to these neuropathologies by these centenarians.

To better understand the resilience and resistance mechanisms in centenarian brains, this thesis then continues with investigating brain proteomics in the context of the degree of AD pathology (Braak stages) as well as age. As a first characterization, clusters of Braak stage-related and age-related proteins are identified that separately are associated with specific biological processes. Some Braak stage-related proteins demonstrate a deviated abundance in centenarians compared to AD (at the Braak stage IV), indicating that these proteins may contribute to the resilient mechanisms of tau accumulation in centenarian brains. A remarkable finding regarding the age-related proteins is that centenarian brains are, in a median of, 18-years "younger" in their protein expression, when compared with non-demented controls, again hinting towards a resilience to age-related diseases.

To further explore the possible role of aging behind AD, this thesis studies the extend and locations of brain somatic mutations. We show that the number of excitatory neuron specific-somatic mutations increases with age, but there is no significant difference between AD and non-demented individuals. Interestingly, certain somatic mutations occurred more frequently in the brains of AD patients.

Concluding, this thesis demonstrates the value of cognitive healthy centenarians in studying brain aging and neurodegenerative diseases. In doing so, it reveals that the re-

lationship between brain aging and neurodegeneration is extremely complex and deeply entangled. Nevertheless, basic processes that are altered during brain aging are identified, which brings targets to counteract the molecular disorder that leads to neurodegeneration, including AD, closer.

SAMENVATTING

Een opmerkelijke prestatie van de mens in de afgelopen eeuwen is de verlenging van de gemiddelde levensduur, bijvoorbeeld door verbeterde levensomstandigheden. Een keerzijde van deze prestatie is dat aan veroudering gerelateerde ziekten, zoals de ziekte van Alzheimer (AD), zijn toegenomen en als gevolg daarvan het leven van een steeds groter deel van de mensen beïnvloeden. Hoewel velen concluderen dat veroudering de belangrijkste risicofactor is voor AD, is het misschien beter om te zeggen dat AD een facet van veroudering is. Inderdaad, de grenzen tussen hersenveroudering en AD zijn grotendeels vloeiend bij ouderen. Toch zijn dementie en AD niet onvermijdelijk. In feite bereikt een klein deel van de bevolking (< 0,1%) minstens 100 jaar oud met behoud van een gezonde cognitie. Deze zeldzame maar opmerkelijke honderdjarigen zouden licht kunnen werpen op hoe je aan ouderdomsziekten kunt ontsnappen.
Dit proefschrift bestudeert cognitief gezonde honderdjarigen als extreme controles in de context van ouder worden en AD. Gebaseerd op een groot aantal gegevens, laat dit proefschrift inderdaad zien dat sommige honderdjarigen ontsnapten aan de opbouw van bepaalde neuropathologieën, wat wijst op resistentie tegen deze neuropathologieën. In tegendeel, dit proefschrift laat ook zien dat de gemiddelde niveaus van AD-geassocieerde neuropathologieën toenemen met de leeftijd bij niet-demente individuen, terwijl deze neuropathologieën afnemen met de leeftijd bij AD-gevallen. Het meest intrigerende is dat dit proefschrift laat zien dat sommige honderdjarigen met de hoogste cognitieve prestaties, de hoogste niveaus van sommige neuropathologieën hebben verzameld, maar cognitief gezond zijn gebleven. Dit proefschrift speculeert vervolgens dat deze waarnemingen wijzen op veerkracht bij deze honderdjarigen tegen deze neuropathologieën.
Om de veerkracht- en weerstandsmechanismen in honderdjarige hersenen beter te begrijpen, gaat dit proefschrift vervolgens verder met het onderzoeken van hersenproteomics in de context van de mate van AD-pathologie (Braak-stadia) en leeftijd. Als eerste karakterisering worden clusters van Braak-stadiumgerelateerde en leeftijdsgebonden eiwitten geïdentificeerd die afzonderlijk geassocieerd zijn met specifieke biologische processen. Sommige aan het Braak-stadium gerelateerde eiwitten vertonen een afwijkende overvloed bij honderdjarigen in vergelijking met AD (in het Braak-stadium IV), wat aangeeft dat deze eiwitten kunnen bijdragen aan de veerkrachtige mechanismen van tauaccumulatie in honderdjarige hersenen. Een opmerkelijke bevinding met betrekking tot de leeftijdsgerelateerde eiwitten is dat honderdjarige hersenen gemiddeld 18 jaar "jonger"zijn in hun eiwitexpressie, in vergelijking met niet-demente controles, wat opnieuw wijst op een veerkracht tegen leeftijdsgerelateerde ziekten.
Om de mogelijke rol van veroudering achter AD verder te onderzoeken, bestudeert dit proefschrift de omvang en locaties van somatische mutaties in de hersenen. We laten zien dat het aantal prikkelende neuron-specifieke-somatische mutaties toeneemt met

de leeftijd, maar er is geen significant verschil tussen AD en niet-demente individuen. Interessant is dat bepaalde somatische mutaties vaker voorkwamen in de hersenen van AD-patiënten.
Concluderend toont dit proefschrift de waarde aan van cognitief gezonde honderdjarigen bij het bestuderen van hersenveroudering en neurodegeneratieve ziekten. Daarbij laat het zien dat de relatie tussen hersenveroudering en neurodegeneratie uiterst complex en diep verweven is. Desalniettemin worden basisprocessen geïdentificeerd die veranderen tijdens hersenveroudering, wat doelen dichterbij brengt om de moleculaire stoornis tegen te gaan die leidt tot neurodegeneratie, waaronder AD.

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LIST OF PUBLICATIONS

4. A.B. Ganz[*], **M. Zhang**[*], F. Koopmans, K.W. Li, S.S.M. Miedema, A.J.M. Rozemuller, P. Scheltens, J.J.M. Hoozemans, M.J.T. Reinders, A.B. Smit and H. Holstege, *Neuroproteomics of cognitively healthy centenarians in the context of aging and disease.* (Under preparation)

3. **M. Zhang**, G.A. Bouland, H. Holstege and M.J.T. Reinders, *Identifying Aging and Alzheimer Disease–Associated Somatic Variations in Excitatory Neurons From the Human Frontal Cortex*, Neurology Genetics (2023), 9(3).

2. **M. Zhang**[*], A.B. Ganz[*], S. Rohde, M.J.T. Reinders, P. Scheltens, M. Hulsman, J.J.M. Hoozemans and H. Holstege, *The correlation between neuropathology levels and cognitive performance in centenarians*, Alzheimer's & Dementia (2023); 1-12.

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