Biochemical and clinical characterization of *CNTN5* and *CLU* as genetic risk factors of Alzheimer's disease

Marina Tedeschi Dauar

Integrated Program in Neuroscience, McGill University, Montreal

September 2024

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Table of Contents

Abstract

Alzheimer's disease (AD) is the most common cause of dementia worldwide. Although extensive research has been done since the disease was first described in 1906, and the first disease modifying treatments have just become available, we still do not fully understand the pathophysiology of AD and the current treatments are far from satisfactory.

AD can be inherited either as an autosomal dominant disorder or as a complex trait (also called sporadic AD). Sporadic AD corresponds to over 97% of the cases and is caused by interactions between genetic and environmental risk factors. But even though environment plays an important role, the heritability of sporadic AD is still notably high, ranging between 50-80%. Over 70 single nucleotide polymorphisms (SNPs) associated with the risk of sporadic AD have been identified, but they account for only about 35% of the phenotypic variation, which means that most genetic determinants are still unknown. The identification of these missing variants is an important strategy to fill in the gaps that still exist in our knowledge of AD. Characterizing new genes could bring insights into AD pathophysiology, improve diagnosis accuracy especially in the early and asymptomatic stages and help identify different treatment strategies.

With the goal of identifying the missing genetic determinants, a genome-wide association study was performed in the Québec Founder Population, a well-characterized population isolate. Twelve genetic variants were found to be associated with AD risk. In this thesis, we explore in depth the role of variants found in the genes contactin 5 (*CNTN5*) and clusterin (*CLU*), throughout the AD spectrum. The objective is to confirm their role as risk/protective factors and to understand how they influence the pathophysiology of AD.

We report, for the first time in the literature, the rs1461684 G variant in the *CNTN5* gene as a risk factor for AD which we confirm across different patient cohorts. Contactin 5 is a protein that acts on synaptogenesis and axonal arborization during neurodevelopment. Our results suggest that this gene is particularly involved in the early presymptomatic phase of the disease when the rs1461684 G variant is associated with faster disease progression and decreased cortical gene expression, and contactin 5 protein levels progressively increase in the cerebrospinal fluid (CSF).

Clusterin, on the other hand, is a neuroprotective apolipoprotein and one of the main cholesterol transporters in the brain. Variants in the *CLU* gene are recognized risk/protective factors associated with AD. We investigated the mechanism of action of the protective variant *CLU* rs11136000 T in different phases of AD. Our findings suggest that this variant's protective role is more important in the later disease stages and is mediated by increases in cerebral gene expression. *APOE-ε4* carriers experience greater increases in gene expression, indicating a potential compensatory mechanism to enhance their cholesterol transport efficiency.

In summary, this work describes a new genetic risk factor for AD and provides important new insights into the mechanism of action of the two studied genes *CLU* and *CNTN5*. Overall, our results highlight the importance of compensatory mechanisms that allow the brain to respond to injury or pathological harm and suggest that they play a meaningful role in AD pathophysiology.

Résumé

La maladie d'Alzheimer (MA) est la cause de démence la plus fréquente dans le monde. Bien que des recherches approfondies aient été menées depuis la première description de la maladie en 1906 et que les premiers traitements modificateurs de la maladie viennent tout juste d'être disponibles, nous ne comprenons toujours pas pleinement la physiopathologie de la MA et les traitements actuels sont loin d'être satisfaisants.

La MA peut être héritée soit comme une maladie autosomique dominante, soit comme un trait complexe (également appelé MA sporadique). La MA sporadique correspond à plus de 97 % des cas et est causée par des interactions entre facteurs de risque génétiques et environnementaux. Mais même si l'environnement joue un rôle important, l'héritabilité de la MA sporadique reste particulièrement élevée, comprise entre 50 et 80 %. Plus de 70 SNP associés au risque de MA sporadique ont été identifiés, mais ils ne représentent qu'environ 35 % de la variation phénotypique, ce qui signifie que la plupart des déterminants génétiques sont encore inconnus. L'identification de ces variantes manquantes est une stratégie importante pour combler les lacunes qui existent encore dans nos connaissances sur la maladie d'Alzheimer. La caractérisation de nouveaux gènes pourrait apporter des informations sur la physiopathologie de la MA, améliorer la précision du diagnostic, en particulier aux stades précoces et asymptomatiques, et aider à identifier différentes stratégies de traitement.

Dans le but d'identifier les déterminants génétiques manquants, une étude d'association pangénomique a été réalisée dans la population fondatrice du Québec, un isolat de population bien caractérisé. Douze variantes génétiques ont été associées au risque de MA. Dans cette thèse, nous

explorons en profondeur le rôle des variants trouvés dans les gènes contactine 5 (CNTN5) et clusterine (CLU), tout au long du spectre de la MA. L'objectif est de confirmer leur rôle en tant que facteurs de risque/protection et de comprendre comment ils influencent la physiopathologie de la MA.

Nous rapportons, pour la première fois dans la littérature, le variant rs1461684 G du gène CNTN5 comme facteur de risque de MA, ce que nous confirmons dans différentes cohortes de patients. La contactine 5 est une protéine qui agit sur la synaptogenèse et l'arborisation axonale au cours du développement neurologique. Nos résultats suggèrent que ce gène est particulièrement impliqué dans la phase présymptomatique précoce de la maladie lorsque le variant rs1461684 G est associé à une progression plus rapide de la maladie et à une diminution de l'expression des gènes corticaux, et que les niveaux de protéine contactine 5 augmentent progressivement dans le liquide céphalo-rachidien (LCR).

La clusterine, quant à elle, est une apolipoprotéine neuroprotectrice et l'un des principaux transporteurs de cholestérol dans le cerveau. Les variantes du gène CLU sont des facteurs de risque/de protection reconnus associés à la MA. Nous avons étudié le mécanisme d'action de la variante protectrice CLU rs11136000 T dans différentes phases de la MA. Nos résultats suggèrent que le rôle protecteur de cette variante est plus important aux stades ultérieurs de la maladie et est médié par une augmentation de l'expression des gènes cérébraux. Les porteurs d'*APOE-ε4* connaissent une augmentation plus importante de l'expression génique, ce qui indique un mécanisme compensatoire potentiel pour améliorer l'efficacité du transport du cholestérol.

En résumé, ce travail décrit un nouveau facteur de risque génétique de la MA et fournit de nouvelles informations importantes sur le mécanisme d'action des deux gènes étudiés, CLU et CNTN5. Dans l'ensemble, nos résultats mettent en évidence l'importance des mécanismes compensatoires qui permettent au cerveau de réagir à une blessure ou à un préjudice pathologique et suggèrent qu'ils jouent un rôle significatif dans la physiopathologie de la MA.

Acknowledgments

Completing a PhD requires the support and contribution of many people. Several people were essential to the execution of this work, while others gave me the knowledge and support to begin this journey in the first place. To all of them, I owe these words of gratitude and appreciation.

First and foremost, I would like to express my sincere gratitude to my supervisor Judes Poirier. Your considerable knowledge and experience combined with your reliability, patience to teach and love for science were invaluable for my experience and helped me grow substantially as a scientist. Thank you for the opportunity to be part of your lab, for sharing your knowledge, for teaching us so much about research and the scientific world and for always showing empathy and kindness towards the students. You are a real role mode, and I will always be thankful for your mentorship and guidance and for the impact you had in my career.

I would like to thank Dr Cynthia Picard and Anne Labonté for the impeccable work they do for lab. Thank you for all you taught me and for always being so helpful every time I needed. Your collaboration was vital for the completion of this thesis. I want to acknowledge Doris Dea for the help with lab and animal work. Special thanks to Dr Nathalie Nilsson, Dr Justin Miron and Dr Marie-Elyse, for the guidance with the early challenges of the PhD. Isabel Sierra-Flores, Gabriel Aumont-Rodrigue, Jiarui Ao, Marc Quesnel, Isabel Sarty, Julia Loncke and Laurence Maligne Bruneau, thank you for the discussions, collaboration and feedback. Above all, I thank all my colleagues from the Poirier lab for their friendship and support.

To my committee members Dr Sylvia Villeneuve and Dr Tak Pan and my mentor Dr Salah El Mestikawy, thank you for the feedback and ideas that greatly improved this work. I would like to thank Dr Pedro Rosa-Neto for giving me my first opportunity at McGill University. Your enthusiasm for science and all you taught me had a significant impact in my career. Thanks for opening this door for me and for your continuous support. I am also thankful for the opportunity of learning from two brilliant clinicians-scientists, Dr Serge Gauthier and Dr John Breitner. I thank the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Dr Judes Poirier for the financial support that allowed me to be here. To the patients and families who selflessly donated their time and health information, thank you for your contribution.

To my mentor, professor, and friend, Dr Paulo Henrique Ferreira Bertolucci. Your passion and enthusiasm for cognitive neurology inspired me to follow this path and I owe you most of my experience in clinical dementia. Your support and guidance since residency were invaluable. Thank you for being a mentor and friend I know I can always count on.

Finally, I would like to thank my family. My mother Eloisa and my father Demetrio, thank you for all the opportunities you gave me, for fostering in me a passion for learning and for the continuous support. To my brother Marcos, thanks for the friendship, and for always being by my side. To my husband Roberto, thank you for the efforts and sacrifices you made to embark in this journey and help me fulfill my dream. Thank you for being my biggest supporter, for celebrating with me the happy moments and helping me through the difficult ones. To our daughter, Isabella, our most precious little ray of sunshine, thank you for the joy and love you bring into our lives, and for constantly reminding us of what truly matters.

Contribution to Original Knowledge

Chapter 2: In this project we describe, for the first time in the literature, the *CNTN5* rs1461684 G variant as a risk factor for Alzheimer's disease. Additionally, we provide original insights into the mechanism of action of this gene in the pathophysiology of AD by showing that the presence of the risk variant is associated with decreased gene expression and faster disease progression (particularly in the early disease stages) and that the contactin 5 protein in the CSF increases progressively in asymptomatic subjects but subsequently decreases in MCI and AD.

Chapter 3: In this chapter we demonstrate that contactin 5 and the apolipoproteins involved in brain cholesterol transport are significantly associated in the CSF and show significant changes in their gene expression during the degeneration/reinnervation process that follows hippocampal deafferentation in an animal model of hippocampal degeneration/reinnervation. This work suggests that contactin 5 and apolipoproteins are involved together in the compensatory changes that occur in the brain in response to pathological harm to promote remodeling and reinnervation. These findings bring important additional evidence for a rarely explored pathological pathway in AD and give further original insights into the role of the newly identified AD genetic risk factor *CNTN5* rs1461684 G.

Chapter 4: In this project we present important new knowledge about the role of the protective variant *CLU* rs11136000 T in the pathophysiology of AD. Our findings suggest that this variant plays a more significant role in the later phases of AD by increasing the gene expression of its neuroprotective protein. *CLU* gene expression is greater in *APOE*-ε4 carriers, which is believed to serve as a compensatory mechanism to improve the deficient cholesterol transport caused by Apoe-ε4.

Contribution of Authors

Chapter 2:

Marina Tedeschi Dauar: study design, data analysis, writing, revising and editing the manuscript.

Judes Poirier: study design, contribution to data analysis, writing, revising and editing the manuscript.

Anne Labonté: data collection and quality control, data compilation, manuscript review.

Cynthia Picard: pan-genomic analysis of DNA samples and risk assessments, contribution to data analysis, manuscript review.

Justin Miron: data collection, contribution to data analysis, manuscript review.

Pedro Rosa-Neto: data collection, contribution to data analysis, manuscript review.

Henrik Zetterberg: data collection and quality control, data compilation, manuscript review.

Kaj Blennow: data collection and quality control, data compilation, manuscript review.

Sylvia Villeneuve: data collection, development of algorithms for data analysis, manuscript review.

Chapter 3:

Marina Tedeschi Dauar: study design, data analysis, writing, revising and editing the manuscript.

Judes Poirier: study design, data analysis and curation, funding acquisition, writing revising and editing the manuscript.

Cynthia Picard: data analysis and quality control, contribution to study design, manuscript review.

Anne Labonté: data analysis and quality control, manuscript review.

John Breitner: assisted in the project design and administration, data curation, funding acquisition, manuscript review

Pedro Rosa Neto: data collection and manuscript review.

Sylvia Villeneuve: data collection and analysis, data curation, funding acquisition, manuscript review.

Chapter 4:

Marina Tedeschi Dauar: study design, data analysis, writing, revising and editing the manuscript.

Judes Poirier: study design, data analysis and curation, funding acquisition, writing and editing the manuscript.

Cynthia Picard: data analysis and quality control, manuscript review.

Henrik Zetterberg: data collection and quality control, data curation, manuscript review.

Ann Brinkmalm: data collection and quality control, manuscript review.

Kaj Blennow: data collection and quality control, data curation, manuscript review.

Sylvia Villeneuve: data collection and analysis, data curation, funding acquisition, manuscript review.

List of Abbreviations

Aβ: amyloid beta

$A\beta_{40}$: amyloid beta 40 amino acid peptide

$A\beta_{42}$: amyloid beta 42 amino acid peptide

AA: Alzheimer's Association

AD: Alzheimer's disease

ADAS-Cog: Alzheimer's Disease Assessment Scale–Cognitive Subscale

APOE-ε4: apolipoprotein E (ε4 allele)

APP: amyloid precursor protein

ARIA: amyloid-related imaging abnormalities

CCNA: Canadian Consortium on Neurodegeneration in Aging

CDR: Clinical Dementia Rating

CERAD: Consortium to Establish a Registry for Alzheimer's Disease

CLU: clusterin

CNS: central nervous system

CNTN5: contactin 5

CSF: cerebrospinal fluid

CTL: controls

DNA: deoxyribonucleic acid

ELISA: enzyme-linked immunosorbent assay

FDG: fluorodeoxyglucose

GFAP: glial fibrillary acidic protein

GWAS: Genome-wide association study

IL: Interleukin

LC/MS/MS: liquid chromatography and mass spectroscopy

MCI: mild cognitive impairment

MMSE: Mini-Mental State Examination

MoCA: Montreal Cognitive Assessment

MRI: magnetic resonance imaging

NFL: neurofilament light chain

NFT: neurofibrillary tangle

NIA: National Institute of Aging

NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders

mRNA: messenger ribonucleic acid

PET: Positron emission tomography

PREVENT-AD: Pre-Symptomatic Evaluation of Novel or Experimental Treatment for Alzheimer's Disease

PS1: presenilin 1

PS2: presenilin 2

p-tau: phosphorylated-tau

p-tau181: tau protein phosphorylated at amino acid residue threonine 181

p-tau217: tau protein phosphorylated at amino acid residue threonine 217

p-tau231: tau protein phosphorylated at amino acid residue threonine 231

QFP: Québec Founder Population

RBANS: Repeatable Battery for Assessment of Neuropsychological Status

RNA: ribonucleic acid

ROS-MAP: Religious Orders Study and the Memory and Aging Project

RT-PCR: real-time polymerase chain reaction

SNP: single nucleotide polymorphism

TNF: tumor necrosis factor

t-*tau* : total tau protein

List of Tables

List of Figures

General Introduction

1 A review of the advances in the scientific knowledge of Alzheimer's disease: from the first documented case to the dawn of the era of disease modifying treatments.

1.1 From a "rare and peculiar disease" to the most common cause of dementia

The first documented case of Alzheimer's disease (AD) is well known in the medical literature. As reported by Alois Alzheimer, the psychiatrist in charge, Auguste Deter was a female patient, who, at the age of 51, started to present symptoms of memory loss and difficulty performing daily activities, accompanied by severe neuropsychiatric symptoms characterized by delusion, aggressive behavior, and agitation (1, 2). Auguste Deter was admitted for treatment at the Frankfurt Mental Hospital where, in the following years, her clinical picture progressively deteriorated until she became completely dependent (1, 2). She died due to septicemia at the age of 55 (2).

At the time of Deter's death, Alois Alzheimer had left Frankfurt and was working in Munich as part of Emil Kraepelin's team. In addition to being a psychiatrist, Alzheimer was also an anatomist and, following on a previous agreement with Deter's family, her brain was sent to him for detailed pathological examination. The post-mortem examination revealed an atrophic brain, atherosclerotic changes in large vessels, miliary foci throughout the cortex (which would later be known as amyloid-ß plaques), and numerous thick fibrils with unique silver staining impregnation located inside neurons (which would later be named neurofibrillary tangles) (1). Alzheimer concluded that Auguste Deter had a yet unidentified illness and that her symptoms were associated

with the pathological brain changes he had found (1). Nowadays it is recognized that Auguste Deter may have had the rare autosomal dominant form of the disease, possibly caused by a mutation in the presenilin 1 (PS1) gene (3).

Alois Alzheimer presented the case of Auguste Deter for the first time in 1906 at the 37th meeting of South-West German Psychiatrists on a talk entitled "on a peculiar disease of the cerebral cortex" (4), which unfortunately generated very little interest in the audience (4). Despite the initial indifference to his findings, it is recognized today that Alzheimer's work was pioneering, not only for identifying a new disease, but also for accurately linking the neurological symptoms to the pathological brain changes. His discoveries had an undeniable importance in the way we understand AD and to the progresses in research, diagnosis and treatment that we see today. In 1910 Emil Kraepelin published the eighth edition of his Textbook of Psychiatry where he named this newly identified dementia *Alzheimer's disease* after his pupil, and described it as a dementia with young age of onset (5).

For many decades AD remained considered a rare disorder with young age of onset, while progressive memory loss in the elderly was known as "senile dementia" or "senility". However, continuous progress in neuropathological and clinical research started to challenge these concepts revealing that senile plaques and neurofibrillary tangles were very common findings in the elderly and were associated with cognitive changes (5). In 1976, Robert Katzman published an editorial on Archives of Neurology asserting that there was enough evidence to conclude that senile dementia and AD were in fact the same disorder, and that AD was actually the most prevalent form of dementia at any age and the 4th or 5th cause of death in the United States (6). This finally

consolidated AD as the most common neurodegenerative dementia worldwide, contextualized the existing knowledge on AD and senile dementia and endorsed the importance of research focused on clarifying the etiology of AD and on searching for better diagnostic and treatment strategies.

1.2 The pathophysiology of AD and the assessment of pathological hallmarks through *in vivo* biomarkers

Significant progress has been made in the understanding of AD pathophysiology since the disease was first described more than a hundred years ago. Nonetheless, the miliary foci in the cortex (amyloid-ß plaques), and the thick fibrils inside neurons (neurofibrillary tangles) first described by Alois Alzheimer remain pathological hallmarks of the disease, while other processes such as neuroinflammation and cholesterol metabolism have also been recognized as prominent features of AD pathology. The combination of these pathological processes culminates with synapse and neuronal loss, which after an individual threshold will lead to cognitive decline (7). The amyloid cascade hypothesis, although controversial to some, is still the dominant view of the pathological progression in AD and it states that amyloid-β (Aβ) is the initial trigger that leads to neurofibrillary tangle (NFT) formation/accumulation and all the other downstream events that will cause neurodegeneration (8, 9). Although this view has been challenged in more recent years with a focus on tangle deposition serving as a primary event in the cascade (10), the presence of both amyloid plaques and neurofibrillary tangles are still required for the pathological diagnosis of AD (11).

In the past decade, the field of AD biomarkers has made remarkable advances. Biomarkers are defined as parameters that can be objectively measured, in biological fluids and with positron emission tomography (PET) imaging, which reflect a normal or a pathogenic biological process or

the response to an intervention (12). Progress in biomarkers have allowed *in-vivo* assessment of the AD pathology described above which brought significant insights into the time course, natural history and interrelations of the different pathological and clinical manifestations of AD. The biomarkers available for each pathological feature will be described in this section as well. The typical progression of the main biomarkers in AD is shown in figure 4.

1.2.1 Amyloid-β

The amyloid-β (Aβ) protein originates from the processing and cleavage of the amyloid precursor protein (APP). APP is a membrane protein highly expressed in synapses and neurons, and it has been shown to act on the formation and maintenance of synapses and dendrites (13). As part of its normal processing APP can undergo two different pathways, the amyloidogenic pathway and the non-amyloidogenic pathway (14) (Figure 1A). In both pathways, APP is cleaved in its transmembrane domain by γ-secretase (14). In the non-amyloidogenic pathway, APP is also cleaved by α-secretase generating the sAPPα fragment and avoiding the formation of Aβ (14). In the amyloidogenic pathway, APP is instead cleaved by ß-secretase generating Aβ peptides that vary in length from 38-43 aminoacids (14), with the 42 aminoacids-long Aβ peptide being the most toxic and prone to aggregation (15). These Aβ monomers aggregate forming soluble oligomers that further aggregate into fibrils and finally into amyloid plaques that form deposits in the brain (14). The deposition of amyloid plaques usually follows a spatial distribution pattern with the first deposits seen in the neocortex, followed by the allocortex (16). With disease progression, diencephalic nuclei, striatum, and cholinergic nuclei of the basal forebrain are also affected, followed by brainstem nuclei and lastly the cerebellum (16).

Aβ pathology can be staged using The Consortium to Establish a Registry for Alzheimer's Disease (CERAD) system which is frequently used for diagnostic and research purposes (17). CERAD score is a semi quantitative measurement of NP density in the frontal, temporal, and parietal regions that leads to a pathological diagnosis of AD. A CERAD score of 0 indicates no plaques present and corresponds to a diagnosis of no-AD (17). CERAD 1 indicates the presence of sparse plaques and corresponds to a diagnosis of possible AD (17). On CERAD 2 there are moderate plaques present indicating a probable AD diagnosis and CERAD 3 shows frequent plaques corresponding to a diagnosis of definite AD (17).

Figure 1: APP processing and brain deposits of neuritic plaque

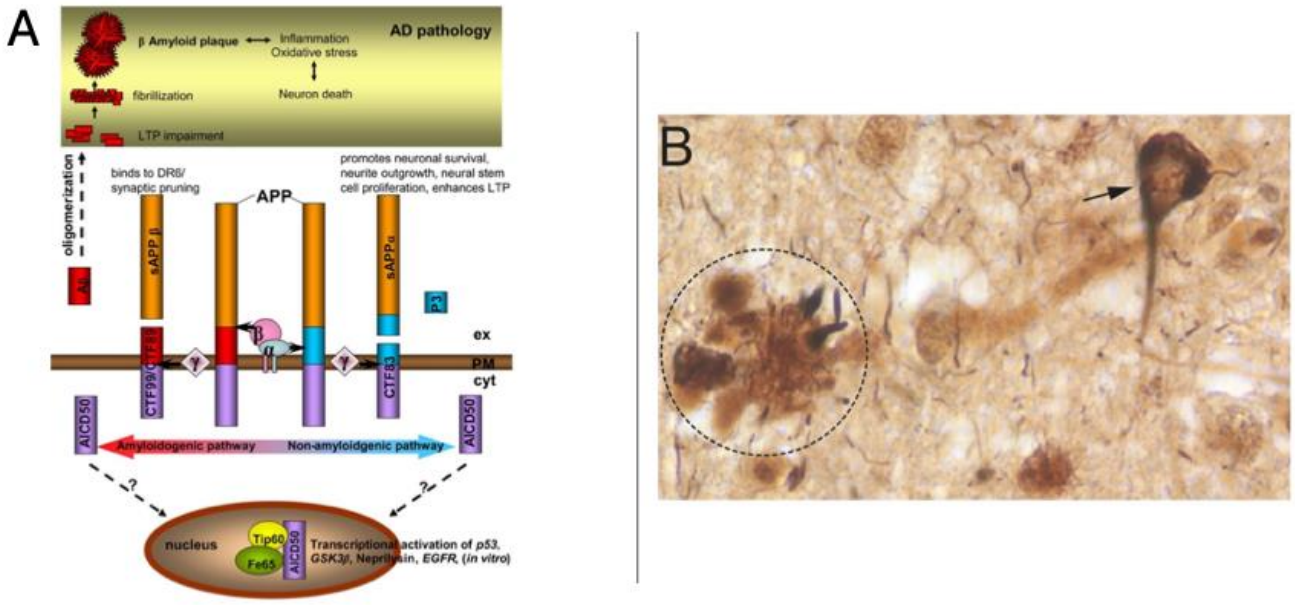


Figure 1: A) Schematic representation of APP processing and Aβ plaque formation, showing the non-amyloidogenic and the amyloidogenic pathways. Adapted from Chow *et al.*, 2010. (14); B) Neuritic plaque (circle) in the frontal cortex of a human brain (Bielschowsky's silver stain). Adapted from Moncaster *et al.,* 2022 (18).

Biomarkers of Aβ:

The soluble oligomeric form of Aβ can be measured in the cerebrospinal fluid (CSF) and used as a biomarker. The deposition of Aβ as plaques in the brain leads to its reduced availability in the CSF, thus CSF Aβ levels decline with the progression of the disease (19). CSF levels of Aβ1-42 peptide and the Aβ1-42/ Aβ1-40 ratio have been extensively used as biomarkers as they have been shown to be significantly decreased in mild cognitive impairment (MCI) and AD (20, 21), and to inversely correlate with brain amyloid deposition seen on PET or autopsy (19, 21, 22).

The fibrillary form of Aβ deposited as plaques in the brain (Figure 1B) can be detected using positron emission tomography (PET) scans. The main tracers that bind to amyloid are [11C]Pittsburgh compound B ([11C]PIB), [18F]florbetapir, [18F]florbetaben, [18F]flutemetamol, and [18F]AZD4694. All radiotracers show comparable results (23, 24), but the [18F]-labeled radiotracers are more widely used due to their longer half-life.

1.2.2 Tau

Tau is a microtubule-associated protein that acts on the maintenance of neuronal structure and on intracellular transport (25, 26). It associates with tubulin to form microtubules playing an essential role in the formation and stabilization of the cytoskeleton (25). Tau also has an important role in the microtubule-dependent cellular transport of several substances such as neurotransmitters and organelles (26). In AD, abnormal phosphorylation of tau protein leads to its aggregation and accumulation inside neurons forming neurofibrillary tangles (NFT) (Figure 2A) (25). This

intracellular accumulation of NFT causes disruptions in microtubule structure and axonal transport which leads to synaptic damage and neuronal death (25, 26).

Figure 2: The process of NFT formation and its deposition in the brain

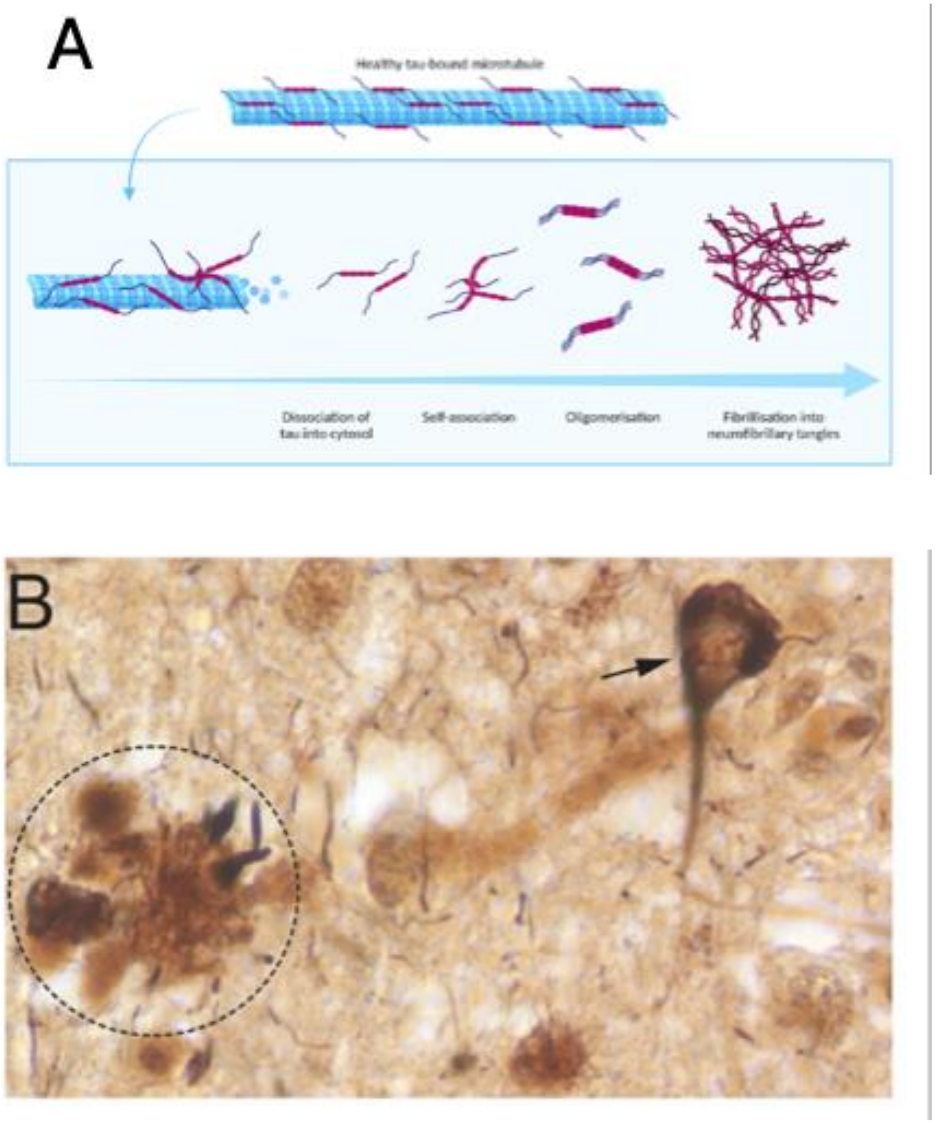


Figure 2: A) Schematic representation of tau phosphorylation and NFT formation. Adapted from Mamsa *et al.* (27); B) NFT (arrow) deposit in the frontal cortex of a human brain (Bielschowsky's silver stain). Adapted from Moncaster *et al.* (18)

Tau pathology follows a consistent spatial pattern that can be measured by the Braak staging (Figure 3) (28). NFT begins to accumulate in the entorhinal and transentorhinal regions (Braak stages I-II), followed by the limbic region (Braak stages III-IV) and then spreading to the neocortex (Braak stages V-VI) (28).

Figure 3: Braak staging for the progression of tau pathology in Alzheimer's disease

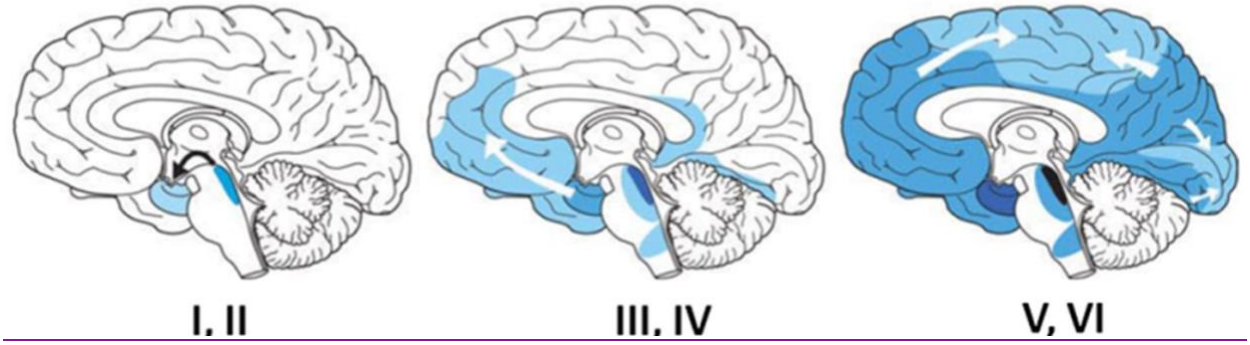


Figure 3: Progression of tau pathology as proposed by Braak and Braak (28). Adapted with permission from Jouanne *et al.(29)*

Tau biomarkers:

CSF levels of phosphorylated-tau (p-tau) can be used as biomarkers of tau pathology and in AD p-tau levels are significantly increased (30). Tau can be phosphorylated at various sites, but the forms used as biomarkers for AD are the ones phosphorylated at threonine 181 (p-tau181), 217 (p-tau217) or 231 (p-tau231) (30). Brain deposition of p-tau (Figure 2B) can also be measured by PET scans. Several radiotracers that bind to tau have been developed and were shown to recapitulate p-tau spread in AD brain (31).

1.2.3 Neuroinflammation

It has long been known that neuroinflammation plays a role in AD however, it was only more recently that it was recognized as one of the main pathological processes in the disease. This involvement is reinforced by the association between several polymorphisms in genes related to immunological activity and increased risk of AD (32-35).

Many different components of the neuro-immune system have shown to be involved in AD. Microglial activation has a protective role by promoting Aβ clearance, but this function seems to decrease with age (36). At the same time, increased levels of cytokines may be induced by Aβ deposition and contribute to neurodegeneration (37-39). The complement system also plays an important role. Aβ and tau have been shown to activate the complement system, which is initially beneficial in the removal of pathological proteins, but the chronic activation of the complement cascade can lead secondary neuronal injuries (40).

The inflammatory system is incredibly complex, with many different cells, molecules and biochemical processes involved that interact with each other. Although its precise mechanisms in AD are not completely understood, it is accepted that neuroinflammation plays a crucial role in the progression of the disease. Most of the evidence available today indicates that neuroinflammatory processes have an initial protective role by clearing pathological proteins, but its continuous activation may lead to neuronal damage and contribute to neurodegeneration.

Biomarkers of neuroinflammation

Classical markers of inflammation such as interleukin-1 (IL1), IL-6 and tumor necrosis factor (TNF) have been shown to be associated with amyloid plaques in the brain parenchyma in AD, whereas alterations in CSF concentration were shown to emerge before cognitive deficits in at-risk asymptomatic subjects (41). More recently, increased levels of astrocyte-specific glial fibrillary acidic protein (GFAP) in the plasma and CSF have been shown to serve as emerging new biomarkers of AD (30). However, GFAP is a biomarker not specific of AD pathophysiology and should not be used for diagnosis alone, but rather for staging (in combination with amyloid and phospho-tau markers), prognosis or as an indicator of treatment effect (30).

1.2.4 Lipid metabolism:

The brain is the organ with the highest concentration of cholesterol and phospholipids, which are mostly used to form myelin sheets or the membranes of neurons and astrocytes (42). In a normal healthy brain, lipid metabolism is independent from the periphery and cholesterol is essential for synaptic and dendritic formation, maintenance and function, both during development and adult life (42). Therefore, all steps involved in the synthesis, transport and storage of lipids need to operate properly to ensure brain health.

APOE and its ε4 variant, remains the most important genetic risk factor ever identified for sporadic AD (43, 44). It is one of the main cholesterol transporters both in the periphery and in the brain. The presence of the *APOE*-ε4 allele leads to lower levels of apoE and a less effective cholesterol transport (45-47). Studies have shown that *APOE* gene expression is elevated in the reinnervation

phase in a mouse model of hippocampal deafferentation (48-50), suggesting that its role as a cholesterol transporter is required after an injury in order to promote reinnervation and repair. Additionally, apolipoproteins J (clusterin), B and D, which are also essential for cholesterol transport in the brain, have also been shown to be associated with AD pathology and genetics (34, 51-53).

1.2.5 Neurodegeneration.

Neurodegeneration is the process of progressive loss of synapsis and neurons that leads to brain atrophy and neurological symptoms (54). It is a general process that occurs in other neurodegenerative diseases as well as in AD. In AD, neurodegeneration is the final result of the pathophysiological processes described above, and it is associated with cognitive decline (55, 56).

Biomarkers of neurodegeneration:

Increased levels of neurofilament light chain (NFL) in the CSF or plasma can be used as biomarkers of neurodegeneration (30). Decreased brain volume in anatomic magnetic resonance imaging (MRI) and hypometabolism in fluorodeoxyglucose (FDG) PET are also biomarkers of neurodegeneration (30). Biomarkers of neurodegeneration are not specific to AD pathology as they can be seen in other neurodegenerative diseases as well, and should be used only for staging, prognosis or to indicate treatment effect (30).

Figure 4: Typical evolution of biomarkers in AD

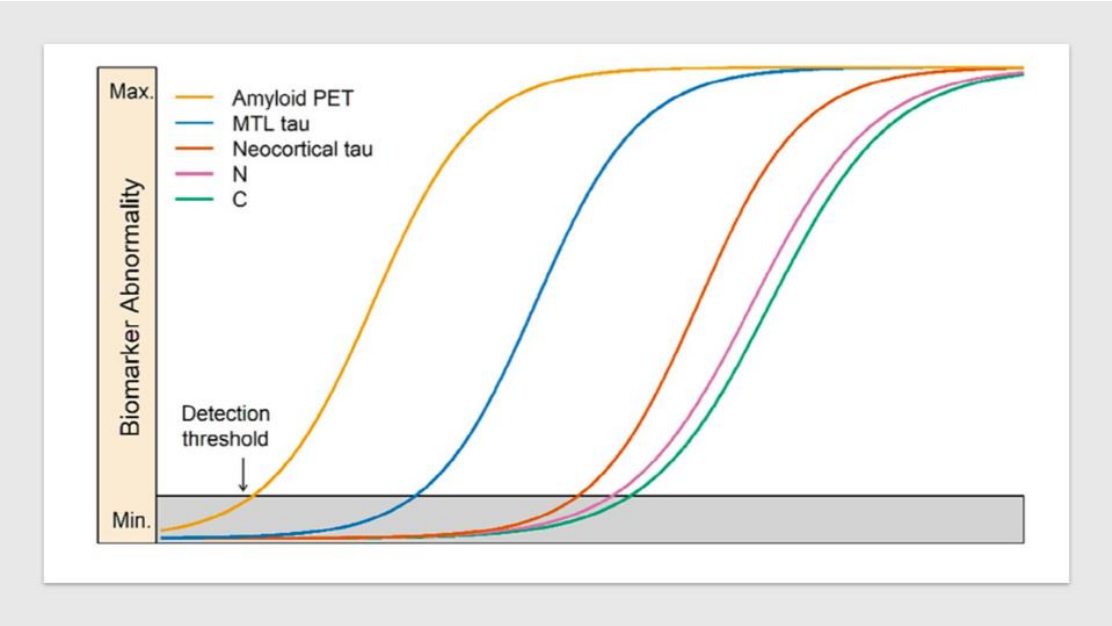


Figure 4: This figure shows the typical progression of biomarkers in an individual with only AD neuropathology. MTL: medial temporal lobe uptake on tau PET; N; neurodegeneration; C: clinical symptoms. Adapted from Jack et al. (30).

1.3 The clinical presentation of AD and the evolution of diagnostic criteria.

The identification of AD as a distinct entity and the most common cause of neurodegenerative dementia encouraged researchers to focus on better understanding the uniqueness of its clinical presentation, natural history and pathophysiology. Concurrent developments in neuropsychology, genetics, biochemistry, neuroimaging and neuropathology were crucial to improve the characterization of the disease. From the clinical perspective, progresses in neuropsychology and the development of standardized tests were fundamental to understand the clinical spectrum of AD. Several validated measurement scales such as the Mini-Mental State examination (MMSE)

(57), the Montreal Cognitive Assessment (MoCA) (58), the Clinical Dementia Rating (CDR) (59), the Alzheimer's Disease Assessment Scale–Cognitive Subscale (ADAS-Cog) (60) and The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) (61) among others were developed and are still widely used today. These measurements allowed a more precise identification of the clinical symptoms and their severity (for clinical and research purposes) and facilitated clinical-pathological correlations.

The clinical presentation of the typical (most common) form of AD is characterized by deficit in episodic memory often accompanied by deficits in other cognitive domains such as executive function, attention, abstract thinking, language or visuospatial abilities (62). Neuropsychiatric symptoms like depression, anxiety, delusions, agitation and aggressiveness are also common (62). Symptoms are mild in the beginning and worsen progressively over a period of 7-15 years (62). At the end stage of the disease patients are fully dependent, unable to communicate and often bedridden (62) and usually die due to bronchopneumonia or ischemic heart disease (63).

In addition to the typical form of AD, clinical-pathological analysis revealed other less common clinical syndromes that may be caused by AD pathology (which are referred to as atypical AD) but can also be due to other etiologies. *Posterior Cortical Atrophy* is a clinical syndrome characterized by early visuoperceptual-visuospatial symptoms with other cognitive domains being affected as disease progresses (64). In *Primary progressive aphasia – logopenic variant* the predominant clinical feature is language deficit characterized by deficits in word finding, phonemic paraphasia and circumlocution and it may also be accompanied by impairment in other cognitive domains (65). The *Frontal Variant* form comprises the dysexecutive and the behavioral variants of AD (66).

The *dysexecutive subtype* is characterized by deficits in areas such as planning, organizing and executing tasks and decision-making with little impairment of other cognitive functions or behavior symptoms (65, 66). The *behavioral subtype* presents with early changes in behavior and personality and common symptoms are disinhibition, apathy, impulsivity and agitation (65, 66). *Motor Variant – Corticobasal Syndrome due to Alzheimer's disease:* Cortical basal syndrome is diagnosed by the presence of two major diagnostic criteria (a. limb rigidity or akinesia, b. limb dystonia, c. limb myoclonus) plus two minor diagnostic criteria (d. orobuccal or limb apraxia, e. cortical sensory deficit, f. alien limb phenomena) (67). Motor symptoms are asymmetric (67) and frontoparietal degeneration predominates in the contralateral hemisphere of the affected limb (65).

Continuous progresses in the understanding of AD clinical presentation and neuropathology required the development of official diagnostic criteria to harmonize diagnosis in different centers across the world, allow earlier and more accurate recognition of the disease and enable comparisons of cases for clinical and research purposes. Significant developments in the field, especially in the area of biomarkers led to updates and new diagnostic criteria being developed throughout the years with a particular acceleration in the last decade(68).

In 1984, the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) work group published their first diagnostic criteria for AD (69). At that time biomarkers were far from being considered a possibility, therefore it was stressed that the diagnosis of AD was made exclusively on a clinical basis (69). Neuropsychological tests were used to corroborate the diagnosis and to follow disease progression and laboratory tests were to be used only for the purpose of ruling out

other diseases (69). The rationale of this early diagnostic criteria was to classify AD according to the degree of certainty of diagnosis. The diagnosis of **Definite AD** was reserved for when there was clinical diagnosis of probable AD combined with histopathological confirmation of the disease (69), and therefore it was only done postmortem. **Probable AD** was diagnosed when there was a deficit in at least two areas of cognition with progressive worsening and absence of other diseases that could justify the symptoms (69). **Possible AD** was diagnosed when there were atypical features in the presentation or progression of the disease, when a single cognitive domain was affected, or when other diseases that could contribute to the dementia symptoms were present (69).

Continuous clinical observation identified that some patients with cognitive decline did not fulfill the criteria for dementia or AD and did not always progress to dementia. This led to the development of the concept of MCI, which describes individuals who have a cognitive complaint that is noticeable and confirmed by objective tests but does not interfere significantly with daily activities (70). MCI is a heterogeneous entity that includes any cognitive decline not only memory and can be caused by different pathologies. MCI became a diagnosis widely used in clinical practice and it was shown that individuals with this diagnosis have a higher chance of progressing to AD or other dementias (71, 72).

With the continuous progress in the understanding of pathophysiology, clinical presentation and biomarkers of AD, in 2011 the National Institute of Aging (NIA) and the Alzheimer's Association (AA) released new diagnostic criteria that were compatible with the scientific knowledge at the time. In these new diagnostic criteria, AD dementia was still classified as probable or possible (73). However, the use of biomarkers (PET scan, CSF and MRI) and the presence of causative

mutations were incorporated as a way to enhance the certainty of AD being the underlying pathology (73). The use of biomarkers was not recommended for routine diagnosis, but rather for research purposes or when the clinician considered appropriate (73). These new criteria also recognized the importance of the non-amnestic forms of AD including them in the clinical presentation of the disease (73).

The 2011 NIA-AA criteria introduced formal criteria for the diagnosis of MCI for clinical and research purposes. The clinical criteria required complaint of cognitive decline in one or more domains confirmed by a formal assessment, preserved independence and no significant impairment in activities of daily life, social and occupational functioning (74). For research purposes, biomarkers (PET, CSF and MRI) were recommended to assess the level of certainty of underlying AD pathology (74). In this case, MCI was classified as: MCI due to AD with high likelihood, MCI due to AD with intermediate likelihood and MCI unlikely due to AD (74).

The most innovative aspect of the 2011 NIA-AA criteria, was the definition of a preclinical stage of AD (75). Created exclusively for research purposes, this classification recognized the fact that the pathophysiological process of AD began years before the clinical symptoms and categorized individuals in three stages according to the biomarkers present (75). *Stage 1- Asymptomatic cerebral amyloidosis* included individuals with biomarker evidence of amyloid accumulation with no evidence of neurodegeneration or clinical symptoms (75). *Stage 2 - Amyloid positivity with synaptic dysfunction and/or neurodegeneration*: this stage included individuals with evidence of amyloid accumulation as in stage 1 combined with markers of neurodegeneration (75). *Stage 3: Amyloid positivity with evidence of neurodegeneration and subtle cognitive decline:* this was the

latest preclinical stage before MCI where individuals presented biomarker evidence of amyloid deposition and neurodegeneration combined with subtle cognitive decline (75). Although at that time the field was already moving from a clinically predominant towards a more pathophysiological view that put dementia at the end of the disease spectrum, the official recognition of a preclinical stage accelerated this mindset change and provided a uniform framework for AD research in clinically asymptomatic subjects.

In 2016, the American Academy of Neurology expanded on the biomarker classification of AD and proposed new research criteria that relied mostly on the presence of biomarkers and less on clinical symptoms. In this classification the biomarkers were divided into three categories: 1. biomarkers of Aß deposition (high ligand retention on amyloid PET or low levels of CSF $A\beta_{42}$), 2. biomarkers of tau pathology (increased levels of CSF p-tau and high ligand retention on tau PET) and 3. biomarkers of AD neurodegeneration (elevated CSF t-tau, low retention of [18F]-fluorodeoxyglucose (FDG) PET, atrophy in AD relevant regions on MRI) (76). Subjects were classified as A+/–, T+/– and N+/– according to their biomarkers profile and then, depending on their clinical presentation (clinically normal, MCI or AD) they were further classified according to the likelihood of their symptoms be due to AD pathology, which depended on the combination of biomarkers present (76). This classification helped researchers better categorize patients according to symptoms and pathology and was important to improve our understanding of biomarker progression and their correlation with clinical diagnosis of AD and other dementias.

A few months ago, due to rapid developments in the field of plasma biomarkers, The National Institute on Aging and the Alzheimer's Association updated the 2016 research criteria moving even

further in the goal of diagnosing AD through biological markers rather than clinical symptoms. Amyloid and tau biomarkers are divided into Core 1 and Core 2 biomarkers where an abnormal Core 1 biomarker is sufficient to establish the diagnosis of AD, while a Core 2 biomarker is used to support a diagnosis when AD is suspected (30). These new criteria also add biomarkers of neuroinflammation and neurodegeneration which are classified as biomarkers non-specific of AD pathophysiology and are recommended to be used for disease staging, prognosis, and to assess treatment effect (30). Biomarkers of vascular brain injury and α-synuclein were also included as indicators of copathology (30) (Figure 5).

Figure 5: Biomarker evolution in a case of AD with the effect of coexisting pathology

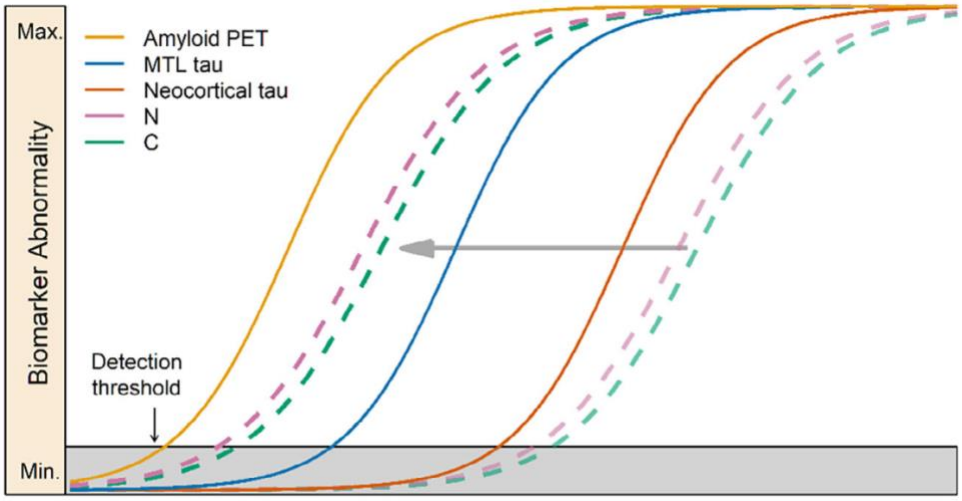


Figure 5: Example of the effect of copathology in a person with AD showing a leftward shift of neurodegeneration (N) and clinical symptoms (C) in comparison with a case of pure AD. This reflects that both N and C are more severe than expected for the degree of tauopathy, which is the result of coexisting pathologies. Adapted from Jack et al. (30)

1.4 Pharmacological treatment of Alzheimer's disease

For over 2 decades, treatments available for AD were only symptomatic. In 2019, a breakthrough led to the approval of the first anti-amyloid drug expected to be a disease modifying treatment, which was followed by the approval of two other similar drugs. Despite controversies related to approval, efficacy, cost and side effects, these drugs inaugurate a new era of AD treatment that will hopefully lead to treatments with a better efficacy and side effect profile, and to the development of treatments targeting different aspects of AD pathophysiology.

1.4.1 Symptomatic drugs.

One of the first systems affected by AD pathology is the cholinergic system. Early research demonstrated that loss of cholinergic neurons was significant, occurred in the early phases of the disease and correlated with memory loss (77-79). Therefore, the first drugs developed were cholinesterase inhibitors, which, as the name indicates, inhibit cholinesterase, the enzyme that metabolizes acetylcholine thereby increasing the availability of this neurotransmitter in the synapses (79). The three cholinesterase inhibitors available are rivastigmine, galantamine, and donepezil. These drugs are used for mild to severe stages and provide temporary improvement or stabilization of symptoms (79). Cholinesterase inhibitors are considered symptomatic drugs, and some studies have suggested that they are effective only in *APOE*-ε4 non-carriers (80). However, since until recently they were one of the only two classes of drugs available, they continue to be widely used.

Memantine is another symptomatic drug used in the treatment of AD and it is approved for the moderate and severe stages. Another process that occurs in AD is neurotoxicity due to excessive excitatory activation of the glutamatergic system (81). Memantine inhibits the NMDA glutamatergic receptor thereby preventing the effects of glutamatergic-induced neurotoxicity (81).

1.4.2 Disease modifying treatments.

After decades of failed clinical trials, dealing with amyloid synthesis inhibitors, depolymerizing agents, vaccines, aggregation inhibitors …, the first anti-amyloid disease modifying drugs were finally approved by the FDA, but not by European agencies. All three drugs are human monoclonal antibodies that target slightly different forms of Aß: Aducanumab binds to aggregated forms of Aβ (oligomers and insoluble fibrils) (82), Donanemab binds to insoluble β-amyloid in amyloid plaques (83) and Lecanemab targets soluble Aβ protofibrils (84). They are all intravenous drugs, indicated for early stages of dementia and require confirmation of increased brain amyloid pathology by PET or CSF amyloid biomarkers. The most serious side effect is amyloid-related imaging abnormalities (ARIA) that occurs more frequently in *APOE*-ε4 carriers (82-84).

1.5 The current state of knowledge in AD

The developments in our knowledge of clinical presentation, pathophysiology and biomarkers of AD that were described in the previous sessions gives us enough evidence that the pathophysiological process begins decades before clinical symptoms and that we are becoming increasingly capable of recognizing this pathology in asymptomatic yet high-risk subjects. The

accelerated development of biomarkers has been essential in this process, and they exist in different forms such as PET scans, CSF biomarkers or plasma biomarkers. While PET scans offer the unique advantage of assessing not only the levels but also spatial distribution of neuropathology, they are expensive and need to be performed in specialized centers, therefore being more suitable for research purposes. CSF is less expensive and more widely available, but it is still an invasive procedure that many patients are not willing to do. The recent developments of more accurate blood biomarkers however are a great promise to facilitate access to AD biomarkers worldwide. At this point I believe that there is no doubt that soon AD we will be accurately diagnosed by biomarkers years before the onset of symptoms. This development, together with new progresses in disease modifying treatments will put us in a good position to finally be able to provide effective clinical management for AD and maybe, even prevention.

2 Genetics and Alzheimer's disease

2.1 The importance of genetics for the study of health and disease.

The understanding of human genetics has evolved greatly and has brought crucial developments to our knowledge of normal physiology and pathophysiology of disease. The identification of causative mutations of serious diseases for instance has been critical not only to help better understand the disease itself and improve treatment perspectives, but also to ensure early and accurate diagnosis allowing patients to make important life and reproductive decisions. More recently, advances in genetic therapy introduced innovative, life changing treatments to severe illnesses such as spinal muscle atrophy (85), sickle cell disease (86) and Hemophilia (87) among others.

In Alzheimer's disease, the identification of causative mutations involved in amyloid processing helped solidify the role of APP and presenilin in the disease progress, both being correlated with Aβ generation (9). Additionally, it has allowed the identification of asymptomatic carriers and large family kindreds who have played a crucial role in AD research (88, 89). The *APOE-ε4* allele, which is the most important genetic risk factor in sporadic AD (43, 44) has also helped our understanding of the disease. As a cholesterol transporter, *APOE* highlights the importance of cholesterol metabolism in the pathophysiology of AD underscoring the relevance of metabolic pathways outside of amyloid and tau pathology.

In this section we will describe in detail the genetics of AD and how a thorough knowledge of the genetics factors involved could improve our understanding of the pathophysiology of the disease which is essential for the advancement of diagnostic and treatment strategies.

2.2 Genetics of Alzheimer's disease

From the genetics perspective, AD can be divided into familial and sporadic forms. The familial form is rare accounting for about 1% of the cases and it is caused by autosomal dominant mutations with 100% penetrance (90). The sporadic form, which represents up to 99% of cases, is caused by a combination of environmental and genetic risk factors. Although the environmental risk factors play an important role in sporadic AD the heritability of the disease is still high and ranges between 50-80% (91, 92). However, the genes identified so far account for only about 30% (92, 93) of this phenotypic variance with *APOE-ε4* being the major contributor (about 20-25% of the 30%) and

all other variants combined playing a much smaller role in AD heritability (93). These numbers show that most of the risk variants involved in sporadic AD have not yet been identified.

2.2.1 The autosomal dominant form of AD

The familial form of AD is caused by fully penetrant autosomal dominant mutations in the genes APP, Presenilin-1 (PS1) and Presenilin-2 (PS2) (90, 94). This form of the disease normally begins at an earlier age, tends to have a more aggressive course and a higher frequency of non-cognitive neurological symptoms such as seizures and extrapyramidal signs (95).

The identification of these autosomal dominant mutations has allowed preclinical diagnosis of asymptomatic subjects and the identification of large groups of mutation carriers and family kindreds that have played a unique role in AD research. Studies of these subjects have allowed follow-up of individuals since their asymptomatic stage which greatly helped the understanding of the natural history, the pathological and biomarker progression and the specific clinical features of this form of the disease.

Of great importance it was the identification, in the Colombian family kindred of PS1 mutation carriers, of a 70-year-old woman who, despite having the dominant mutation only developed MCI in her seventies (three decades after the expected age) and did not develop high levels of tau pathology despite having abnormally high levels of amyloid (96). Although it is not confirmed, studies suggest that she did not develop AD due to the presence of two copies of the *APOE3* Christchurch mutation (96). If this fact is confirmed it could bring new treatment perspectives for

AD. Moreover, it highlights the importance of the study of genetics of AD and the inclusion of less represented racial groups.

2.2.2 The sporadic form of AD

The sporadic form of AD corresponds to about 99% of the cases and is inherited as a complex trait that results from interactions between genetic and environmental risk factors (97). The most important environmental modifiable risk factors for sporadic AD are low education, cardiovascular risk factors (hypertension, obesity, diabetes, smoking and dyslipidemia), physical inactivity, depression, traumatic brain injury, air pollution, visual and hearing loss, social isolation and excessive alcohol intake (98). But even though these factors play an important role, it has been demonstrated that the heritability of AD is very high, ranging between 50-80% (91, 92). Genome-wide association studies (GWAS) involving hundreds of thousands of patients have identified polymorphisms in about 75 genome-wide significant loci associated with AD risk (99). However, the variants identified so far account for only about 30% of the global phenotypic variance (92, 93), which means that a significant part of the risk alleles for sporadic AD remain to be identified. The identification of these missing variants could bring important insights into the pathophysiology of AD which is fundamental for the search for new diagnostic and treatment strategies.

2.2.3 The *APOE*-ε4 variant and its role in sporadic AD

The ε4 allele in the *APOE* gene is widely known as the most important genetic risk factor for sporadic AD (43, 100). Two SNPs in the *APOE* gene lead to 3 different alleles *APOE-ε2*, *APOE-*

ε3 and *APOE-ε4* which results in three isoforms of the apolipoprotein E (101). *APOE-ε2* is considered protective while the presence of *APOE-ε4* significantly increases the risk of AD by up to eight times in homozygote individuals (102). *APOE-ε4* is the major AD risk factor in all populations but its role is more significant in the Caucasian than among other races (103, 104).

Apolipoprotein E is the main cholesterol transporter in the brain. It forms HDL particles that have an essential role in the transport of cholesterol and phospholipids necessary for neuronal and synaptic turnover and processing. The presence of the *APOE-ε4* allele leads to lower protein levels and less effective cholesterol transport therefore impairing one of the most important roles of apolipoprotein E in the brain (47). This protein reduction is not caused by reduced gene expression but more likely by faster rate of degradation of the *ε4 variant* (105).

There is still not a consensus about the role *APOE-ε4* plays in AD. One line of research states that *APOE-ε4* increases Aβ deposition and impairs its clearance therefore leading to increased amyloid load (46, 106). On the other hand, a significant amount of evidence supports an impairment in the role of *APOE-ε4* as a cholesterol transporter. Cholesterol and phospholipids are the main components of neuronal membrane, synapses and myelin; therefore, cholesterol transport is essential for processes of neuronal and synaptic turnover and regeneration particularly following neuronal damage (48). The *APOE-ε4* variant leads to lower protein levels and less effective transport of cholesterol and phospholipids in the brain (45, 47). This decreased efficacy in cholesterol transport may lead to a lower ability of the brain to promote neuronal remodeling and reinnervation following a pathological injury such as the one that occurs in AD (47). In fact, studies have shown that in mouse models of hippocampal deafferentation there is an increase in *APOE*

gene expression during the reinnervation phase (48, 49), likely to increase cholesterol uptake and delivery to reinnervating neurons.

2.2.4 The search for the missing heritability of AD.

As described above, the genetic polymorphisms identified so far explain only a small part of the phenotypic variability in AD, meaning that most genetic variants involved in the risk of AD have not yet been identified. Additionally, as we have shown, the study of genetics is an important strategy to improve our knowledge about a disease and its treatment and has played a crucial role in the developments we had in AD research so far.

With the goal of identifying some of the unknown genetic risk factors of AD, the Poirier lab performed a GWAS in the genetically homogenous Quebec Founder Population (QFP) cohort, a well-characterized population isolate based in North America (107). The QFP is a unique population isolate from eastern Canada, for which genealogical information is available for almost four centuries. This population descends from the French settlers that founded Nouvelle France in the 17th and 18th centuries (107). The migration and isolated nature of the settlements created a founder effect giving rise to a population with large linkage disequilibrium blocks and low genetic heterogeneity which leads to a lower genetic noise that is highly advantageous for genetic studies (108).

The original GWAS included at total of 751 pairs of Case/Control subjects from the QFP cohort matched for sex, age and especially region of birth and mapped 535,000 polymorphisms in all subjects.

This study identified polymorphisms in the following genes as risk factors for AD ($p < 0.00001$): *APOE, CLU (ApoJ), CDK5-RAP2, HIVEP3, RGS17, ACAT2, PPP2R1A, Intergen/ODZ4, STIL, CNTN5, ZNF10.*

In a second stage replication analysis all risk factors identified in the QFP cohort were reassessed and validated in a French Case/Control population using France-based subsample from IGAP study (34). A total of 2032 AD cases and 5328 age-matched controls were included in the replication phase, and all variants identified in the QFP cohort were confirmed to be statistically associated with AD ($p< 0.05$).

For this thesis, two of the variants identified by the GWAS were selected to a) confirm their role in AD and b) to understand how they are involved with the pathophysiology of the disease. For this purpose, we chose two variants located in the *CNTN5* and in the *CLU* genes. No polymorphism in the *CNTN5* gene had ever been associated with AD before, therefore by extensively studying *CNTN5* in different populations, we aim to unravel its mechanism of action in AD pathophysiology and broaden our understanding of the disease. In contrast, *CLU* is an established risk gene for sporadic AD. Our goal is to use different population cohorts to provide more insights in the mechanism of action of this gene in the different phases of the disease. These genes were chosen for further studies also because they showed relatively high odds ratio in the QFP GWAS, and because they were significantly correlated with aspects of AD pathophysiology in preliminary studies (table 1).

Table 1: Effect of the top 12 risk variants: Impact of risk allele on classical pathological markers of AD

Rs#	Gene	Association -LOG(P)	Allele	Odd Ratio	Association SNP*	Association NFT^	Association ChAT Act.#
429358	ApoE	54.00			p < 0.001	p < 0.001	p < 0.05
11136000	ApoJ	3.92	T	0.75			p < 0.001
10984186	CDK5-RAP2	4.66	A	1.41		p < 0.05	
10493098	HIVEP3	4.60	C	1.37		p < 0.05	p < 0.05
317104	RGS17	3.90	G	0.71	p < 0.05		
9658625	ACAT2	3.66	G	1.64			
10406151	PPP2R1A	4.30	C	1.39	p < 0.05		
10898006	Intergenic (~ODZ4)	3.63	G	1.46			
10789507	STIL	5.20	A	2.3		p < 0.01	
7973103	Intergenic(~)	3.92	T	1.42	p < 0.05		
1461684	CNTN5	4.66	G	1.48	p < 0.05		
11609382	ZNF10	3.64	A	1.36		p < 0.05	p = 0.06

* : Total senile plaque density for 5 brain regions / n=118
^ : Total neurofibrillary tangle density for 5 brain regions / n=118
"#: Choline acetyltransferase activity in the hippocampus in AD / n=20
Significance was assessed by ANOVA

2.2.5 The *CNTN5* rs1461684 G variant

Contactins are a family of proteins that contains 6 members, Contactin-1 to Contactin-6. These membrane proteins are cell adhesion molecules that play an important role during neurodevelopment by acting on processes such as neuronal migration, axon guidance, synaptogenesis, myelin formation and regulation of neurite outgrowth (109, 110).

The *CNTN5* gene codes for contactin 5 which is a membrane protein mainly involved in neurite outgrowth and axonal arborization (111) and synaptic formation (112). Contactin 5 has its peak expression in the first weeks after gestation and is mainly found in the olfactory bulb, cerebral cortex and in the thalamus (109). One possible association of contactin 5 with AD pathology is that, since contactins lack the intracellular region that would allow them to communicate across the membrane, contactin 5 binds to APP and its precusor-like variants (APLP1) in their cytoplasmic regions, thereby using amyloid-dependent signal transduction pathways to relay information across the membrane (113).

Single nucleotide polymorphisms and copy number variants in the *CNTN5* gene have been associated with increased risk of neuropsychiatric disorders particularly the neurodevelopmental ones such as autism spectrum disorder (114, 115) and attention-deficit hyperactivity disorder (116). Only one other GWAS in AD reported a risk-associated polymorphism (rs10501927) in the *CNTN5* gene but it did not reach genome-wide significance (117). Ours was the first GWAS so far to find a significant association between a *CNTN5* variant (rs1461684 G) and AD risk. Our purpose in this thesis is to confirm the *CNTN5* rs1461684 G variant as a risk factor for AD and to investigate what role the gene, variant and protein play in the pathophysiology of the disease.

2.2.6 The *CLU* rs 11136000 T variant

The *CLU* gene is already established as one of the main genetic risk factors of AD (118) with several variants been associated with disease risk or protection (33, 34, 117, 119). *CLU* codes for the clusterin (or ApoJ) protein which is a glycoprotein ubiquitously expressed in the brain and in most body tissues and with a wide range of physiological functions (120).

Among the many physiological processes clusterin is involved in, the main ones are cholesterol transport and mobilization (121, 122), regulation of cell survival and apoptosis (123, 124), protection against oxidative stress (125, 126) and acting as a molecular chaperone (127). As a consequence of its ubiquitous presence and wide range of functions, clusterin has been associated with a variety of pathological processes such as cardiovascular diseases (128), different neurological disorders (129, 130), and promotion of tumorigenesis and chemoresistance (131-133).

Several studies have confirmed the association of different *CLU* variants with risk of AD (33, 34, 117, 134) and some variants are associated with increased risk (134), while others are associated with lower risk (34, 135). However, even though *CLU* polymorphisms have been consistently associated with AD in Caucasian populations (34, 117, 136) these associations are not always found among other populations such as Asians (119, 134, 137), Hispanics (135, 138), and populations of African descent (135, 138).

The precise mechanism of action of *CLU* in AD pathology has not yet been established and the main possibilities include: it is involved in Aß aggregation and clearance (139-141), it has a neuroprotective role by acting on oxidative stress and neuroinflammation (142, 143), it regulates cholesterol transport for membrane and synaptic remodeling (49). The variant identified in our GWAS is the rs11136000 T which is associated with a lower risk of AD (34, 135) (table 1). The purpose of this thesis is to provide further insights into the role of this important gene, the rs11136000 T protective variant and the clusterin protein in the pathophysiology of AD.

3. Thesis Rational and Objectives

As thoroughly explained in the previous sessions, there are still significant gaps in our knowledge of AD pathophysiology which hinder our ability to provide earlier diagnosis and develop better treatment and prevention strategies. Genetics play an important role in the sporadic form of AD and a detailed knowledge of the genetic background is an important pathway to better understand the pathophysiology of a disease. Since only a small part of the genetic variants associated with

the risk of AD are currently known, a GWAS was performed by our lab to uncover new genetic polymorphisms associated with AD. Twelve variants were identified in the QFP GWAS and for this thesis we chose to study in depth the variants identified in the *CNTN5* and *CLU* genes. The general goal is to confirm the association of the *CNTN5* polymorphism with risk of AD and to investigate the mechanism of action of both genes in AD pathophysiology throughout the different phases of the disease process.

To accomplish these goals, we first aimed to confirm the association of the newly identified *CNTN5* rs1461684 G variant with increased risk of AD by investigating the effect of this variant on the risk of developing AD in different population cohorts. We then proceeded to study the mechanism of action of the *CNTN5* and *CLU* genes, the rs1461684 G and rs11136000 T variants and the contactin 5 and clusterin proteins throughout the different phases of the disease. We did that by investigating the association of the variants and their proteins with different clinical and pathological measurements of the disease in several patient cohorts that encompassed the different phases of the disease spectrum. The pathological aspects of AD were assessed both in living patients with the use of biomarkers and in brain samples from autopsied patients. Autopsied AD patients and controls from different cohorts were also used to assess the effect of the variants and the presence of AD on *CNTN5* and *CLU* gene expression. Finally, we aimed to investigate the role of *CNTN5* and *CLU* during the neuronal degeneration/reinnervation process that follows neuronal injury, to further understand the mechanism of action of these genes and their possible role in AD. For that we used a mouse model of hippocampal deafferentation and measured CNTN5 and CLU gene expression at different time-points for 40 days covering both the deafferentation and the reinnervation periods.

Manuscript 1: Characterization of the contactin 5 protein and its risk-associated polymorphic variant throughout the Alzheimer's disease spectrum

Marina Tedeschi Dauar[1,2,3,4], Anne Labonté[1,2], Cynthia Picard[1,2], Justin Miron[1,2,3], Pedro Rosa-Neto[1,2,3,5], Henrik Zetterberg[6,7,8,9,10], Kaj Blennow[6,7], Sylvia Villeneuve[1,2,3,5], Judes Poirier[1,2,3,5]

1. Douglas Mental Health University Institute, Montréal, Canada
2. Centre for the Studies in the Prevention of Alzheimer's, Douglas Mental Health University Institute, Montréal, Canada
3. McGill University, Montréal, Canada
4. CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil
5. Department of Psychiatry, McGill University, Montréal, Canada
6. Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
7. Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden
8. Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK
9. UK Dementia Research Institute at UCL, London, UK
10. Hong Kong Center for Neurodegenerative Diseases, Hong Kong, China

Correspondence:
Judes Poirier, Centre for the Studies in the Prevention of Alzheimer's Disease, Douglas Mental Health University Institute, 6875, Lasalle, Montréal, Quebec H4H 1R3, Canada. E-mail: judes.poirier@mcgill.ca

Published in Alzheimer's & Dementia®. 2023 Jul;19(7):2816-2830. (doi: 10.1002/alz.12868. PMID: 36583624)

1. Preface

The identification of genetic risk factors that influence the risk of AD is an important step to better understand the disease. However, it is essential to also understand the role these genetic variants play in the pathological process of AD in order to fill in the gaps that still exist in our knowledge of AD pathophysiology.

In this chapter, we aim to investigate the role of the *CNTN5* rs1461684 G variant in AD. Since this was the first time this variant was associated with AD, we started by confirming its role as a risk factor in different populations. We followed by looking into the role of the *CNTN5* rs1461684 G variant and the contactin 5 protein in the different phases of AD. By using population cohorts that encompass the different stages of the AD spectrum (from cognitively unimpaired subjects to autopsy confirmed AD cases) we investigate the role of the *CNTN5* rs1461684 G variant on gene expression, contactin 5 levels and clinical and pathological hallmarks of the disease. We also studied the association of CSF contactin 5 with different AD biomarkers and with disease progression. We believe that with this investigation we can describe a new genetic risk factor for AD and gain insights into its role in the disease.

2. Abstract

Introduction: We investigate the *CNTN5* rs1461684 G variant and the contactin 5 protein in sporadic Alzheimer's disease (sAD).

Methods: Contactin 5, sAD biomarkers, and synaptic markers were measured in the cerebrospinal fluid (CSF). Amyloid and tau deposition were assessed using positron emission tomography. Contactin 5 protein and mRNA levels were measured in brain tissue.

Results: CSF contactin 5 increases progressively in cognitively unimpaired individuals and is decreased in mild cognitive impairment and sAD. CSF contactin 5 correlates with sAD biomarkers and with synaptic markers. The rs1461684 G variant associates with faster disease progression in cognitively unimpaired subjects. Cortical full-length and isoform 3 *CNTN5* mRNAs are decreased in the presence of the G allele and as a function of Consortium to Establish a Registry for Alzheimer's Disease stages.

Discussion: The newly identified rs1461684 G variant associates with sAD risk, rate of disease progression, and gene expression. Contactin 5 protein and mRNA are affected particularly in the early stages of the disease

3. Introduction

Alzheimer's disease (AD) is one of the most important causes of cognitive decline in the elderly population (1,2). Pathologically, AD is characterized by progressive accumulation of amyloid plaques and neurofibrillary tangles, and by synaptic and neuronal degeneration and loss (3,4). Several genetic variants have already been identified as common risk factors for sporadic AD (sAD), with the most important one being the ε4 allele of the apolipoprotein E (*APOE*) gene (5,6). However, it is estimated that the single nucleotide polymorphism (SNP)–based heritability of sAD

ranges from 13% to 33%, indicating that a significant part of the risk alleles remains to be identified (5,7).

The *CNTN5* gene encodes the contactin 5 protein, which is a cell-surface protein with multiple isoforms that belongs to the contactin family. Contactins are a family of cell-adhesion molecules that contain six members: contactin 1 to contactin 6. These proteins play an important role in neurodevelopment through the regulation of neurite outgrowth, neuronal migration, axon guidance, synaptogenesis, myelin formation, neuron—glia interactions, and cell survival (8,9).

Single nucleotide polymorphisms and copy number variants in the *CNTN5* gene have been associated with increased risk for several neuropsychiatric disorders such as autism spectrum disorder (10,11), anorexia (12), and attention deficit hyperactivity disorder (13). In sAD, a risk-associated polymorphism in the *CNTN5* gene (rs10501927) was reported by Harolds et al. (14) but it failed to reach genome-wide significance (odds ratio [OR]: 1.18; $P = 2.0 \times 10{-}6$, $n = 11789$). Using a unique population isolate from eastern Canada, we report here the presence of a distinct risk variant (rs1461684 G) in the *CNTN5* gene, which is associated with increased risk for sAD in this cohort, as well as in other heterogenous genetic studies.

In the present work, our objective is to characterize the role of contactin 5 (soluble in the cerebrospinal fluid [CSF] and membrane-bound to neurons in the frontal cortex) and its association with different pathological biomarkers of sAD in both the asymptomatic and symptomatic stages of the disease. In parallel, we examined the newly identified rs1461684 risk variant G throughout the spectrum of sAD. More specifically, we investigate the role of this common polymorphism (minor allele frequency [MAF]: 0.16) in the risk of developing sAD and its effect in the clinical and pathological progression of the disease using four different cohorts.

4. Methods

This study used four different patient cohorts. All of them received local approval from the research ethics committee or institutional review boards of the participating centers.

4.1 PREVENT-AD

4.1.1 Study participants

The Pre-symptomatic Evaluation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) cohort is composed of cognitively unimpaired individuals over the age of 55, who have a first degree relative diagnosed with sAD (15). There are 373 active participants (or subset of participants) who are followed annually with clinical and cognitive assessments, blood and CSF biomarkers, structural and functional magnetic resonance imaging (MRI) and brain positron emission tomography (PET) scans to assess amyloid beta (Aß) and tau deposition. Data used in this article were obtained from data release 6.0 (2020, https://openpreventad.loris.ca/).

4.1.2 CSF

Lumbar punctures were performed in a subset of volunteers (n = 170) in the morning after an overnight fast using a Sprotte 24-gauge atraumatic needle. CSF samples were centrifuged within 4 hours, cells and insoluble material were excluded, and samples were aliquoted and stored at -80ºC. The AD biomarkers phosphorylated tau (p-tau)181, total tau (t-tau), and Aß42 were measured following procedures developed by the BIOMARKAPD consortium (16), using validated Innotest enzyme-linked immunosorbent assay kits (P(181)-tau Cat.# 81581, T-tau Cat.# 81579, and Aβ42 Cat.# 81583) from Fujirebio. CSF contactin 5 levels were measured using Olink's proximity extension assay and the neurology panel. Synaptic markers were quantified in the CSF using immunoprecipitation followed by mass spectroscopy as described previously (17–20).

4.1.3 Neuroimaging acquisition and processing

18F-NAV4694 (Navidea Biopharmaceuticals) was used to quantify Aß accumulation. Scans were performed 40 to 70 minutes after injection in a subgroup of PREVENT-AD subjects (n = 129). Flortaucipir ([18F]AV1451) was used to measure tau deposition and scans were acquired 80 to 100 minutes post injection as described previously (21). Standardized uptake value ratios (SUVRs) were obtained using the cerebellum as a reference region for Aß-PET and the inferior cerebellum gray matter for flortaucipir (21). AD-related tau deposition was assessed by averaging flortaucipir SUVR in the entorhinal cortex, fusiform, parahippocampal, and lingual gyri (21,22).

4.1.4 Alzheimer Progression Score

The composite Alzheimer Progression Score (APS) was developed by our team to map the progression of the disease in the absence of visible cognitive deficits in the PREVENT-AD cohort, for the purpose of prevention drug trials (23). The APS is a composite that incorporates multimodal imaging, neurosensory, cognitive, and CSF markers, based on an assumption that change in each of these arises from a single underlying latent process (i.e., AD pathogenesis). Its scores are scaled as a standard normal distribution, with higher scores denoting increasing severity. Constituent measures are summarized in greater detail in Leoutsakos et al. (24).

4.1.5 Genotyping

Automated DNA extraction from buffy coat samples was performed using the QIAsymphony DNA mini kit (Qiagen). Genotyping was performed using the Omni2.5-8 BeadChip (Illumina). PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) was used to filter sex mismatches, filter missingness at sample level (< 5%) and SNP level (< 5%), assess sample heterozygosity, and filter SNPs in

Hardy–Weinberg disequilibrium ($P > 0.001$). Only post-imputed SNPs with an info score > 0.7 were kept.

4.2 COMPASS-ND cohort

4.2.1 Study participants

The Comprehensive Assessment of Neurodegeneration and Dementia (COMPASS-ND) study is enrolling 1650 memory-impaired/concerned subjects from 31 centers across Canada. Participants typically undergo comprehensive baseline evaluation including clinical and neuropsychological assessment, biospecimen collection, polymorphisms mapping, and MRI neuroimaging (25). Data are made available to investigators in the Canadian Consortium on Neurodegeneration in Aging (CCNA) as well as others through the Longitudinal Online Research and Imaging System (LORIS) database at https://ccna-ccnv.ca/national-platforms/. CSF collection and measurements are performed as described above for the PREVENT-AD cohort. No genotype information is available for this cohort at the moment.

4.3 ROS-MAP

The Religious Orders Study (ROS) was established in 1994, and it includes nuns, priests, and brothers from across the United States (26). The Rush Memory and Aging Project (MAP) started in 1997 and includes lay people from the state of Illinois. Participants from the cohorts were cognitively normal at enrolment and were followed annually with neuropsychological evaluation and blood test and consented to genotyping and brain donation (26). *Post mortem* evaluation was performed to assess AD pathology using Consortium to Establish a Registry for Alzheimer's Disease (CERAD) and Braak staging. ROS-

MAP datasets and protocols used in the present study are summarized in the supporting information 1.

4.3.1 Tandem mass tag proteomic data

Three hundred forty cortical prefrontal brain tissue samples from the community-based aging ROS-MAP cohort were analyzed by a mass spectrometry-based protein quantification approach, using isobaric multiplex tandem mass tags (TMT) as described previously by Ping et al (27). Briefly, TMT labeling with synchronous precursor selection (SPS)-MS3 for reporter ion quantitation was used to achieve comprehensive global quantitation of 100 mg (wet tissue weight) pre-frontal cortex from healthy controls and sAD cases. In total, 127,321 total unique peptides were identified from >1.5 million peptide spectral matches (PSMs), which mapped to 11,840 unique proteins groups, representing 10,230 gene symbols, which map to ≈65% of the protein coding genes in the brain. Two major isoforms of *CNTN5* expressed in the brain are available in the ROS-MAP dataset: the full length (O94779) wild type variant and the isoform type 3 (O94779-4, missing amino acids 912–1100), which are particularly prevalent in the central nervous system (CNS).

4.4 The QFP cohort

The Quebec Founder Population (QFP) cohort is a population isolate from eastern Canada that descends from 3000 French settlers that founded Nouvelle France in the 17th and 18th centuries (28). The migration and the isolated nature of settlements created a founder effect, which resulted in a population with less genetic heterogeneity, large linkage disequilibrium blocks, and low genetic noise, which is highly advantageous for genetic studies (28,29), especially for genome-wide association study (GWAS) case/control studies in which age, sex, and especially place of

birth are used to control the sample's demographic characteristics. Genealogical information for this population, for almost four centuries, is available in the BALSAC database (30).

4.5 Statistical analyses

Analyses of the demographics were done using analysis of variance (ANOVA) and the significant main effects were decomposed using Tukey's post hoc test. The progression of CSF contactin 5 levels over time was tested using mixed linear model adjusted for sex, age, and *APOE* ε4. The correlation of CSF or plasma contactin 5 with CSF biomarkers, synaptic proteins, and amyloid and tau PET in the PREVENT-AD cohort was assessed using linear regression adjusted for sex, age, and *APOE* ε4. For this analysis we used only data acquired at baseline visit. The effect of the *CNTN5* variant on NAV4694 and flortaucipir SUVR retention in the PREVENT-AD cohort was assessed using linear regression adjusted for sex, age, and *APOE* ε4. ANOVA was used to assess the difference in APS scores and CSF contactin 5 according to *CNTN5* genotype, the difference in CSF contactin 5 according to clinical diagnosis (cognitively unaffected, mild cognitive impairment [MCI], and AD), the levels of contactin 5 isoforms in the prefrontal cortex according to Braak stages and the difference in *CNTN5* mRNA and proteins levels in asymptomatic and MCI subjects according to the number of *CNTN5* rs1461684 G variants; all analyses were adjusted for sex, age, and *APOE* ε4 genotype. Changes in contactin 5 levels in the CSF of controls versus MCI and sAD subjects were decomposed using Tukey's post hoc test. The association between contactin 5 mRNA levels and Braak and CERAD stages in the ROS-MAP cohort was assessed using ordinal logistic regression and adjusted for age, sex, and *APOE* ε4. For this cohort, the exact age is not specified for participants over the age of 90. Because there is a significant number of participants older than 90, age was considered using age groups: < 80, 80 to 84, 85 to 89, > 90.

5. Results

5.1 Demographics:

Table 2 summarizes the demographic characteristics of the main cohorts used to analyze the impact of the *CNTN5* variant on CSF protein level (PREVENT-AD) and on cortical protein and mRNA prevalence (ROS-MAP) at different stages of sAD's spectrum. There was no difference in age, sex, education, and *APOE* ε4 status between the *CNTN5* genetic subgroups.

Table 2: PREVENT-AD and ROS-MAP cohort demographics

	PREVENT - AD Cohort					ROSMAP - Cohort				
		CNTN5 rs1461684-G Allele Dose					CNTN5 rs1461684-G Allele Dose			
	Overall N=373	0 N=259	1 N = 103	2 N = 11	p-value	Overall N = 1,118	0 N= 821	1 N=269	2 N=28	p-value
AGE	63.2 ± 5.2	63.2 ± 5.2	62.9 ± 5.2	64 ± 5.3	**0.77**	89.0 (84.7, 90.0)	89.0 (84.7 - 90.0)	88.9 (84.7 - 90.0)	88.3 (83.3 - 90.0)	**0.3**
< 80						102 (9.1%)	69 (8.4%)	30 (11%)	3 (11%)	
80 - 84						196 (18%)	145 (18%)	43 (16%)	8 (29%)	
85 - 89						329 (29%)	240 (29%)	80 (30%)	9 (32%)	
> 90						491 (44%)	367 (45%)	116 (43%)	8 (29%)	
SEX					**0.64**					**0.4**
male	111/373 (30%)	76/259 (29%)	33/103 (32%)	2/11 (18%)		377 (34%)	279 (34%)	92 (34%)	6 (21%)	
female	262/373 (70%)	183/259 (71%)	70/103 (68%)	9/11 (82%)		741 (66%)	542 (66%)	177 (66%)	22 (79%)	
APOE4					**0.13**					**>0.9**
APOE4 -	230/373 (62%)	168/259 (65%)	57/103 (55%)	5/11 (45%)		824 (74%)	603 (74%)	200 (74%)	21 (75%)	
APOE4 +	143/373 (38%)	91/259 (35%)	46/103 (45%)	6/11 (55%)		293 (26%)	217 (26%)	69 (26%)	7 (25%)	

Abbreviations: *APOE*, apolipoprotein E; PREVENT-AD, Pre-symptomatic Evaluation of Experimental or Novel Treatments for Alzheimer's Disease; ROS-MAP, Religious Orders Study Rush Memory and Aging Project.

5.2 PREVENT-AD cohort: contactin 5 protein levels in the CSF and plasma

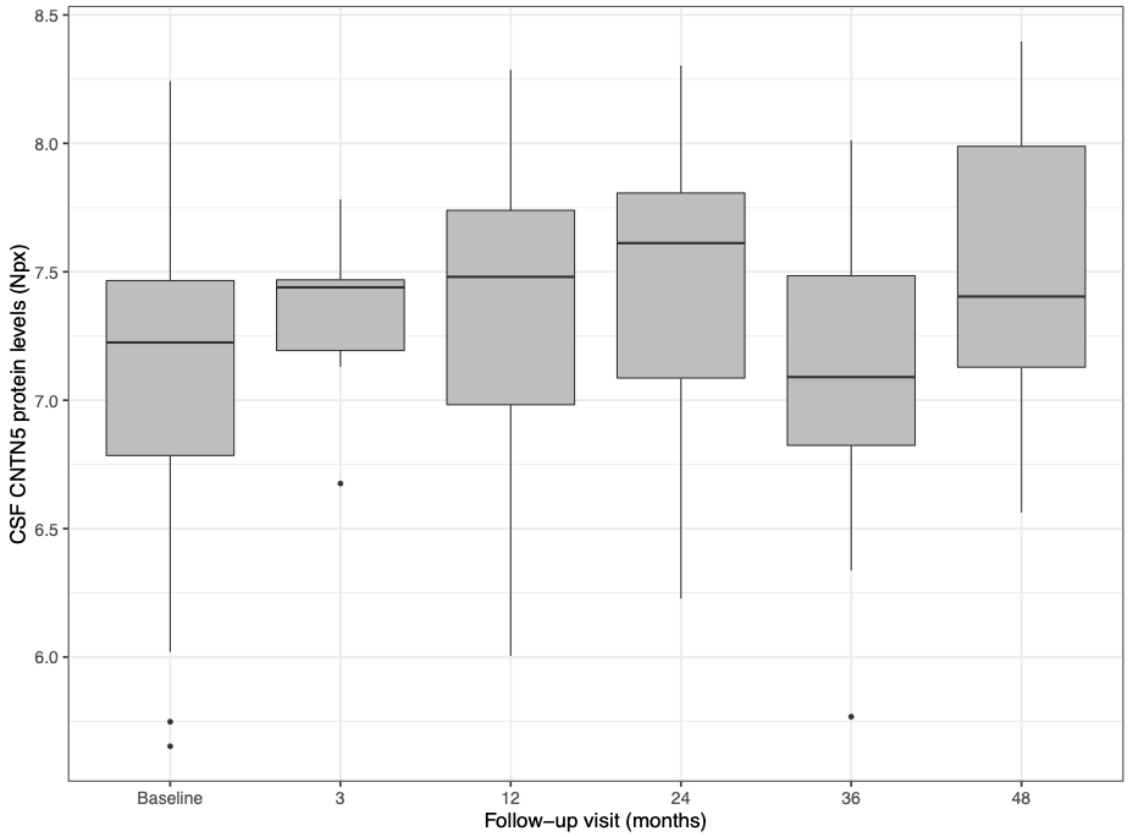
CSF contactin 5 levels were measured in cognitively unimpaired participants from the PREVENT-AD cohort. Longitudinal measures of CSF contactin 5 showed a progressive increase in the levels of this protein over time, with significant alterations at follow-up visits at 12 ($P = 0.025$), 24 ($P = 0.018$), and 48 months ($P = 8 \times 10^{-5}$; Figure 6).

CSF contactin 5 measures were contrasted with sAD pathological biomarkers in the same cognitively unimpaired subjects. A positive correlation was found between CSF contactin 5 and CSF Aß42 ($r^2 = 0.26$; $P = 0.00049$), Aß40 ($r^2 = 0.13$; $P = 0.002$), t-tau ($r^2 = 0.47$; $P = 1.4 \times 10^{-11}$), and p-tau ($r^2 = 0.51$; $P = 6.6 \times 10^{-12}$; Figure 7). There was a trend toward a positive correlation between CSF contactin 5 and tau deposition in the entorhinal cortex measured by PET ($r^2 = 0.23$; $P = 0.065$) in these asymptomatic subjects.

CSF contactin 5 levels were also positively correlated with synaptic proteins GAP43 ($r^2 = 0.50$; $P = 1.4 \times 10^{-06}$), neurogranin ($r^2 = 0.37$; $P = 0.0004$), Syt1 ($r^2 = 0.43$; $P = 2.7 \times 10^{-05}$), and Snap 25 long ($r^2 = 0.46$; $P = 1.6 \times 10^{-05}$; Figure 6) in cognitively unimpaired subjects from the PREVENT-AD cohort.

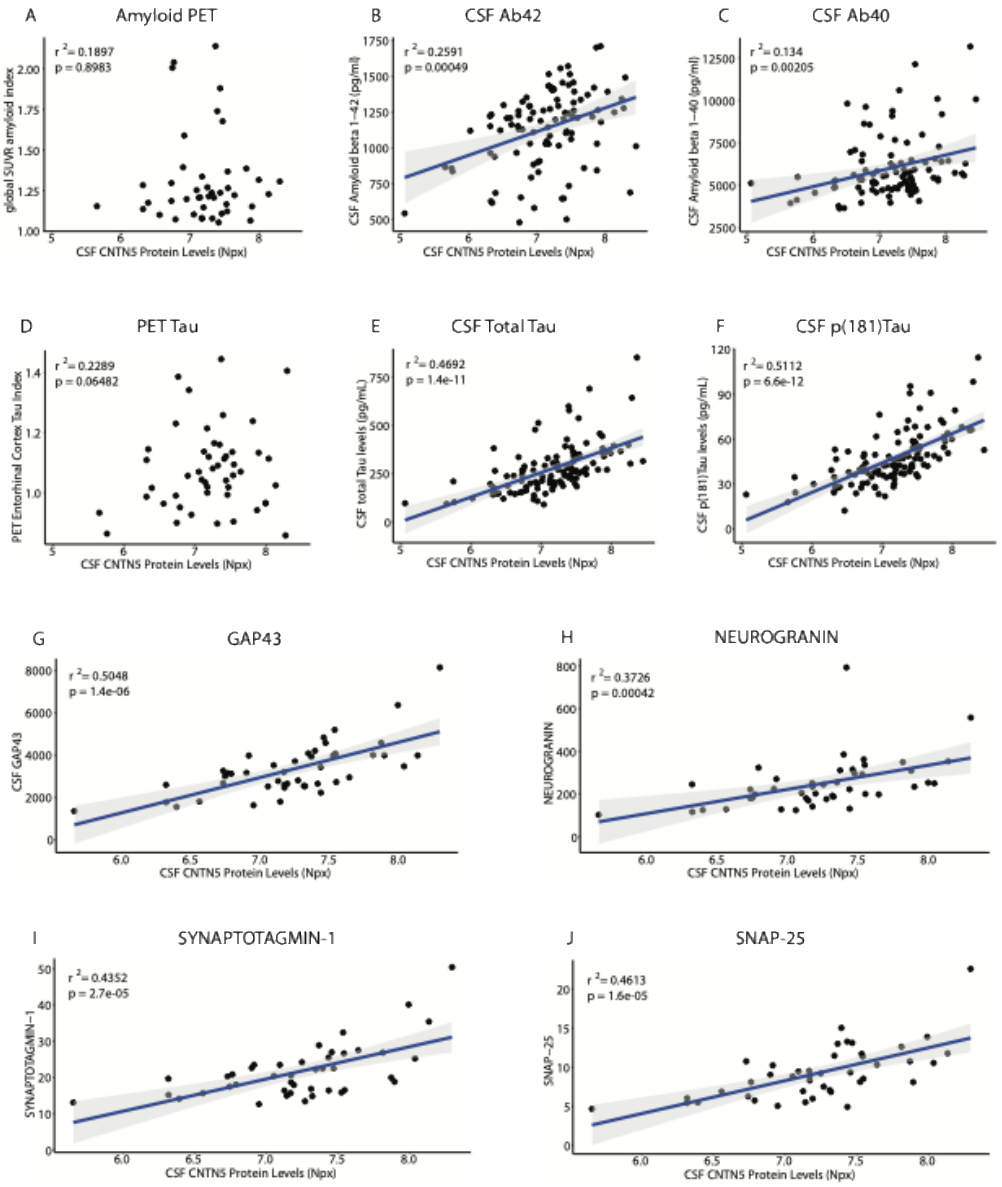
No association was found between CSF and plasma levels of contactin 5 protein nor between plasma contactin 5 and CSF sAD biomarkers (not shown).

Figure 6: Cerebrospinal fluid (CSF) contactin 5 levels over consecutive assessments in cognitively unimpaired subjects from the PREVENT-AD cohort



Contactin 5 protein was measured in the CSF using Olink's proximity extension assay. Assessments were done at baseline and on follow-up visits. CSF contactin 5 is significantly increased on follow-up visits at 12 (P = 0.025, n = 25), 24 (P = 0.018, n = 25), and 48 months (P = 8e-5, n = 15) compared to baseline (n = 33)

Figure 7: Association between CSF contactin 5 and sAD biomarkers or synaptic proteins in the PREVENT-AD cohort.



Contactin 5 protein was measured in the CSF using Olink's proximity extension assay. Global SUVR amyloid index (A) was measured with [18F]NAV4694 PET (n = 44). CSF AD biomarkers Aß 1-42 (B; n = 99), Aß 1-40 (C; n = 96), total tau (E; n = 113) and p-tau (F; n = 113) were measured by enzyme-linked immunosorbent assay according to the procedures from the BIOMARKAPD consortium of the EU Joint Program in Neurodegenerative Diseases. Tau deposition in the entorhinal cortex (D) was measured with flortaucipir PET (n = 48). The synaptic markers GAP43 (G; n = 45), neurogranin (H; n = 45), synaptotagmin-1 (I; n = 43) and SNAP-25 (J; n = 43) were quantified using selective reaction monitoring mass spectroscopy. Significant linear regressions are represented with a blue confidence region of the fitted line. R squares and P values are shown in the top left corners of each figure. Analyses were adjusted for age, sex and apolipoprotein E ε4. Aß, amyloid beta; AD, Alzheimer's disease; BIOMARKAPD, Biomarkers for Alzheimer's Disease and Parkinson's Disease; CSF, cerebrospinal fluid; PET, positron emission tomography; p-tau, phosphorylated tau; sAD, sporadic Alzheimer's disease; SUVR, standardized uptake value ratio

5.3 Effect of the *CNTN5* rs1461684 risk G variant on clinical progression and on CSF contactin 5 protein levels in the pre-symptomatic PREVENT-AD cohort

Searching for polymorphic variants affecting AD risk level in the QFP, a GWAS was performed in case and control subjects matched for sex, age, and especially place of birth (31,32). More than two thirds of the GWAS subjects saw their disease status confirmed by a pathologist at autopsy, thus reducing diagnostic uncertainties. Among the top variants found to be associated with AD in the QFP was the minor allele (G) of polymorphism rs1461684 (MAF 0.16), a variant found in intron 1 of the contactin 5 (*CNTN5*) gene. Table 3 summarizes results for the QFP and replication analyses obtained from the International Genomics of Alzheimer's and Alzheimer's Disease Genetics Consortium cohorts. This variant is distinct from the genetic polymorphism reported in the *CNTN5* gene (rs10501927 variant) by Harold et al.'s original UK GWAS (14). While both *CNTN5* SNPs are in linkage equilibrium (D': 0.21, r^2: 0.04, $P = 0.006$), the low r^2 and modest D' indicates that the SNPs cannot substitute each other. This is mostly likely due to the low prevalence of the rs10501927 variant found in the QFP.

In the asymptomatic PREVENT-AD cohort, *CNTN5* rs1461684 risk variant G is significantly associated with a much faster rate of progression compared to G-negative subjects as assessed by the APS in the cognitively unimpaired participants (Figure 8A, $P = 0.01$). Figure 8B illustrates the CSF contactin 5 protein levels measured as a function of *CNTN5* rs1461684 G allele: no significant difference was detected.

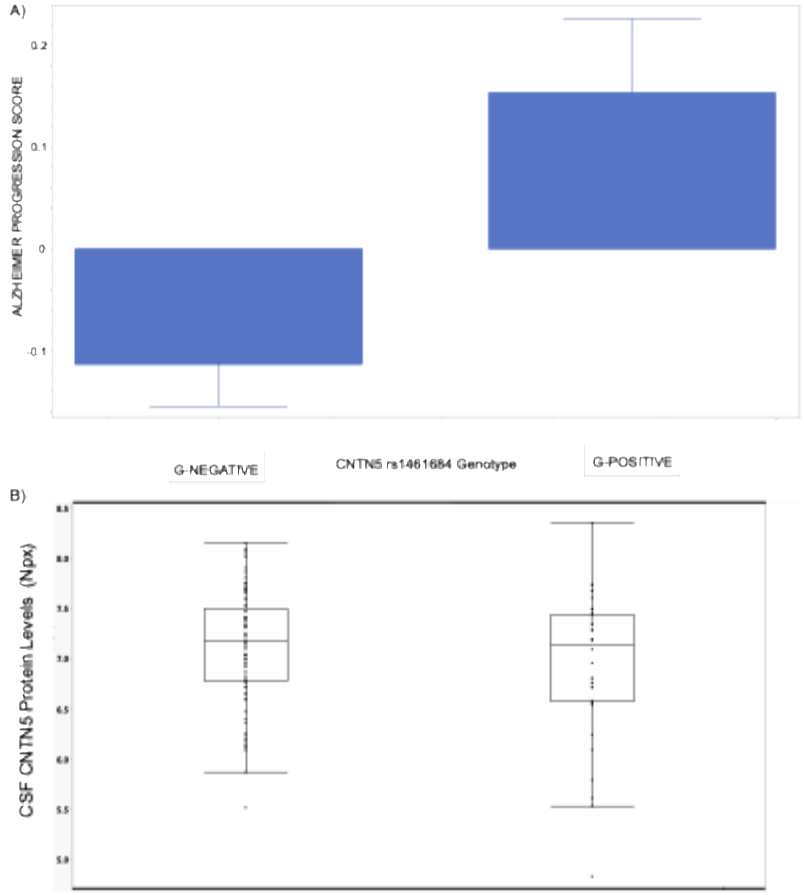
Table 3 - *CNTN5* risk variant in different cohorts

		Cohorts		
GWAS disease association	**rs1461684/ Chromosome 11:99225748 (GRCh38)**	**Beta/OR**	**P Values**	**N**
Quebec Founding Population isolate*	G carriers	.222 / 1.25	0.0001	1502 matched for age, gender and place of birth
	GG carriers	1.18 / 3.28	0.0000002	
IGAP Stage 1 **	G carriers	0.0581/1.14	0.0025	94497
IGAP Stage 2 **	G carriers	0.0487/1.12	0.0012	
ADGC Stage 1 ***	G carriers	na	0.0008	15675
ADGC Stage 2 ***	G carriers	na	0.0006	7096

*: Original GWAS description from Hu et al., 2011.
**: Derived from Kunkle et al. 2019. Note: the IGAP cohort includes a large subset of subjects from France where the QFP originates.
***: Original GWAS description from Naj et al., 2011.

Abbreviations: ADGC, Alzheimer's Disease Genetics Consortium; GWAS, genome-wide association study; IGAP, International Genomics of Alzheimer's; na, not applicable; OR, odds ratio; QFP, Quebec Founder Population.

Figure 8: Association between the CNTN5 rs1461684 G variant and Alzheimer Progression Score in the PREVENT-AD cohort.



A) Association between the presence of the rs1461684 G allele and the Alzheimer's disease progression score in the PREVENT-AD cohort (P = 0.01, n = 418). B) Association between the presence of the rs1461684 G allele on cerebrospinal fluid (CSF) contactin 5 protein levels at baseline (non-significant, n = 103). Analyses were adjusted for age and sex

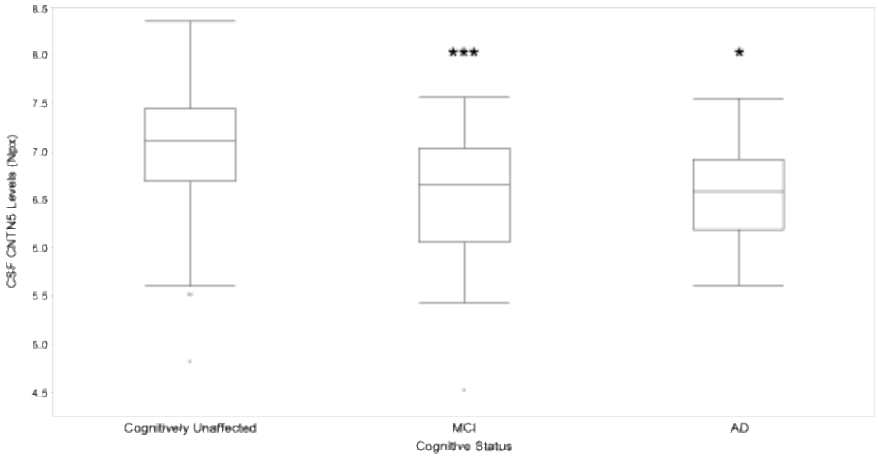
5.4 Effect of the *CNTN5* rs1461684 G variant on PET biomarkers

We investigated the effect of the *CNTN5 rs1461684* G variant on 18F- NAV4694 and flortaucipir PET uptake in the asymptomatic PREVENT- AD cohort and found no significant association with either amyloid or tau deposition in these cognitively unaffected subjects (not shown).

5.5 CSF contactin 5 levels in cognitively unimpaired, mild cognitively impaired, and sAD subjects

Using PREVENT-AD cognitively unimpaired subjects, we contrasted CSF contactin 5 concentrations with subjects from the COMPASS- ND cohort who have received a diagnosis of MCI and sAD. Figure 9 illustrates the modest cross-sectional reduction in CSF contactin 5 levels in MCI and sAD relative to the cognitively unimpaired subjects. Unfortunately, the COMPASS-ND does not have a functional genetic component yet and we were unable to examine the effects of *APOE* ε4 or *CNTN5* variants on CSF contactin 5 protein concentrations in these subjects. Of interest, the reduction was significant when the pre-symptomatic stage and MCI were contrasted, but not between MCI and AD stages, consistent with an early time-specific pathophysiological role.

Figure 9: Association between CSF contactin 5 and cognitive status in the PREVENT-AD and CCNA cohorts.



Contactin 5 protein was assessed using Olink's proximity extension assay in subjects who are cognitively unimpaired (PREVENT-AD cohort: $N = 105$) or diagnosed with mild cognitive impairment (CCNA's MCI: $N = 28$) or Alzheimer's disease (CCNA's AD: $n = 14$). CSF contactin 5 is significantly decreased in MCI ($P < 0.0001$) and AD ($P < 0.02$) relative to cognitively unaffected individuals.

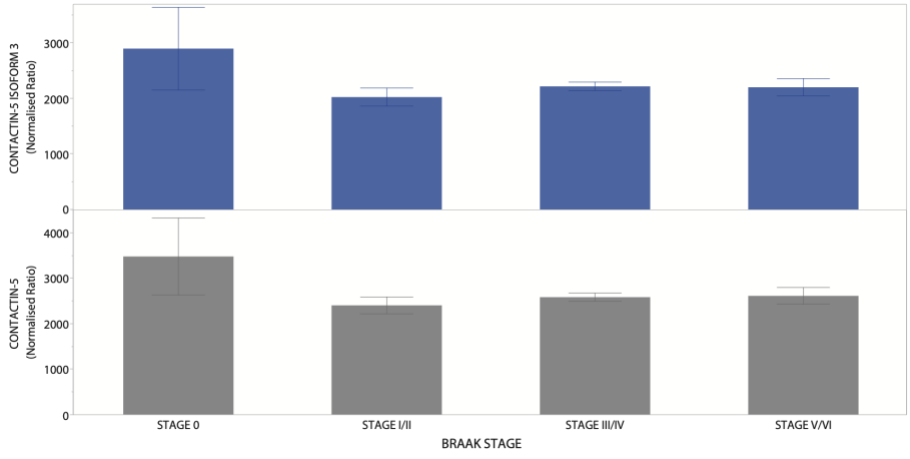
5.6 Contactin 5 protein levels in the prefrontal cortex in the different stages of the AD spectrum

Tissue levels of two contactin 5 protein isoforms were obtained from the ROS-MAP TMT proteomic database, which is described in detail in the Ping et al. (27). Cortical levels of the two major isoforms of contactin 5 expressed in the brain were obtained for some 288 subjects for which we also have corresponding *CNTN5* genotype information, the full length (O94779) wild type variant and the truncated isoform type 3 (O94779-4) protein, which are particularly prevalent in the CNS. Stratification of the two contactin 5 protein variants by Braak stages reveals a modest (but not significant) reduction of cortical contactin 5 levels between stages 0 and the later pathological stages (Figure 10). This is consistent with the CSF *CNTN5* changes reported in living patients with emerging cognitive deficits in the COMPASS-ND cohort described above.

Parallel analyses of the mRNA prevalence for the wild type and spliced isoform 3 variants were performed in the ROS-MAP subjects who underwent cortical RNA sequencing profiling. While we did not observe any significant changes across Braak stages (Figure 11 A, B), a significant reduction of the isoform 3 variant was observed as a function of CERAD staging ($P = 0.027$, Figure 11 D).

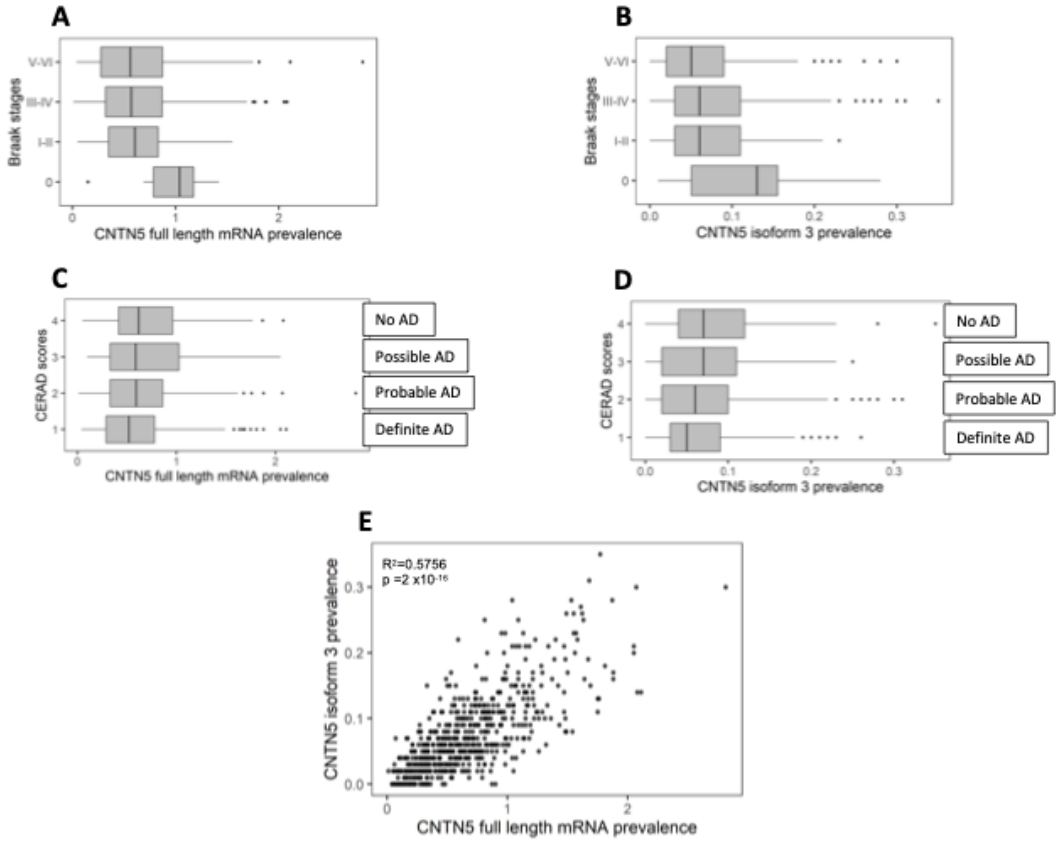
Finally, stratification of the *CNTN5* mRNA variants (full length and isoform 3) as a function of rs1461684 G risk variant reveals a strong allele-dependent reduction of the mRNA prevalence of both isoforms in the cortex of asymptomatic and MCI (early stage, $n = 152$) cases (Figure 12 A). A concomitant modest reduction (trend only) of the protein concentrations was observed in G allele carriers (Figure 12 B). The small sample size of the asymptomatic group ($n = 6$) and of the homozygous G allele carriers ($n = 3$) greatly limits our genomic analysis in the early phase of the disease process in this cohort.

Figure 10: Cortical contactin 5 protein levels at different Braak stages in the ROS-MAP cohort.



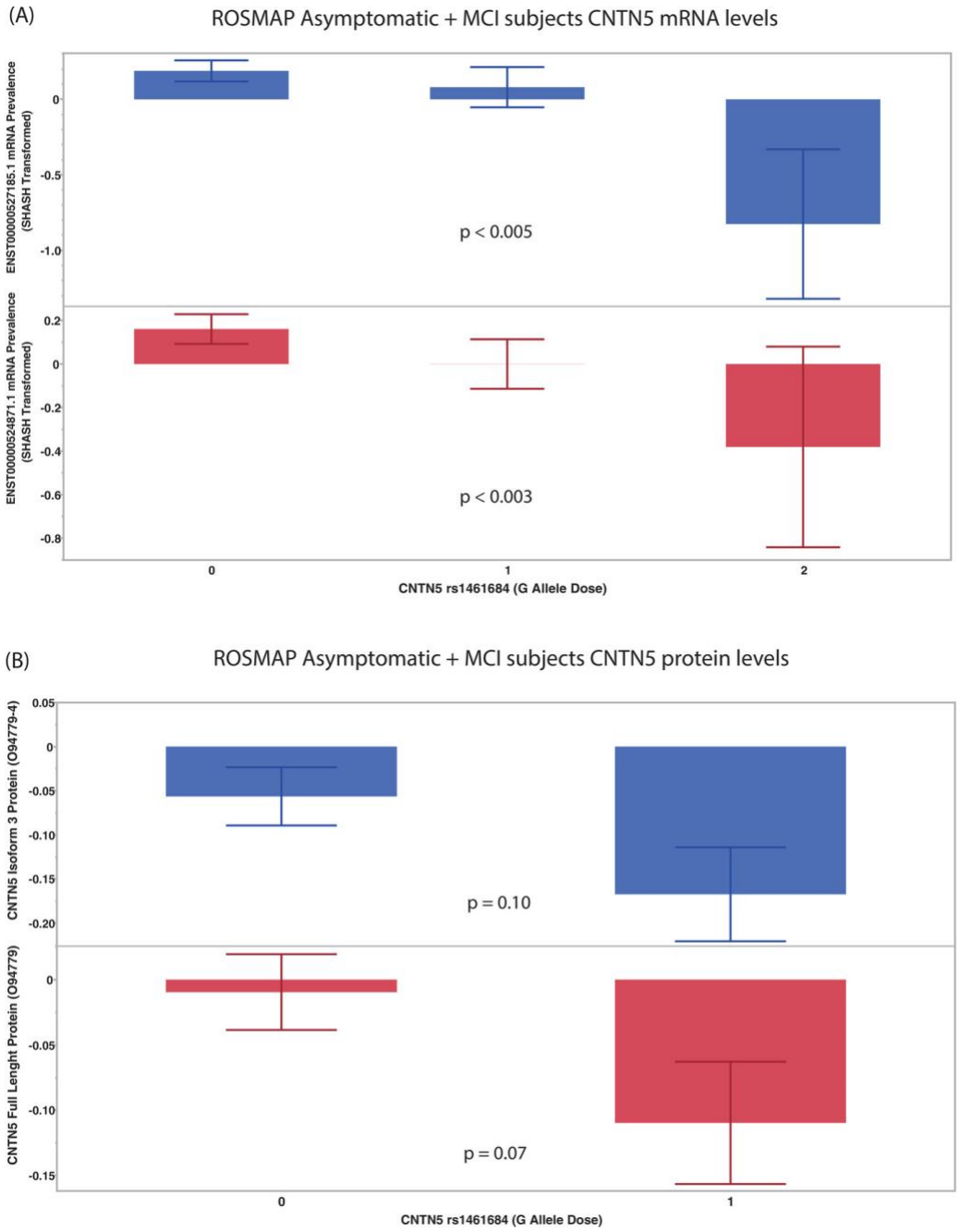
Contactin 5 protein was assessed using tandem mass tags proteomic data in subjects from the ROS-MAP cohort. Normalized cortical contactin 5 (full length and isoform 3) ratios are not significantly affected by tau pathology accumulation ($P > 0.05$, $n = 288$)

Figure 11: Association between cortical *CNTN5* mRNA splice variants and AD pathology (CERAD and Braak stages) in the ROS-MAP cohort.



CNTN5 splice variants were obtained from the ROS-MAP RNAseq database. A) There was no association between ENST00000524871.6 full length mRNA and Braak stages (P = 0.2, n = 608). B) There was no association between ENST00000527185.5 isoform 3 variant prevalence and Braak stages (P = 0.2, n = 608). C) There was a weak association (trend) between ENST00000524871.6 full length mRNA and CERAD scores (P = 0.066, n = 615). D) ENST00000527185.5 isoform 3 variant prevalence is associated with worse CERAD scores (P = 0.027, n = 615). E) There is a significant association between cortical levels of ENST00000524871.6 full length mRNA and enst00000527185_1 isoform 3 variant (r^2 = 0.5756, P = 2e-16). Analyses were adjusted for age, sex, and apolipoprotein E ε4.

Figure 12: Association between the CNTN5 rs1461684 G variant and cortical CNTN5 mRNA and proteins levels in asymptomatic and mild cognitive impairment (MCI) subjects from the ROS-MAP cohort



A) Association between the presence of the rs1461684 G allele and *CNTN5* mRNA prevalence for the full length (bottom: ENST524871, r2: 0.13, $P < 0.003$, $n = 301$) and isoform 3 (top: ENST524185, r2 = 0.07, $P < 0.005$, $n = 301$) variants. B) Association between the presence of the rs1461684 G allele and contactin 5 protein levels (full length O94779, $P = 0.10$, $n = 103$ and isoform O094779-4, $P = 0.07$). Analyses of variance were adjusted for age, apolipoprotein E ε4, and sex.

6. Discussion

In the present study, *CNTN5* gene expression and protein (contactin 5) alterations were investigated in both the brain tissue and CSF throughout the sAD spectrum. Contactins represent major proteins involved in neuronal development, formation of dendrites, and synaptic contacts. Indeed, their roles in neuritogenesis, fasciculation of neurons, axonal and dendritic targeting, fine tuning of synapse formation, and synaptic plasticity have been demonstrated in multiple situations (33,34). Contactins are neural cell adhesion molecules that encode axon-target specificity during the patterning of the developing CNS, and also in response to neuronal injury and damage (35).

CNTN5 is specifically implicated in the specification of dendritic arbors. Recent studies further examining the coreceptor function of contactins with the amyloid precursor proteins (APP) have shown that contactin 4 and contactin 5 can bind to APP and its precusor-like variants (APLP1) when they co-opt their cytoplasmic regions to relay information across the plasma membrane using amyloid-dependent signal transduction pathways (36,37). These observations suggest a significant interplay between the different contactins and APP metabolism during dendritic remodeling and synaptic formation during neuronal response to injury. This could explain, at least in part, the observed positive correlation between contactin 5, Aß42, and Aß40 in the CSF in the pre-symptomatic phase of the disease (Figure 7).

Figure 6 shows that cognitively unimpaired elderly subjects with a parental history of AD display a slow but steady longitudinal increase in contactin 5 protein level in the CSF over the course of 4 years. These subjects typically carry a 2- to 3-fold risk of developing AD compared to subjects without a familial history (38). CSF t-tau and p-tau in these subjects display a significant

association with contactin 5 with correlation coefficients of 0.47 and 0.51, respectively. Interestingly, the associations do not translate into significant deposition in the brain when PET scanning was used for total brain amyloid ($P = 0.89$) and entorhinal tau depositions ($P = 0.06$; Figure 7). One possible interpretation for these discrepancies is the fact that CSF amyloid and tau changes precede by several years (sometime a decade) tangle deposition detected by PET imaging (39). However, the weak association observed between contactin 5 and PET tau in the entorhinal cortex ($P = 0.06$), the brain region typically used for the detection of AD-specific early tau deposition (40) is certainly consistent with the known spreading of tau pathological cascade. An upcoming second round of PET imaging in these subjects will most likely provide a more definitive answer to this question.

Contactin 5 is known to play an important role in the formation of glutamatergic synapses in the rodent central auditory system during postnatal development and to interact with the E1 domain of APP/APLP1 in the presynaptic compartment (41). This prompted us to examine the possible association between contactin 5 and well-established soluble biomarkers of synaptic integrity in the CSF, namely GAP43, neurogranin, synaptotagmin-1, and SNAP-25. Figure 6 illustrates the highly significant associations between contactin 5 and all four synaptic biomarkers in the CSF of our cognitively unimpaired subjects: consistent with tau-mediated, contactin 5–associated modulation of synaptic pathology in the pre-symptomatic phase of the disease.

These findings led us to examine the situation later in life, in subjects in which symptoms emerge (MCI) in response to markedly compromised synapses and, later when neuronal damage and cortical atrophy takes a toll (sAD). Cross-sectional analysis of the CSF contactin 5 level in

cognitively unimpaired subjects, MCI, and sAD cases reveals a disease-dependent reduction in the COMPASS-ND cohort. The modest but significant reduction of contactin 5 protein levels observed in the CSF ($P < 0.01$) of our living subjects and, in cortical tissue (trends only, ROS-MAP) of autopsied MCI and AD subjects markedly contrast with the t-tau and p-tau alterations observed in the CSF in the asymptomatic (pre-symptomatic?) stage of the disease in our cognitively unimpaired subjects with a parental history of AD.

Together, these results suggest that the strong association between contactin 5 and tau/synaptic biomarkers prior to the emergence of symptoms serves as an index of early neuronal damage. As synaptic and neuronal structures become more compromised with emerging cognitive symptoms, contactin 5 levels in the brain decrease both in tissues and CSF, presumably in parallel to the ongoing neuronal loss.

As we further examined the pre-symptomatic phase of the disease, the analysis of the rs1461684 G risk variant in the asymptomatic PREVENT-AD cohort led to an interesting finding when used in conjunction with APS to map disease progression in the absence of obvious cognitive deficit (Figure 8). Presence of the G allele was found to be significantly associated with a faster rate of progression ($P = 0.01$) compared to G-negative subjects (Figure 8A), but it does not affect contactin 5 levels in the CSF. So, if the soluble form of contactin 5 found in the CSF is not affected by the presence of the G allele, what about the tissue concentration?

In this context, we used the ROS-MAP cohort to explore the cortical expression of the two major mRNA isoforms of *CNTN5* transcripts found in the CNS, that is, the full length

(ENST00000524871.6) and the isoform 3 (ENST00000527185.5), which is truncated at the 3′ end. Figure 11 summarizes the findings. Using CERAD and Braak staging, we examined *CNTN5* gene expression throughout the AD spectrum. *CNTN5* isoform 3 prevalence is associated with worsening on the CERAD scores ($P = 0.027$) but not on the Braak scores ($P = 0.636$; Figure 10 B, D). There was a modest association (trend only, $P = 0.066$) between *CNTN5* full length mRNA prevalence and CERAD scores but no correlation with Braak staging ($P = 0.437$; Figure 10 A, C).

When mRNA results from early-stage ROS-MAP subjects (asymptomatic + MCI) were pooled and stratified by rs1461684-G risk variant, we observed a strong allele–dose reduction of the CNTN5 isoform 3 (Figure 12A; $P < 0.004$) and full-length mRNA transcripts ($P < 0.005$) in cortical tissues. Using a similar approach to stratify cortical contactin 5 protein levels as a function of the G allele, we only found modest reductions (trends only, full-length $P = 0.07$, isoform 3 $P = 0.10$) of tissue concentrations. As stated above, in contrast to the mRNA dataset, the ROS-MAP proteomic dataset that overlaps with the subjects enrolled simultaneously with the GWAS contains a relatively small number of subjects in the asymptomatic group for which we have rs1461684 genetic information, thus explaining the small sample size.

Together, these results indicate that *CNTN5* gene expression contributes, at least in part, to the observed reduction of brain contactin 5 protein levels when amyloid-associated staging (CERAD) is used to map the disease progression in symptomatic subjects in ROS-MAP. The presence of the G allele contributes to a marked reduction of the *CNTN5* full-length and isoform 3 mRNA variant in cortical tissue.

Isoform 3 lacks amino-acid sequence 912–1100. While speculative, one could suggest that G-allele mediated decrease in isoform 3 mRNA leads to a protein variant of contactin 5 that is missing a portion of the cytoplasmic region, which is required to relay information across the plasma membrane using the amyloid-dependent signal transduction pathways described previously (36, 37). Whether the result is a cause, or a consequence of neuronal loss associated with the tau/contactin 5 interaction detected in the extracellular space remains to be elucidated. Additional molecular studies are now planned to examine the role of isoform 3 and its relationships to APP, amyloid, and tau metabolism.

Altogether, these results suggest that *CNTN5* plays a prominent role in the early phase of the disease, in the pre-symptomatic stage, when tangle pathology emerges but amyloid and tau deposition are still limited. The strong association of CSF contactin 5 protein with synaptic markers, especially pre-synaptic ones, is consistent with its neurodevelopmental role in the regulation of dendritic arborization remodeling and synaptic connectivity. We know that the brain is not static in the face of early neuronal loss and that compensatory mechanisms exist to limit the loss of synaptic input and to facilitate dendritic remodeling and synaptic reorganization from intact neuronal circuits. The presence of a relatively common *CNTN5* risk variant that affects this cascade was found to significantly affect the disease progression on the APS scale in the pre-symptomatic phase, *CNTN5* tissue mRNA prevalence in the early stage of the disease, affecting the CERAD scale in symptomatic subjects from ROS-MAP.

7. References

1. Boyle PA, Wang T, Yu L, et al. To what degree is late life cog- nitive decline driven by age-related neuropathologies? *Brain*. 2021;144(7):2166-2175.
2. Robinson JL, Richardson H, Xie SX, et al. The development and convergence of co-pathologies in Alzheimer's disease. *Brain*. 2021;144(3):953-962.
3. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science*. 1992;256(5054):184-185. (New York, N.Y.).
4. Duyckaerts C, Delatour B, Potier M-C. Classification and basic pathology of Alzheimer disease. *Acta Neuropathol*. 2009;118(1):5– 36.
5. Ridge PG, Hoyt KB, Boehme K, et al>. Assessment of the genetic variance of late-onset Alzheimer's disease. *Neurobiol Aging*. 2016; 41:200.e13-200.e20.
6. Poirier J, et al. Apolipoprotein E polymorphism and Alzheimer's dis- ease. *Lancet*. 1993;342(8873):697-699.
7. Brainstorm C, Bulik-Sullivan B, Finucane HK, et al. Analysis of shared heritability in common disorders of the brain. *Science*. 2018(6395):360.
8. Oguro-Ando A, Zuko A, Kleijer KTE, Burbach JPH. A current view on contactin-4, -5, and -6: implications in neurodevelopmental disorders. *Molecular and Cellular Neurosciences*. 2017;81:72-83.
9. Mohebiany AN, Harroch S, Bouyain S. New insights into the roles of the contactin cell adhesion molecules in neural development. *Advances in Neurobiology*. 2014;8:165-194.
10. Nava C, Keren B, Mignot C, et al. Prospective diagnostic analysis of copy number variants using SNP microarrays in individuals with autism spectrum disorders. *European journal of human genetics: EJHG*. 2014;22(1):71-78.
11. van Daalen E, Kemner C, Verbeek NE, et al. Social Responsiveness Scale-aided analysis of the clinical impact of copy number variations in autism. *Neurogenetics*. 2011;12(4):315-323.
12. Nakabayashi K, Komaki G, Tajima A, et al. Identification of novel candidate loci for anorexia nervosa at 1q41 and 11q22 in Japanese by a genome-wide association analysis with microsatellite markers. *J Hum Genet*. 2009;54(9):531-537.
13. LionelAC,CrosbieJ,BarbosaN,etal.Rarecopynumbervariationdis- covery and cross-disorder comparisons identify risk genes for ADHD. *Sci Transl Med*. 2011;3(95):95-75.
14. Harold D, Abraham R, Hollingworth P, et al. Genome-wide associ- ation study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet*. 2009;41(10):1088-1093.
15. Breitner JCS, Poirier J, Etienne PE, Leoutsakos JM. Rationale and structure for a new center for studies on prevention of Alzheimer's Disease (StoP-AD). *The Journal of Prevention of Alzheimer's Disease*. 2016;3(4):236-242.

16. Lleó A, Alcolea D, Martínez-Lage P, et al. Longitudinal cerebrospinal fluid biomarker trajectories along the Alzheimer's disease continuum in the BIOMARKAPD study. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*. 2019;15(6):742-753.
17. Brinkmalm A, Brinkmalm G, Honer WG, et al. SNAP-25 is a promis- ing novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Mol Neurodegener*. 2014;9:53.
18. OhrfeltA,BrinkmalmA,DumurgierJ,etal.Thepre-synapticvesicle protein synaptotagmin is a novel biomarker for Alzheimer's disease. *Alzheimers Res Ther*. 2016;8(1):41.
19. Portelius E, Olsson B, Höglund K, et al. Cerebrospinal fluid neuro- granin concentration in neurodegeneration: relation to clinical phe- notypes and neuropathology. *Acta Neuropathol*. 2018;136(3):363– 376.
20. Sandelius A, Portelius E, Källén Åsa, et al. Elevated CSF GAP-43 is Alzheimer's disease specific and associated with tau and amyloid pathology. *Alzheimers Dement*. 2019;15(1):55-64.
21. McSweeneyM,BinetteAP,MeyerP-F,etal.Intermediateflortaucipir uptake is associated with Aβ-PET and CSF tau in asymptomatic adults. *Neurology*. 2020;94(11):e1190-e1200.
22. Ossenkoppele R, Rabinovici GD, Smith R, et al. Discriminative accuracy of [18F]flortaucipir positron emission tomography for Alzheimer disease vs other neurodegenerative disorders. *JAMA*. 2018;320(11):1151-1162.
23. Meyer PF, Tremblay-Mercier J, Leoutsakos J, et al. INTREPAD: a randomized trial of naproxen to slow progress of presymptomatic Alzheimer disease. *Neurology*. 2019;92(18):e2070-e2080.
24. Leoutsakos JM, Gross AL, Jones RN, Albert MS, Breitner JCS. Alzheimer's progression score': development of a Biomarker Sum- mary Outcome for AD Prevention Trials. *J Prev Alzheimers Dis*. 2016;3(4):229-235.
25. Chertkow H, Borrie M, Whitehead V, et al. The comprehensive assess- ment of neurodegeneration and dementia: canadian Cohort Study. *Can J Neurol Sci*. 2019;46(5):499-511.
26. Bennett DA, Buchman AS, Boyle PA, Barnes LL, Wilson RS, Schneider JA. Religious orders study and rush memory and aging project. *Journal of Alzheimer's disease: JAD*. 2018;64(s1): S161-S189.
27. Ping L, Duong DM, Yin L, et al. Global quantitative analysis of the human brain proteome in Alzheimer's and Parkinson's Disease. *Sci Data*. 2018; 5:180036.
28. Laberge AM, Michaud J, Richter A, et al. Population history and its impact on medical genetics in Quebec. *Clin Genet*. 2005;68(4):287- 301
29. Shifman S, Darvasi A. The value of isolated populations. *Nat Genet*. 2001;28(4):309-310.
30. Nakai M, Kawamata T, Taniguchi T, Maeda K, Tanaka C. Expression of apolipoprotein E mRNA in rat microglia. *Neurosci Lett*. 1996;211(1):41- 44.
31. Miron J, Picard C, Nilsson N, et al. CDK5RAP2 gene and tau pathophys- iology in late-onset sporadic Alzheimer's disease. *Alzheimers Dement*. 2018;14(6):787-796.

32. Hu X, Pickering E, Liu YC, et al. Meta-analysis for genome-wide association study identifies multiple variants at the BIN1 locus asso- ciated with late-onset Alzheimer's disease. *PLoS One*. 2011;6(2): e166 16.
33. Murai KK, Misner D, Ranscht B. Contactin supports synaptic plas- ticity associated with hippocampal long-term depression but not potentiation. *Curr Biol*. 2002;12(3):181-190.
34. Stoeckli ET. Neural circuit formation in the cerebellum is controlled by cell adhesion molecules of the Contactin family. *Cell Adh Migr*. 2010;4(4):523-526.
35. ChoH, Shimazaki K, Takeuchi K, etal. Biphasicchangesin F3/contactin expression in the gerbil hippocampus after transient ischemia. *Exp Brain Res*. 1998;122(2):227-234.
36. Muller UC, Deller T, Korte M. Not just amyloid: physiological func- tions of the amyloid precursor protein family. *Nat Rev Neurosci*. 2017;18(5):281-298.
37. Karuppan SJ, Vogt A, Fischer Z, et al. Members of the vertebrate contactin and amyloid precursor protein families interact through a conserved interface. *J Biol Chem*. 2022;298(2):101541.
38. GreenRC,CupplesLA,GoR,etal.Riskofdementiaamongwhiteand African American relatives of patients with Alzheimer disease. *JAMA*. 2002;287(3):329-336.
39. GrootC,SmithR,StomrudetE,etal.Phospho-tauwithsubthreshold tau-PET predicts increased tau accumulation rates in amyloid-positive individuals. *Brain*. 2022;287(3):329-336
40. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathol*. 1991;82(4):239-259.
41. Toyoshima M, Sakurai K, Shimazakiet K, et al. Preferential local- ization of neural cell recognition molecule NB-2 in developing glu- tamatergic neurons in the rat auditory brainstem. *J Comp Neurol*. 2009;513(4):349-362.
42. Kunkle, B. W., et al. (2019). Genetic meta-analysis of diagnosed Alzheimer's disease identifies new risk loci and implicates Abeta, tau, immunity and lipid processing. *Nat Genet* 51(3): 414–430.
43. Naj, A. C., et al. (2011). Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat Genet* 43(5): 436–441.

Manuscript 2: Contactin 5 and Apolipoproteins Interplay in Alzheimer's Disease

Marina Tedeschi Dauar[a,b,c,d], Cynthia Picard[a,b], Anne Labonté[a,b], John Breitner[a,b,c,e], Pedro Rosa-Neto[c,f,g], Sylvia Villeneuve[a,b,c,e] and Judes Poirier[a,b,c,e,*] for the PREVENT-AD Research Group

a. Douglas Mental Health University Institute, Montreal, Canada
b. Centre for the Studies in the Prevention of Alzheimer's Disease, Montréal, Canada
c. McGill University, Montreal, Canada
d. CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil
e. Department of Psychiatry, McGill University, Montreal, Canada
f. Translational Neuroimaging Laboratory, McGill University Research Centre for Studies in Aging, Alzheimer's Disease Research Unit, Douglas Research Institute, Verdun, Canada
g. Department of Neurology and Neurosurgery, McGill University, Montre´al, Canada

Correspondence:
Judes Poirier, Deputy Director, Centre for the Studies in the Prevention of Alzheimer's Disease, Douglas Mental Health University Institute, 6875, Lasalle, Montréal, Quebec H4H 1R3, Canada. Tel.: +1 514 761 6131 Ext. 6153, Fax: +1 514 888 4094; E-mail: judes.poirier@mcgill.ca

Published in Journal of Alzheimer's disease®. 2024 98(4):1361-1375. (doi: 10.3233/JAD-231003. PMID: 38578887).

1. Preface

We identified, for the first time, a variant in the CNTN5 gene as associated with increased risk for AD. Contactin 5 is a protein involved in axonal arborization and synaptic formation during neurodevelopment, but this protein is not known to be directly associated with the amyloid cascade, inflammatory pathways, or cholesterol metabolism. Therefore, we believe that it is crucial to continue to investigate contactin 5 in AD, to better understand the role the *CNTN5* gene and its protein play in the pathophysiology of the disease.

In this chapter, we investigate the associations between contactin 5 and apolipoproteins in the presymptomatic phase of the disease. Several apolipoproteins are known risk factors of AD and also play a role in synaptic and neuronal remodeling. Our objective was to understand how these two different classes of proteins that share similarities in mechanism of action and as risk factors for AD interact in the extracellular compartment during the presymptomatic phase of the disease. We followed this investigation by measuring gene expression in pathologically confirmed AD and control patients, to understand if the associations found could be due to changes in gene expression.

Finally, to understand if the mechanism of action of these proteins in AD could be associated with their role in processes of neuronal and synaptic formation and remodeling, we measured gene expression in the hippocampus of a mouse model of hippocampal deafferentation for an extended period that covered both the degeneration and reinnervation phases.

2. Abstract

Background: Apolipoproteins and contactin 5 are proteins associated with Alzheimer's disease (AD) pathophysiology. Apolipoproteins act on transport and clearance of cholesterol and phospholipids during synaptic turnover and terminal proliferation. Contactin 5 is a neuronal membrane protein involved in key processes of neurodevelopment.

Objective: To investigate the interactions between contactin 5 and apolipoproteins in AD, and the role of these proteins in response to neuronal damage.

Methods: Apolipoproteins (measured by Luminex), contactin 5 (measured by Olink's proximity extension assay) and cholesterol (measured by liquid chromatography mass spectrometry) were assessed in the cerebrospinal fluid (CSF) and plasma of cognitively unimpaired participants (n=93). Gene expression was measured using polymerase chain reaction in the frontal cortex of autopsied-confirmed AD (n=57) and control subjects (n=31) and in the hippocampi of mice following entorhinal cortex lesions.

Results: Contactin 5 positively correlated with apolipoproteins B ($p=5.4x10^{-8}$), D ($p=1.86x10^{-4}$), E ($p=2.92x10^{-9}$), J ($p=2.65x10^{-9}$) and with cholesterol (p=0.0096) in the CSF, and with cholesterol (p=0.02), HDL (p=0.0143) and LDL (p=0.0121) in the plasma. Negative correlations were seen between *CNTN5*, *APOB* (p=0.034) and *APOE* (p=0.015) mRNA levels in the brains of control subjects. In the mouse model, *apoe* and *apoj* gene expression increased during the reinnervation phase ($p<0.05$), while *apob* (p=0.023) and *apod* (p=0.006) increased in the deafferentation stage.

Conclusions: Extensive interactions were observed between contactin 5 and apolipoproteins and cholesterol, possibly due to neuronal damage. The alterations in gene expression of apolipoproteins suggest a role in axonal, terminal and synaptic remodeling in response to entorhinal cortex damage.

3. Introduction

Alzheimer's disease (AD) is the most common form of degenerative dementia affecting over 33 million people worldwide [1]. AD is defined by the presence of Aß plaques and pathologic tau [2] that leads to cognitive decline. While the majority of research has focused on the pathological processes that lead to Aß and tau deposition and neuronal death, less attention is given to the changes that occur in response to this pathological process in an attempt to promote compensatory changes and reinnervation.

Current literature suggests that apolipoproteins play a central role in the compensatory response to neuronal damage, possibly due to their role in the transport and clearance of cholesterol and phospholipids during synaptic turnover and terminal proliferation [3]. The main apolipoproteins involved in cholesterol transport in the central nervous system are apolipoproteins E (APOE), B (APOB), D (APOD), and J (APOJ). All of them have been previously implicated in different aspects of AD pathophysiology [4-11]. The APOE-ε4 allele is well known as the most important genetic risk factor for sporadic AD [12, 13], and the *CLU* (also called *APOJ*) gene is also currently associated with risk of AD [14, 15]. Rare genetic variants in the *APOB* gene have been associated with increased risk for early-onset familial AD [6]. Furthermore, apoB was found to be higher in the cerebrospinal fluid (CSF) of AD patients and was strongly related to alterations in tau and phospho-tau, as well as changes in synaptic proteins in asymptomatic subjects at risk of dementia [7]. Similarly, apoD was found to be increased in the CSF [5] and in the hippocampus of AD patients [5, 16].

Contactin 5 is another protein recently associated with sporadic AD risk and pathology. Contactin 5 is a neuronal membrane protein that plays crucial roles in the organization of axonal domains, axonal guidance, myelination, neuritogenesis, synaptogenesis and axo-glia interactions [17, 18]. Recently, it was shown that the rs1461684 G variant in the *CNTN5* gene is associated with increased risk and a faster rate of progression throughout the AD spectrum [19]. Moreover, contactin 5 was found to increase progressively with age and to be associated with tau biomarkers and soluble synaptic proteins in the CSF [19].

In the present work, we sought to investigate the interplay between the soluble form of contactin 5 and apolipoproteins in AD. Investigating the connections between these two classes of proteins, which are related to synaptogenesis and synaptic reorganization, may provide us with a better understanding of how neurons respond to pathological harm and increase our understanding of AD pathophysiology.

4. Methods:

4.1 Study Populations:

Data were obtained from the Pre-symptomatic Evaluation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) cohort and from the Quebec Founder Population (QFP) cohort. All procedures were approved by the McGill University Faculty of Medicine Institutional Review Board and complied with the ethical principles of the Declaration of Helsinki.

4.2 PREVENT-AD Cohort:

The PREVENT-AD cohort includes over 365 cognitively unimpaired subjects who have a first-degree relative with AD and therefore are at a higher risk of developing this dementia [20]. Participants are monitored annually with clinical and cognitive assessments, CSF and blood biomarkers and neuroimaging modalities (PET and MRI) [20]. Data used in the preparation of this article were obtained from the PREVENT-AD program *(*https://douglas.research.mcgill.ca/stop-ad-centre*),* data release 6.0.

A complete listing of the PREVENT-AD Research Group can be found in the PREVENT-AD database:https://preventad.loris.ca/acknowledgements/acknowledgements.php?date=2023-04-01.

4.2.1 CSF

Cerebrospinal fluid was collected from a subset of participants of the PREVENT-AD cohort. In this study, samples from 93 subject, for whom we had all necessary measurements, were used. Lumbar punctures were performed in the morning following an overnight fast using a Sprotte 24-gauge atraumatic needle as described in Tremblay-Mercier et al (2021) [21]. CSF was centrifuged for 10 minutes at room temperature, cells and insoluble material were excluded, and aliquots were stored at –80ºC.

4.2.2 Proteins

Apolipoproteins were measured using the apolipoprotein Luminex assay kit (10-plex magneto-fluorescent immunoassays, cat# 12003081, BioRad, USA) as per manufacturer instructions.

Contactin 5 was measured using the neurology panel of Olink's proximity extension assay as described before [19].

4.2.3 Cholesterol

CSF samples were homogenized briefly by sonication in butanol/methanol (3:1) and 4 M KOH in the presence of internal standards, followed by incubation at 37'C for 1.5hr. The second extraction was performed in heptane/ethyl acetate (3:1) and acetic acid (1%). The superior sterol-containing layer was vacuum dried and resuspended in 50uL of methanol (90%) and injected into RP-UPLC/MS. The chromatographic system was a Waters ACQUITY UPLC equipped with a Phenomenex Kinetics C18 column (2.1×150mm 1.7um). The Mass spectrometer is AB Sciex 6500 Qtrap. Data analysis software used is Analyst and Multiquant.

4.2.4 Blood

Blood samples were collected immediately after the lumbar puncture following an overnight fast. Blood was centrifuged for 10 minutes at 4^{o}C, cells and insoluble material were saved, and plasma aliquots were stored at –80^{o}C. Protein and cholesterol measurements were performed in plasma using the methods described above.

4.2.5 DNA extraction and genotyping

Genomic DNA was extracted from buffy coat using the Qiagen EZ1 DNA Kit. Genotype profiling of ApoE 112/158 single nucleotide polymorphisms (which determine the E2, E3, and E4 isoforms) was performed through PCR followed by pyrosequencing. PCR was used for amplification with the following primer pairs: ApoE 112: forward, 5′-ACGGCTGTCCAAGGAGCTG-3′, and reverse, biotin 5′-CACCTCGCCGCGGTACTG-3′; and ApoE 158: forward, 5′-CTCCGCGATGCCGATGAC-3′, and reverse, biotin 5′-CCCCGGCCTGGTACACTG-3′. Genomic DNA (250–500 ng) was amplified with 20 pm of each primer, 1× PCR buffer kit (Qiagen), 0.4 mm dNTP, 1.0 mm MgCl2, DMSO, and 0.01 U of Qiagen Taq polymerase. A Biometra TProfessional Basic Thermocycler was used for amplification with the following conditions for 35 cycles: 30 s at 95°C, 30 s at 58.6°C, or 58.1°C for ApoE 112 or 158 respectively, and 1 min at 72°C. These 35 amplification cycles were preceded by a 2 min hot start at 95°C and were followed by a final 4 min extension to the last cycle at 72°C. PCR products were visualized on a 1.2% agarose gel. The polymorphisms were subsequently determined via an established pyrosequencing protocol with oligo sequencing for ApoE112 (5′-CGGACATGGAGGACG-3′) and ApoE 158 (5′-CGATGACCTGCAGAAG-3′). The analyzed sequence was as follows: TGT/CGCGGCCGCCT for ApoE112 and T/CGCCT/GGCAG for ApoE158.

4.3 Quebec Founder Population Cohort: Autopsy-confirmed case/control subjects

The QFP cohort is a Canadian population that descends from French settlers who founded Nouvelle France in the 17th and 18th centuries. A founder effect was created in this population due to the migration and isolated nature of the settlements, resulting in less genetic heterogeneity, large

linkage disequilibrium blocks and low genetic noise. QFP human brain tissue was obtained from the Douglas Bell Canada Brain Bank. This study had the approval of the Douglas Research Center institutional review board and was performed in conformity with the Code of Ethics of the World Medical Association (McGill/Douglas ethic approvals A05-B16-11B and IUSMD-02-34). The histopathological diagnosis of AD followed the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria [22].

4.3.1 DNA extraction and genotyping.

APOE4 allele determination was performed on brain tissue samples with DNeasy tissue extraction kit (Qiagen Hilden, Germany) and the pyrosequencing protocol described above.

4.3.2 RNA extraction and quality control.

RNA was extracted from frontal cortex tissues (n=88) using the Maxwell® 16 Tissue LEV Total RNA Purification Kit (Promega, WI, USA) on a Maxwell® 16 LEV Instrument (Promega, WI, USA). Then, cDNA was obtained by reverse transcription on a Multigene thermocycler (Labnet International Inc.) using the high-capacity cDNA RT kit (Applied Biosystems, CA, USA) and 200ng of total RNA. The purity and integrity of RNA samples were estimated using the ratio of absorbance values at 260 and 280 nm evaluated on a Biotek Synergy H1 reader (Fisher Scientific, ON, Canada), and the RNA integrity number (RIN) determined with a Bio-Rad's Experion instrument (Bio-Rad, CA, USA). The ratios of absorbance were all over 1.5, while RINs ranged from 2 to 8.4, with 84 % of samples over 5, the cutoff value representing very good total RNA quality [23] for microarray methodology.

4.3.3 Microarray.

The purity and integrity of extracted RNA were estimated using, respectively, the ratio of absorbance values at 260 nm and 280 nm evaluated on a Biotek Synergy H1 reader (Fisher Scientific, ON, Canada), and the RNA integrity number (RIN) determined with a Bio-Rad's Experion instrument (Bio-Rad, CA, USA) before processing with the Applied Biosystem Clariom D microarray according to the manufacturer's protocols. Gene-level expressions of *APOE, CLU, APOD, APOB* and *CNTN5* transcripts in the frontal cortex were estimated using the transcriptome analysis console (Applied Biosystem, USA) following standardization protocols.

4.4 Animal Studies:

4.4.1 Animals:

All animal procedures were performed in conformity with the Canadian Guidelines for Use and Care of Laboratory Animals and were approved by the McGill University Animal Care Committee (approval DOUG-10032). Animals used were 2–3-month-old male C57BL/6J wild-type mice. All mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), housed individually, and fed standard laboratory chow *ad libitum.* Animals were kept in a 12- h-light-dark cycle, with light onset at 07:00 and offset at 19:00.

4.4.2 Unilateral entorhinal cortex lesions:

Unilateral electrolytic lesions were performed on the entorhinal cortex of adult mice as previously described [24]. Animals were anesthetized with isoflurane and placed in a stereotaxic apparatus in a flat skull position. Lesion coordinates were determined from Lambda in the following positions: 1) [AP: 0 mm], [L: –3 mm], [DV: –3 mm, –4 mm]; 2) [AP: 0 mm], [L: –3.5 mm], [DV: –3 mm, –

4 mm]; 3) [AP: +0.5 mm], [L: –4 mm], [DV: –3 mm, –4 mm]; 4) [AP: +1 mm], [L: –4 mm], [DV: –3 mm, –4 mm]. A current of 1mA was applied at each coordinate for 10 seconds. For sham-operated mice, which are used as the control group, the same steps were followed, but the electrode was lowered only 1 mm, and no current was applied. After surgery, subcutaneous physiological saline was given to prevent dehydration and animals were nursed throughout their recovery. Lesioned mice were sacrificed at 2, 7, 14, 21 and 40 days post-lesion (DPL) and sham-lesioned mice were sacrificed on the same day as surgery. Six mice were sacrificed at each time point. Animals were decapitated, the brain was quickly removed, hippocampi contralateral and ipsilateral to EC lesion was dissected and stored at –80ºC.

4.4.3 RNA extraction and quality assessment:

RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany) was used for RNA extraction. RNA quality was assessed at McGill Genome Centre. All RNA samples had RIN > 7.8 and 260/280 ratios > 2.1.

4.4.4 Hippocampal *CNTN5*, *APOE*, *CLU* (*APOJ*), *APOB* and *APOD* mRNA levels:

Hippocampal mRNA levels of *CNTN5*, *APOE*, *APOJ*, *APOB* and *APOD* were measured at different time points (n=6 animals per time point) by the McGill Genome Centre, using the Mouse Clariom^{TM} D genechip assay (Affymetrix, Santa Clara, CA, USA).

4.5 Statistical Analysis:

The differences in population distribution were assessed using the chi-square test. The difference in age between AD subjects and controls was measured using the Student's t-test. The correlational analyses between contactin 5 and apolipoproteins or cholesterol were performed in the plasma and CSF using linear regression models corrected for age, sex and APOE4. The correlation between contactin 5 mRNA and apolipoprotein mRNA in the frontal cortex was measured using linear regression models. The Kruskal-Wallis test was used to evaluate the difference in mean mRNA levels between different days post-lesion in the lesioned mouse model. Significance level was considered at $p<0.05$.

5. Results

5.1 Demographics:

Table 4 summarizes the demographic characteristics of the cohorts used. In the QFP cohort there were no differences in the proportion of males and females or in age between diagnostic groups, but AD subjects were more likely to be APOE ***ε***4 positive compared to controls ($p = 0.01$).

Table 4: PREVENT-AD and QFP demographics

	PREVENT-AD	QFP		
		AD	Controls	*p*
Sex F/M				0.445
Female	66/93 (71.0%)	25/57 (43.8%)	11/31 (35.5%)	
Male	27/93 (29.0%)	32/57 (56.2%)	20/31 (64.5%)	
***APOE* ε4**				0.01
APOE ε–	55/93 (59.1%)	24/57 (42.1%)	22/31 (70.9%)	
APOE ε+	38/93 (40.9%)	33/57 (57.9%)	9/31 (29.1%)	
Age (y) mean ± SD	62.6 ± 5.5	80.68 ± 6.3	77.39 ± 11.3	0.143

5.2 Associations in the CSF:

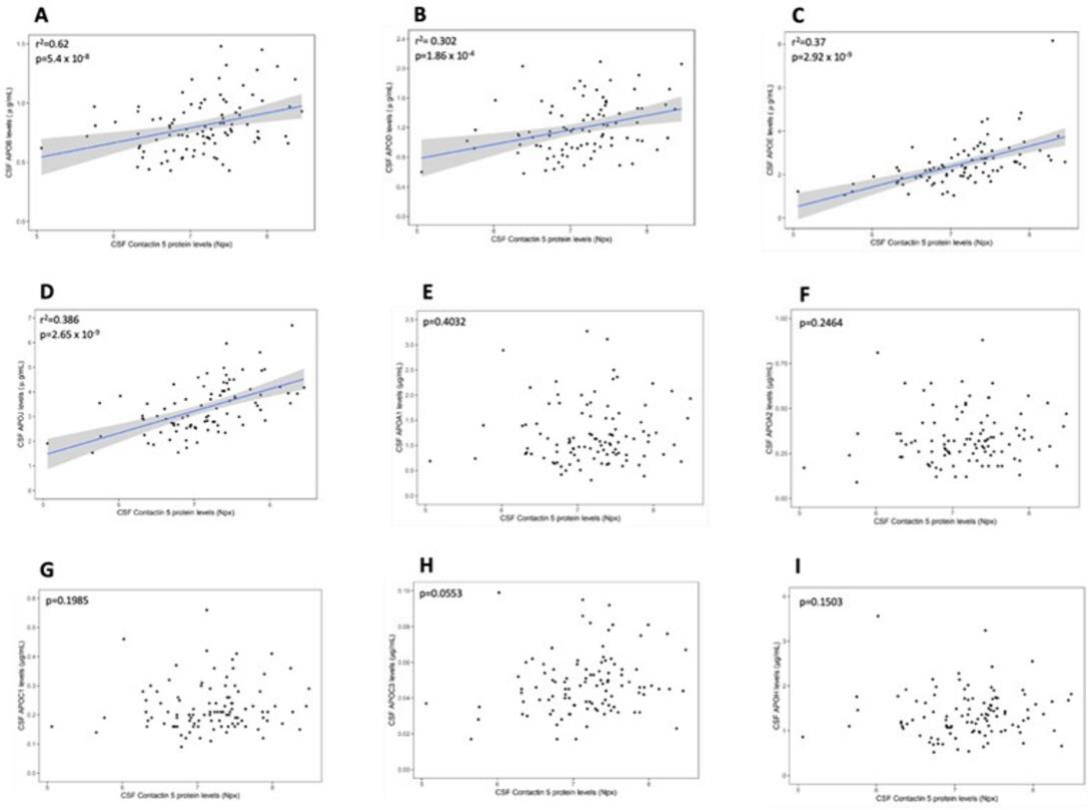
Contactin 5 was found to positively correlate with apolipoproteins B (r^2=0.62, p=5.4x10^{-8}), D (r^2=0.302 p=1.86x10^{-4}), E (r^2=0.370, p=2.92x10^{-9}) and J (r^2=0.386, p=2.65x10^{-9}) in the CSF of cognitively unimpaired subjects from the PREVENT-AD cohort. (Figure 13). In contrast, CSF apolipoproteins A1, A2, C1, C3 and H (all with peripheral origin) displayed no association with contactin 5 (Figure 13).

CSF cholesterol concentration (but not 25-hydroxycholesterol) positively correlated with contactin 5 concentration (r^2=0.1352, p=0.0096, Figure 14).

Stratification by APOE genotype (E4 positive vs E4 negative) was used to assess the effect of the most important genetic risk factor for sporadic AD in the PREVENT-AD cohort (Figure 15). In APOE4 positive subjects, CSF contactin 5 positively correlated with apolipoproteins B (r^2=0.431 p=0.00005), D (r^2=0.323 p=0.04), E (r^2=0.620 p=0.0000003) and J (r^2=0.466 p=0.000374). In APOE4 negative subjects, contactin 5 positively associated with apolipoproteins B (r^2=0.168 p=0.003), D (r^2=0.311 p=0.002), E (r^2=0.326 p=0.00002) and J (r^2=0.437 p=0.0000004) (Figure 15).

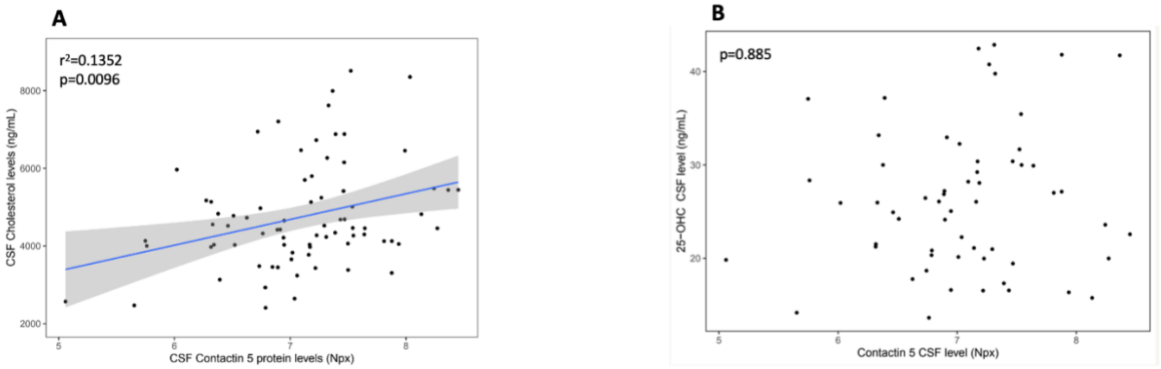
A noteworthy observation is that the trajectory of CSF apoB levels show parallel rather than overlapping trajectories in those who are E4-positive compared to those who are E4-negative (Figure 15A). Other apolipoproteins (D, E and J) display parallel and overlapping slopes (Figure 15B-D): suggesting different lipoprotein compartments from that of apoB.

Figure 13: Association between CSF Contactin 5 and apolipoproteins



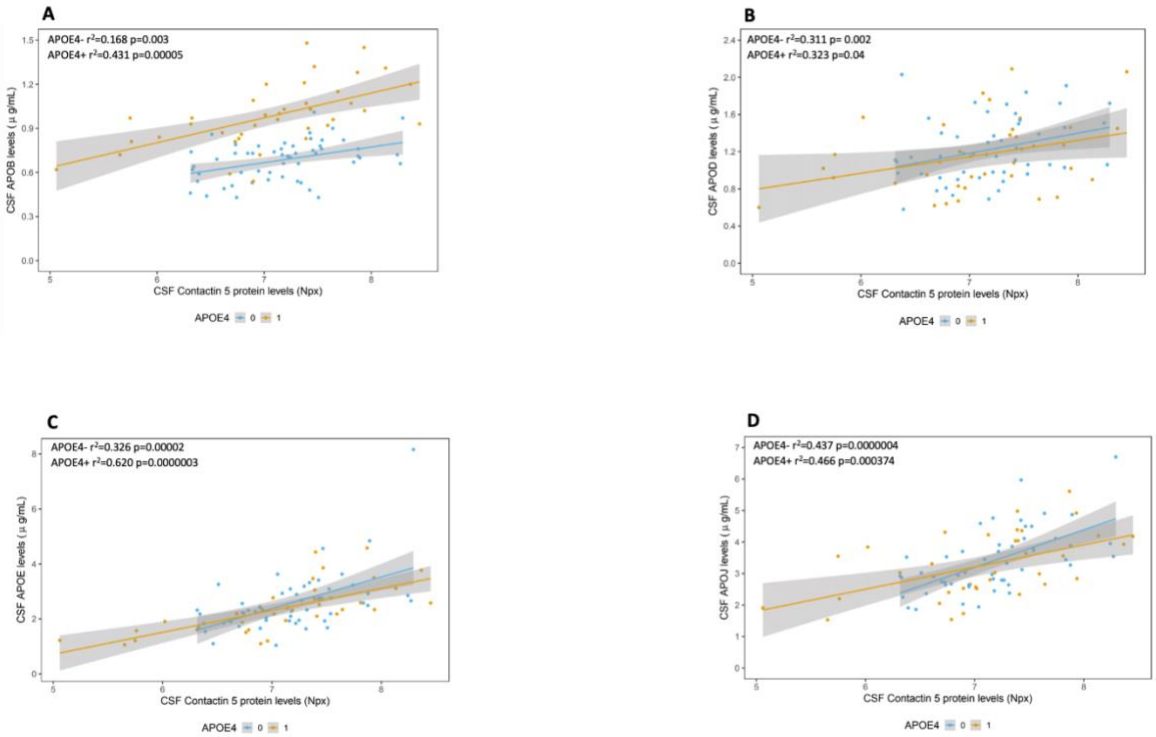
Contactin 5 protein was measured in the CSF using Olink's proximity extension assay. Apolipoproteins were measured in the CSF using Luminex. Significant linear regressions are represented with a gray confidence region of the fitted line. Individual R squares and *p* values are shown in the top left corners of each figure.

Figure 14: Association between CSF Contactin 5 and Cholesterol and between CSF Contactin 5 and 25-OHC.



Contactin 5 protein was measured in the CSF using Olink's proximity extension assay. Cholesterol and 25-OHC were measured using liquid chromatography mass spectrometry. Significant linear regressions are represented with a gray confidence region of the fitted line. Individual R squares and *p* values are shown in the top left corners of each figure.

Figure 15: Association between CSF Contactin 5 and apolipoproteins divided by the presence and absence of the APOE4 allele.

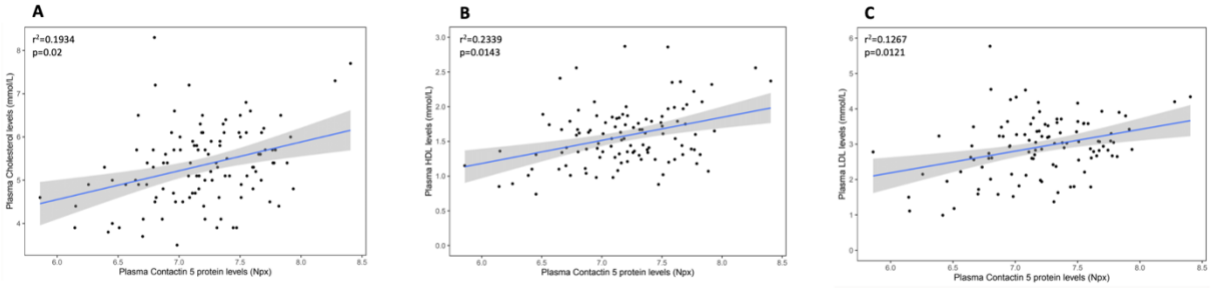


Contactin 5 protein was measured in the CSF using Olink's proximity extension assay. Apolipoproteins were measured using Luminex. Significant linear regressions are represented with a gray confidence region of the fitted line. Individual R squares and *p* values are shown in the top left corners of each figure.

5.3 Associations in the plasma:

In the plasma, contactin 5 positively correlated with peripheral cholesterol (r^2=0.1934, p=0.02), HDL (r^2=0.2339, p=0.0143) and LDL (r^2=0.1267, p=0.0121) concentrations (Figure 16). However, in contrast to the CSF, there were no significant correlations between contactin 5 and apolipoproteins B, D, E or J in the plasma compartment (not shown).

Figure 16: Association between plasma Contactin 5 and cholesterol.

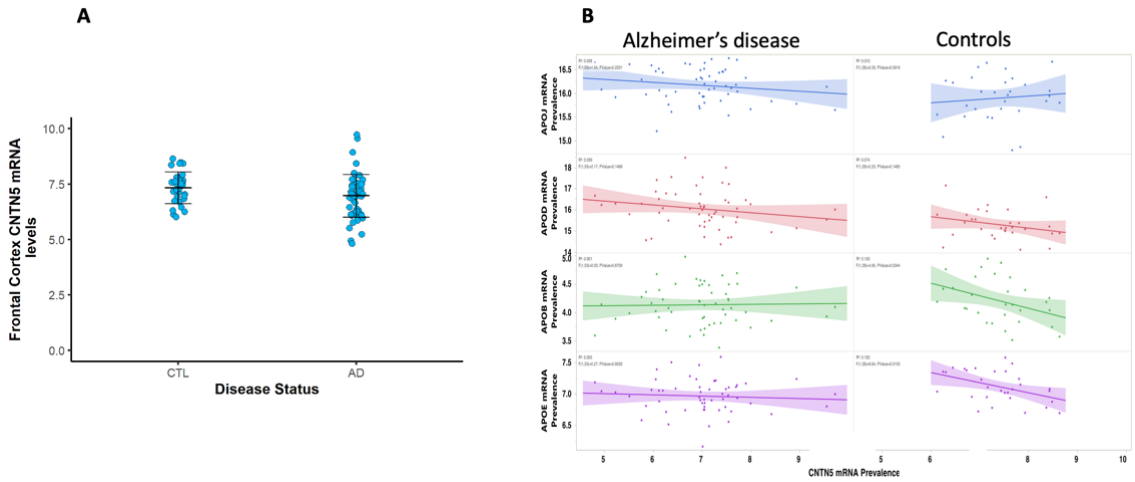


Contactin 5 protein was measured in the plasma using Olink's proximity extension assay. Cholesterol was measured using liquid chromatography mass spectrometry (LCMS/MS). Significant linear regressions are represented with a gray confidence region of the fitted line. Individual R squares and *p* values are shown in the top left corners of each figure.

5.4 Gene expression in the frontal cortex in autopsy-confirmed AD and control cases:

Figure 17 contrasts levels of *APOE, APOB, APOD*, and *CLU* mRNAs as a function of *CNTN5* mRNA prevalence in the frontal cortex of AD and control cases from the QFP cohort. There are no significant differences in cortical CNTN5 gene expression between AD and controls when adjusted for APOE4, sex and age (Figure 17A). Significant negative correlations were seen between *CNTN5*, *APOB* (r^2=0.15, p=0.034) and *APOE* (r^2=0.192, p=0.015) mRNA levels in the control group but not in the AD cases (Figure 17B).

Figure 17: mRNA levels of CNTN5 and apolipoproteins in the frontal cortex of AD and control subjects



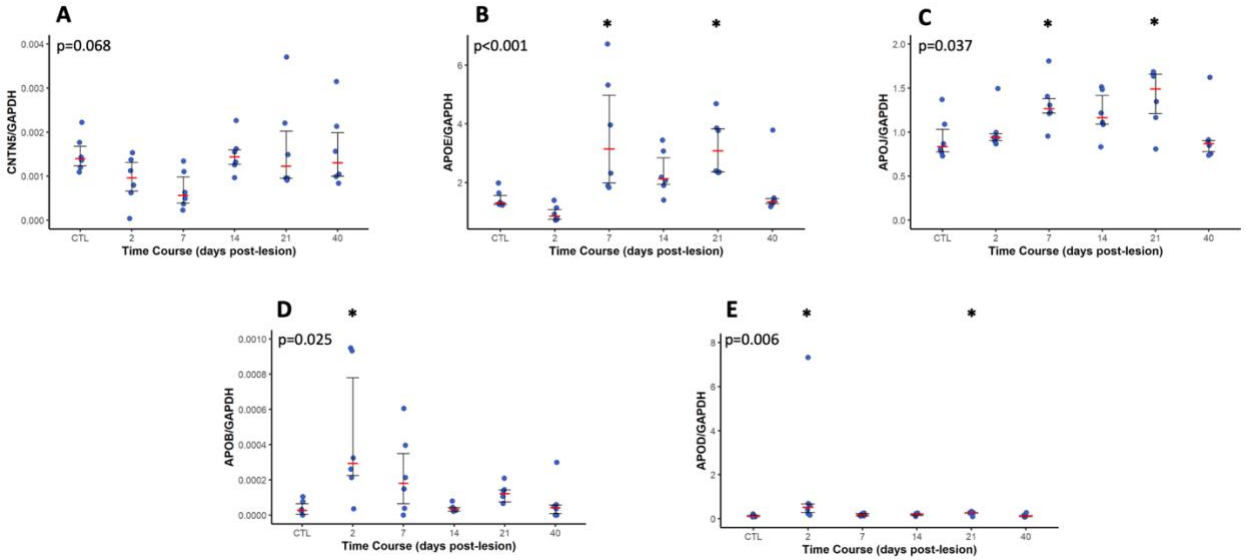
A) Contactin 5 mRNA levels in the frontal cortex of AD (n = 57) and controls (n = 31). mRNA was measured using qRT-PCR. $p > 0.05$. The demarcated lines represent the mean and SD. B) Association between contactin 5 and apolipoproteins mRNA levels in the frontal cortex of AD subjects and controls. mRNA was measured using qRT-PCR. Significant linear regressions are represented with a colored confidence region of the fitted line. Individual R squares and p values are shown in the top left corners of each figure.

5.5 Gene expression in a mouse model of hippocampal deafferentation and reinnervation:

Figure 18 illustrates the time course analysis of the hippocampal mRNA prevalence for *cntn5*, *apoe*, *clu* (apoJ), *apob* and *apod* as a function of deafferentation (0-10 days) and the ensuing reinnervation (10-40 days) process. The ipsilateral modifications are being contrasted with the outcomes from the control group (sham mice). A transient decrease in cntn5 mRNA levels (trends only, p=0.068) can be seen 7 days after the lesioning, which coincides with the peak of neuronal deafferentation in the hippocampus. In contrast, *apoe* and *apoj* gene expression increased at 7 DPL ($p<0.05$) in the early phase of the reinnervation process and remained elevated during the

reinnervation phase until 21 DPL ($p<0.05$). There were transient increases of *apob* and *apod* at 2DPL ($p=0.025$ and $p=0.006$, respectively), which is indicative of the initial stage of the deafferentation process ($p<0.05$).

Figure 18: Gene expression of Contactin 5 and apolipoproteins in a mouse model of entorhinal cortex lesion (ECL).



The dots represent the values of either an apolipoprotein or contactin 5 divided by GAPDH (n = 6 animals per time point). The demarcated lines represent the median and the interquartile range. The p-value for each protein is on the top left corner of each figure. $*$ $p < 0.05$ as compared to day 0.

6. DISCUSSION:

We studied the interaction between contactin 5, apolipoproteins, and cholesterol in elderly individuals who were cognitively unimpaired but carrying a greater risk of developing AD. We observed extensive interactions between these molecules in both CSF and plasma. The recent

identification of several genetic risk factors for sporadic AD by GWAS that belong to key regulatory genes involved in cholesterol metabolism such as *APOE4* and *CLU* (lipoprotein-mediated cholesterol transport), *PICALM*, *SORL1* and *BIN1* (lipoproteins internalization), ABCA1 and *ABCA7* (intracellular cholesterol transport and mobilization) [25-27] further emphasize the importance of such interactions in the mature and aging brains.

Indeed, all CNS-relevant apolipoproteins shown here to interact with contactin 5 had been previously associated with different aspects of AD pathology. For example, apoE is actively involved in cholesterol transport and ß-amyloid catabolism, whereas the *APOE-ε4* variant shows lower affinity for lipids and leads to less effective lipoprotein-mediated cholesterol and amyloid transport and increased Aß production combined to decreased Aß clearance [28, 29]. In addition, APOE and APOA1 were shown to independently protect from amyloid precursor protein carboxy terminal fragment-associated cytotoxicity [30]. *CLU* (apoJ), another important genetic risk factor for AD is a major component of lipoprotein complexes (HDL-like) and has also been involved in amyloid transport and catabolism [31, 32]. Similarly, apoB and apoD participate in cholesterol transport as minor components of HDL-like particles, and both have been found to be elevated in the CSF of individuals with AD and to markedly associate with tau pathology [5, 7].

Variants in the *CNTN5* gene have been associated with increased risk for neurodevelopmental disorders such as attention deficit hyperactivity disorder [33] and autism [34, 35]. *CNTN5* genetic variants have been associated with increased risk for AD (rs1461684 and rs10501927) [19, 26] and a faster rate of progression (rs1461684) [19] in the pre-symptomatic phase of the disease. The level of contactin 5 protein is elevated in the CSF of those who are “at-risk” but have not yet experienced cognitive impairment, while decreased levels have been reported in MCI and AD subjects [19],

similar to other contactin species [36]. Contactin 5 is also associated with CSF tau, phospho-tau levels, and synaptic markers, and to a lesser extent, to amyloid [19].

Contactin 5 is a neuronal membrane protein that acts during neurodevelopment on neuronal migration, axonal guidance, myelin formation and synaptogenesis [37, 38]. It is particularly involved in axonal arborization, synaptic formation and remodeling [17, 18]. In the mice, *cntn5* expression pattern included strong expression in the cerebral cortex in layers II–V, hippocampus and mammillary bodies in addition to previously described brain nuclei of the auditory pathway and the dorsal thalamus [39]. Deletion of the *cntn5* gene in mice leads to defects in the cortical and subcortical auditory pathways and loss of presynaptic inhibitory boutons in multiple brain areas [40, 41]. The association between contactin 5 and apolipoproteins seen in the CSF of these "at-risk" subjects for AD is very significant because not only both families of proteins have been involved in the pathology of AD, but they share complementary functions as they are both involved in synaptic maintenance and remodeling in the CNS.

Synaptic dysfunction and axonal loss are important early events in AD preceding cognitive decline [42]. Given the well-established mechanisms of action of contactin 5 and apolipoproteins in synaptic physiology, it is conceivable that in response to early synaptic damage, lipophilic contactin 5 is released in the extracellular space where it is taken up by lipid-rich lipoproteins and transported to target cells in the CNS and/or eliminated from the CNS. This is consistent with the finding that contactin 5 is also positively associated with cholesterol, HDL and LDL in the plasma as well as with cholesterol in the CSF. The soluble form of contactin-2, a contactin 5 analog, acts as a guiding molecule for the outgrowth of neurites and plays a role in axon extension initiation, axonal guidance and fasciculation [43, 44]. It is thus conceivable that contactin 5, which also acts

as a scaffold on inter-neurons where dendrites of direction-selective neuronal cells can fasciculate, requires both secretion and the presence of lipid-rich HDLs complex to provide reinnervating neurons with the necessary lipids for effective neurite outgrowth and axonal extension [41, 45, 46].

To better understand the possible origin of the association between contactin 5 and apolipoproteins in the CNS, we measured and contrasted their gene expression in the frontal cortex of autopsy-confirmed AD and control subjects. mRNA levels of *CNTN5* negatively associate with *APOB* and *APOE* transcripts in control subjects, but not in AD. These findings show that the associations between these proteins in the CSF compartment are not due to changes in gene expression but are more likely a consequence of the neuronal damage that occurs in the early phase of the neurodegenerative process.

To further investigate the role of contactin 5 and apolipoproteins during axonal sprouting and terminal proliferation in the mature CNS, we measured the gene expression of contactin 5 and apolipoproteins in a well-established model of rodent hippocampal deafferentation/reinnervation: the entorhinal cortex lesioning paradigm [47]. In this model, using wild-type animals, it is possible to examine without the interference of amyloid and tau pathology, the alterations in gene expression that occur in the hippocampus in response to a lesion to the entorhinal cortex and the resulting loss of input due to degeneration of the perforant pathway. It gave us the opportunity to observe how the gene expression alterations of these potential markers reacted to the synaptic loss that occurred in the initial 10 days and the consecutive terminal and synaptic restructuring that occurred in the 14-42 days window after the lesion.

Results show that *apob* and *apod* mRNA levels are increased early on during the deafferentation phase (2 DPL) while the gene expression of the major CNS cholesterol transporters *apoe* and *apoj* increases in the late phase of the deafferentation process and peak during the early stage of the reinnervation process, up to 21 DPL. This is consistent with previous literature that shows peak elevation of apoE mRNA levels between 7 and 14 DPL, during the early phase of the reinnervation process, when terminal and synaptic remodeling begins [47, 48]. Contactin 5 mRNA levels display a time-dependent reduction in the 0-7 DPL window ($p=0.06$ at 7 DPL), with levels returning to normal after day 14. This transient reduction is identical to the time-course alterations reported for other synaptic markers such as GAP-43, synaptophysin and SNAP-25 [47, 49, 50]. These results suggest that the gene expression of apolipoproteins (and possibly contactin 5) does change in response to synaptic deafferentation, but the elevation at different time-points suggests each protein plays a different role in the deafferentation/reinnervation process.

In summary, we show that extensive interactions exist between apolipoproteins and contactin 5 in asymptomatic subjects at high-risk of AD. Additionally, we show that following a lesion to the entorhinal cortex in a mouse model, significant alterations in gene expression of apolipoproteins occur at key moments in the reinnervating hippocampus. The precise role of contactin 5 and apolipoproteins in the pathophysiology of AD is not completely understood at this time, but these results suggest a possible active role in axonal, terminal and synaptic remodeling in response to entorhinal cortex damage due to experimental lesions or, to AD pathology in humans.

Follow-up studies looking into the biochemistry of these proteins throughout the spectrum of AD pathology would be important to better understand the pathophysiology of the disease and more specifically how the brain reacts to this type of pathological damage.

Furthermore, in this initial work into the role of contactin 5 and apolipoproteins following neuronal damage, we aimed to investigate the earliest changes in gene expression. In the animal model of hippocampal deafferentation, that meant focusing on mRNA levels. However, to fully elucidate the role of these molecules on neuronal repair, it is crucial to also understand the changes that occur in protein level and function. Work is currently underway to investigate protein changes in the hippocampus in response to entorhinal cortex deafferentation.

7. References:

[1] World Health Organization (2022) Dementia. https://www.who.int/news-room/fact-sheets/detail/dementia, Last updated September 20, 2022, Accessed on March 10, 2023.

[2] Jack CR, Bennett DA, Blennow K, Carrillo MC, Feldman HH, Frisoni GB, Hampel H, Jagust WJ, Johnson KA, Knopman DS, Petersen RC, Scheltens P, Sperling RA, Dubois B (2016) A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology* **87**, 539-547.

[3] Leduc V, Jasmin-Bélanger S, Poirier J (2010) APOE and cholesterol homeostasis in Alzheimer's disease. *Trends Mol Med* **16**, 469-477.

[4] Poirier J, Delisle MC, Quirion R, Aubert I, Farlow M, Lahiri D, Hui S, Bertrand P, Nalbantoglu J, Gilfix BM, Gauthier S (1995) Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proc Natl Acad Sci U S A* **92**, 12260-12264.

[5] Terrisse L, Poirier J, Bertrand P, Merched A, Visvikis S, Siest G, Milne R, Rassart E (1998) Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. *J Neurochem* **71**, 1643-1650.

[6] Wingo TS, Cutler DJ, Wingo AP, Le NA, Rabinovici GD, Miller BL, Lah JJ, Levey AI (2019) Association of Early-Onset Alzheimer Disease With Elevated Low-Density Lipoprotein Cholesterol Levels and Rare Genetic Coding Variants of APOB. *JAMA Neurol* **76**, 809-817.

[7] Picard C, Nilsson N, Labonte A, Auld D, Rosa-Neto P, Alzheimer's Disease Neuroimaging I, Ashton NJ, Zetterberg H, Blennow K, Breitner JCB, Villeneuve S, Poirier J, group P-Ar (2022) Apolipoprotein B is a novel marker for early tau pathology in Alzheimer's disease. *Alzheimers Dement* **18**, 875-887.

[8] Wisniewski T, Golabek A, Matsubara E, Ghiso J, Frangione B (1993) Apolipoprotein E: binding to soluble Alzheimer's beta-amyloid. *Biochem Biophys Res Commun* **192**, 359-365.

[9] Rodriguez GA, Tai LM, LaDu MJ, Rebeck GW (2014) Human APOE4 increases microglia reactivity at Abeta plaques in a mouse model of Abeta deposition. *J Neuroinflammation* **11**, 111.
[10] Beffert U, Cohn JS, Petit-Turcotte C, Tremblay M, Aumont N, Ramassamy C, Davignon J, Poirier J (1999) Apolipoprotein E and beta-amyloid levels in the hippocampus and frontal cortex of Alzheimer's disease subjects are disease-related and apolipoprotein E genotype dependent. *Brain Res* **843**, 87-94.
[11] Wisniewski T, Drummond E (2020) APOE-amyloid interaction: Therapeutic targets. *Neurobiol Dis* **138**, 104784.
[12] Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S (1993) Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet (London, England)* **342**, 697-699.
[13] Strittmatter WJ, Roses AD (1995) Apolipoprotein E and Alzheimer disease. *Proc Natl Acad Sci U S A* **92**, 4725-4727.
[14] Zhu R, Liu X, He Z (2018) Association between CLU gene rs11136000 polymorphism and Alzheimer's disease: an updated meta-analysis. *Neurol Sci* **39**, 679-689.
[15] Lambert J-C, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, Combarros O, Zelenika D, Bullido MJ, Tavernier B, Letenneur L, Bettens K, Berr C, Pasquier F, Fiévet N, Barberger-Gateau P, Engelborghs S, De Deyn P, Mateo I, Franck A, Helisalmi S, Porcellini E, Hanon O, European Alzheimer's Disease Initiative I, de Pancorbo MM, Lendon C, Dufouil C, Jaillard C, Leveillard T, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossù P, Piccardi P, Annoni G, Seripa D, Galimberti D, Hannequin D, Licastro F, Soininen H, Ritchie K, Blanché H, Dartigues J-F, Tzourio C, Gut I, Van Broeckhoven C, Alpérovitch A, Lathrop M, Amouyel P (2009) Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat Genet* **41**, 1094-1099.
[16] Glockner F, Ohm TG (2003) Hippocampal apolipoprotein D level depends on Braak stage and APOE genotype. *Neuroscience* **122**, 103-110.
[17] Mercati O, Danckaert A, Andre-Leroux G, Bellinzoni M, Gouder L, Watanabe K, Shimoda Y, Grailhe R, De Chaumont F, Bourgeron T, Cloez-Tayarani I (2013) Contactin 4, -5 and -6 differentially regulate neuritogenesis while they display identical PTPRG binding sites. *Biol Open* **2**, 324-334.
[18] Toyoshima M, Sakurai K, Shimazaki K, Takeda Y, Nakamoto M, Serizawa S, Shimoda Y, Watanabe K (2009) Preferential localization of neural cell recognition molecule NB-2 in developing glutamatergic neurons in the rat auditory brainstem. *J Comp Neurol* **513**, 349-362.
[19] Dauar MT, Labonte A, Picard C, Miron J, Rosa-Neto P, Zetterberg H, Blennow K, Villeneuve S, Poirier J (2022) Characterization of the contactin 5 protein and its risk-associated polymorphic variant throughout the Alzheimer's disease spectrum. *Alzheimers Dement*.
[20] Breitner JCS, Poirier J, Etienne PE, Leoutsakos JM (2016) Rationale and Structure for a New Center for Studies on Prevention of Alzheimer's Disease (StoP-AD). *J Prev Alzheimers Dis* **3**, 236-242.

[21] Tremblay-Mercier J, Madjar C, Das S, Pichet Binette A, Dyke SOM, Etienne P, Lafaille-Magnan ME, Remz J, Bellec P, Louis Collins D, Natasha Rajah M, Bohbot V, Leoutsakos JM, Iturria-Medina Y, Kat J, Hoge RD, Gauthier S, Tardif CL, Mallar Chakravarty M, Poline JB, Rosa-Neto P, Evans AC, Villeneuve S, Poirier J, Breitner JCS, Group P-AR (2021) Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. *Neuroimage Clin* **31**, 102733.

[22] Khachaturian ZS (1985) Diagnosis of Alzheimer's disease. *Arch Neurol* **42**, 1097-1105.

[23] Leduc V, Theroux L, Dea D, Dufour R, Poirier J (2016) Effects of rs3846662 Variants on HMGCR mRNA and Protein Levels and on Markers of Alzheimer's Disease Pathology. *J Mol Neurosci* **58**, 109-119.

[24] Blain J-F, Paradis E, Gaudreault SB, Champagne D, Richard D, Poirier J (2004) A role for lipoprotein lipase during synaptic remodeling in the adult mouse brain. *Neurobiol Dis* **15**, 510-519.

[25] Lambert JC, Ibrahim-Verbaas CA, Harold D, Naj AC, Sims R, Bellenguez C, DeStafano AL, Bis JC, Beecham GW, Grenier-Boley B, Russo G, Thorton-Wells TA, Jones N, Smith AV, Chouraki V, Thomas C, Ikram MA, Zelenika D, Vardarajan BN, Kamatani Y, Lin CF, Gerrish A, Schmidt H, Kunkle B, Dunstan ML, Ruiz A, Bihoreau MT, Choi SH, Reitz C, Pasquier F, Cruchaga C, Craig D, Amin N, Berr C, Lopez OL, De Jager PL, Deramecourt V, Johnston JA, Evans D, Lovestone S, Letenneur L, Morón FJ, Rubinsztein DC, Eiriksdottir G, Sleegers K, Goate AM, Fiévet N, Huentelman MW, Gill M, Brown K, Kamboh MI, Keller L, Barberger-Gateau P, McGuiness B, Larson EB, Green R, Myers AJ, Dufouil C, Todd S, Wallon D, Love S, Rogaeva E, Gallacher J, St George-Hyslop P, Clarimon J, Lleo A, Bayer A, Tsuang DW, Yu L, Tsolaki M, Bossù P, Spalletta G, Proitsi P, Collinge J, Sorbi S, Sanchez-Garcia F, Fox NC, Hardy J, Deniz Naranjo MC, Bosco P, Clarke R, Brayne C, Galimberti D, Mancuso M, Matthews F, European Alzheimer's Disease I, Genetic, Environmental Risk in Alzheimer's D, Alzheimer's Disease Genetic C, Cohorts for H, Aging Research in Genomic E, Moebus S, Mecocci P, Del Zompo M, Maier W, Hampel H, Pilotto A, Bullido M, Panza F, Caffarra P, Nacmias B, Gilbert JR, Mayhaus M, Lannefelt L, Hakonarson H, Pichler S, Carrasquillo MM, Ingelsson M, Beekly D, Alvarez V, Zou F, Valladares O, Younkin SG, Coto E, Hamilton-Nelson KL, Gu W, Razquin C, Pastor P, Mateo I, Owen MJ, Faber KM, Jonsson PV, Combarros O, O'Donovan MC, Cantwell LB, Soininen H, Blacker D, Mead S, Mosley TH, Bennett DA, Harris TB, Fratiglioni L, Holmes C, de Bruijn RF, Passmore P, Montine TJ, Bettens K, Rotter JI, Brice A, Morgan K, Foroud TM, Kukull WA, Hannequin D, Powell JF, Nalls MA, Ritchie K, Lunetta KL, Kauwe JS, Boerwinkle E, Riemenschneider M, Boada M, Hiltuenen M, Martin ER, Schmidt R, Rujescu D, Wang LS, Dartigues JF, Mayeux R, Tzourio C, Hofman A, Nöthen MM, Graff C, Psaty BM, Jones L, Haines JL, Holmans PA, Lathrop M, Pericak-Vance MA, Launer LJ, Farrer LA, van Duijn CM, Van Broeckhoven C, Moskvina V, Seshadri S, Williams J, Schellenberg GD, Amouyel P (2013) Meta-analysis of 74,046 individuals identifies 11 new susceptibility loci for Alzheimer's disease. *Nat Genet* **45**, 1452-1458.

[26] Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, Pahwa JS, Moskvina V, Dowzell K, Williams A, Jones N, Thomas C, Stretton A, Morgan AR, Lovestone S, Powell J, Proitsi P, Lupton MK, Brayne C, Rubinsztein DC, Gill M, Lawlor B, Lynch A, Morgan K, Brown KS, Passmore PA, Craig D, McGuinness B, Todd S, Holmes C, Mann D, Smith AD, Love S, Kehoe PG, Hardy J, Mead S, Fox N, Rossor M, Collinge J, Maier W, Jessen F, Schürmann B, Heun R, van den Bussche H, Heuser I, Kornhuber J, Wiltfang J, Dichgans M, Frölich L, Hampel H, Hüll M, Rujescu D, Goate AM, Kauwe JSK, Cruchaga C, Nowotny P, Morris JC, Mayo K, Sleegers K, Bettens K, Engelborghs S, De Deyn PP, Van Broeckhoven C, Livingston G, Bass NJ, Gurling H, McQuillin A, Gwilliam R, Deloukas P, Al-Chalabi A, Shaw CE, Tsolaki M, Singleton AB, Guerreiro R, Mühleisen TW, Nöthen MM, Moebus S, Jöckel K-H, Klopp N, Wichmann HE, Carrasquillo MM, Pankratz VS, Younkin SG, Holmans PA, O'Donovan M, Owen MJ, Williams J (2009) Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. *Nat Genet* **41**, 1088-1093.

[27] Bellenguez C, Kucukali F, Jansen IE, Kleineidam L, Moreno-Grau S, Amin N, Naj AC, Campos-Martin R, Grenier-Boley B, Andrade V, Holmans PA, Boland A, Damotte V, van der Lee SJ, Costa MR, Kuulasmaa T, Yang Q, de Rojas I, Bis JC, Yaqub A, Prokic I, Chapuis J, Ahmad S, Giedraitis V, Aarsland D, Garcia-Gonzalez P, Abdelnour C, Alarcon-Martin E, Alcolea D, Alegret M, Alvarez I, Alvarez V, Armstrong NJ, Tsolaki A, Antunez C, Appollonio I, Arcaro M, Archetti S, Pastor AA, Arosio B, Athanasiu L, Bailly H, Banaj N, Baquero M, Barral S, Beiser A, Pastor AB, Below JE, Benchek P, Benussi L, Berr C, Besse C, Bessi V, Binetti G, Bizarro A, Blesa R, Boada M, Boerwinkle E, Borroni B, Boschi S, Bossu P, Brathen G, Bressler J, Bresner C, Brodaty H, Brookes KJ, Brusco LI, Buiza-Rueda D, Burger K, Burholt V, Bush WS, Calero M, Cantwell LB, Chene G, Chung J, Cuccaro ML, Carracedo A, Cecchetti R, Cervera-Carles L, Charbonnier C, Chen HH, Chillotti C, Ciccone S, Claassen J, Clark C, Conti E, Corma-Gomez A, Costantini E, Custodero C, Daian D, Dalmasso MC, Daniele A, Dardiotis E, Dartigues JF, de Deyn PP, de Paiva Lopes K, de Witte LD, Debette S, Deckert J, Del Ser T, Denning N, DeStefano A, Dichgans M, Diehl-Schmid J, Diez-Fairen M, Rossi PD, Djurovic S, Duron E, Duzel E, Dufouil C, Eiriksdottir G, Engelborghs S, Escott-Price V, Espinosa A, Ewers M, Faber KM, Fabrizio T, Nielsen SF, Fardo DW, Farotti L, Fenoglio C, Fernandez-Fuertes M, Ferrari R, Ferreira CB, Ferri E, Fin B, Fischer P, Fladby T, Fliessbach K, Fongang B, Fornage M, Fortea J, Foroud TM, Fostinelli S, Fox NC, Franco-Macias E, Bullido MJ, Frank-Garcia A, Froelich L, Fulton-Howard B, Galimberti D, Garcia-Alberca JM, Garcia-Gonzalez P, Garcia-Madrona S, Garcia-Ribas G, Ghidoni R, Giegling I, Giorgio G, Goate AM, Goldhardt O, Gomez-Fonseca D, Gonzalez-Perez A, Graff C, Grande G, Green E, Grimmer T, Grunblatt E, Grunin M, Gudnason V, Guetta-Baranes T, Haapasalo A, Hadjigeorgiou G, Haines JL, Hamilton-Nelson KL, Hampel H, Hanon O, Hardy J, Hartmann AM, Hausner L, Harwood J, Heilmann-Heimbach S, Helisalmi S, Heneka MT, Hernandez I, Herrmann MJ, Hoffmann P, Holmes C, Holstege H, Vilas RH, Hulsman M, Humphrey J, Biessels GJ, Jian X, Johansson C, Jun GR, Kastumata Y, Kauwe J, Kehoe PG, Kilander L, Stahlbom AK, Kivipelto M, Koivisto A, Kornhuber J, Kosmidis MH, Kukull WA, Kuksa PP, Kunkle BW, Kuzma AB, Lage C, Laukka EJ,

Launer L, Lauria A, Lee CY, Lehtisalo J, Lerch O, Lleo A, Longstreth W, Jr., Lopez O, de Munain AL, Love S, Lowemark M, Luckcuck L, Lunetta KL, Ma Y, Macias J, MacLeod CA, Maier W, Mangialasche F, Spallazzi M, Marquie M, Marshall R, Martin ER, Montes AM, Rodriguez CM, Masullo C, Mayeux R, Mead S, Mecocci P, Medina M, Meggy A, Mehrabian S, Mendoza S, Menendez-Gonzalez M, Mir P, Moebus S, Mol M, Molina-Porcel L, Montrreal L, Morelli L, Moreno F, Morgan K, Mosley T, Nothen MM, Muchnik C, Mukherjee S, Nacmias B, Ngandu T, Nicolas G, Nordestgaard BG, Olaso R, Orellana A, Orsini M, Ortega G, Padovani A, Paolo C, Papenberg G, Parnetti L, Pasquier F, Pastor P, Peloso G, Perez-Cordon A, Perez-Tur J, Pericard P, Peters O, Pijnenburg YAL, Pineda JA, Pinol-Ripoll G, Pisanu C, Polak T, Popp J, Posthuma D, Priller J, Puerta R, Quenez O, Quintela I, Thomassen JQ, Rabano A, Rainero I, Rajabli F, Ramakers I, Real LM, Reinders MJT, Reitz C, Reyes-Dumeyer D, Ridge P, Riedel-Heller S, Riederer P, Roberto N, Rodriguez-Rodriguez E, Rongve A, Allende IR, Rosende-Roca M, Royo JL, Rubino E, Rujescu D, Saez ME, Sakka P, Saltvedt I, Sanabria A, Sanchez-Arjona MB, Sanchez-Garcia F, Juan PS, Sanchez-Valle R, Sando SB, Sarnowski C, Satizabal CL, Scamosci M, Scarmeas N, Scarpini E, Scheltens P, Scherbaum N, Scherer M, Schmid M, Schneider A, Schott JM, Selbaek G, Seripa D, Serrano M, Sha J, Shadrin AA, Skrobot O, Slifer S, Snijders GJL, Soininen H, Solfrizzi V, Solomon A, Song Y, Sorbi S, Sotolongo-Grau O, Spalletta G, Spottke A, Squassina A, Stordal E, Tartan JP, Tarraga L, Tesi N, Thalamuthu A, Thomas T, Tosto G, Traykov L, Tremolizzo L, Tybjaerg-Hansen A, Uitterlinden A, Ullgren A, Ulstein I, Valero S, Valladares O, Broeckhoven CV, Vance J, Vardarajan BN, van der Lugt A, Dongen JV, van Rooij J, van Swieten J, Vandenberghe R, Verhey F, Vidal JS, Vogelgsang J, Vyhnalek M, Wagner M, Wallon D, Wang LS, Wang R, Weinhold L, Wiltfang J, Windle G, Woods B, Yannakoulia M, Zare H, Zhao Y, Zhang X, Zhu C, Zulaica M, Eadb, Gr@Ace, Degesco, Eadi, Gerad, Demgene, FinnGen, Adgc, Charge, Farrer LA, Psaty BM, Ghanbari M, Raj T, Sachdev P, Mather K, Jessen F, Ikram MA, de Mendonca A, Hort J, Tsolaki M, Pericak-Vance MA, Amouyel P, Williams J, Frikke-Schmidt R, Clarimon J, Deleuze JF, Rossi G, Seshadri S, Andreassen OA, Ingelsson M, Hiltunen M, Sleegers K, Schellenberg GD, van Duijn CM, Sims R, van der Flier WM, Ruiz A, Ramirez A, Lambert JC (2022) New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat Genet* **54**, 412-436.

[28] Dafnis I, Raftopoulou C, Mountaki C, Megalou E, Zannis VI, Chroni A (2018) ApoE isoforms and carboxyl-terminal-truncated apoE4 forms affect neuronal BACE1 levels and Abeta production independently of their cholesterol efflux capacity. *Biochem J* **475**, 1839-1859.

[29] Deane R, Sagare A, Hamm K, Parisi M, Lane S, Finn MB, Holtzman DM, Zlokovic BV (2008) apoE isoform-specific disruption of amyloid beta peptide clearance from mouse brain. *J Clin Invest* **118**, 4002-4013.

[30] Maezawa I, Jin LW, Woltjer RL, Maeda N, Martin GM, Montine TJ, Montine KS (2004) Apolipoprotein E isoforms and apolipoprotein AI protect from amyloid precursor protein carboxy terminal fragment-associated cytotoxicity. *J Neurochem* **91**, 1312-1321.

[31] Narayan P, Orte A, Clarke RW, Bolognesi B, Hook S, Ganzinger KA, Meehan S, Wilson MR, Dobson CM, Klenerman D (2011) The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1-40) peptide. *Nat Struct Mol Biol* **19**, 79-83.

[32] Oda T, Wals P, Osterburg HH, Johnson SA, Pasinetti GM, Morgan TE, Rozovsky I, Stine WB, Snyder SW, Holzman TF (1995) Clusterin (apoJ) alters the aggregation of amyloid beta-peptide (A beta 1-42) and forms slowly sedimenting A beta complexes that cause oxidative stress. *Exp Neurol* **136**, 22-31.

[33] Lionel AC, Crosbie J, Barbosa N, Goodale T, Thiruvahindrapuram B, Rickaby J, Gazzellone M, Carson AR, Howe JL, Wang Z, Wei J, Stewart AFR, Roberts R, McPherson R, Fiebig A, Franke A, Schreiber S, Zwaigenbaum L, Fernandez BA, Roberts W, Arnold PD, Szatmari P, Marshall CR, Schachar R, Scherer SW (2011) Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. *Sci Transl Med* **3**, 95ra75.

[34] Nava C, Keren B, Mignot C, Rastetter A, Chantot-Bastaraud S, Faudet A, Fonteneau E, Amiet C, Laurent C, Jacquette A, Whalen S, Afenjar A, Périsse D, Doummar D, Dorison N, Leboyer M, Siffroi J-P, Cohen D, Brice A, Héron D, Depienne C (2014) Prospective diagnostic analysis of copy number variants using SNP microarrays in individuals with autism spectrum disorders. *European journal of human genetics: EJHG* **22**, 71-78.

[35] van Daalen E, Kemner C, Verbeek NE, van der Zwaag B, Dijkhuizen T, Rump P, Houben R, van 't Slot R, de Jonge MV, Staal WG, Beemer FA, Vorstman JAS, Burbach JPH, van Amstel HKP, Hochstenbach R, Brilstra EH, Poot M (2011) Social Responsiveness Scale-aided analysis of the clinical impact of copy number variations in autism. *Neurogenetics* **12**, 315-323.

[36] Chatterjee M, Del Campo M, Morrema THJ, de Waal M, van der Flier WM, Hoozemans JJM, Teunissen CE (2018) Contactin-2, a synaptic and axonal protein, is reduced in cerebrospinal fluid and brain tissue in Alzheimer's disease. *Alzheimers Res Ther* **10**, 52.

[37] Oguro-Ando A, Zuko A, Kleijer KTE, Burbach JPH (2017) A current view on contactin-4, -5, and -6: Implications in neurodevelopmental disorders. *Mol Cell Neurosci* **81**, 72-83.

[38] Mohebiany AN, Harroch S, Bouyain S (2014) New insights into the roles of the contactin cell adhesion molecules in neural development. *Advances in Neurobiology* **8**, 165-194.

[39] Kleijer KTE, van Nieuwenhuize D, Spierenburg HA, Gregorio-Jordan S, Kas MJH, Burbach JPH (2018) Structural abnormalities in the primary somatosensory cortex and a normal behavioral profile in Contactin-5 deficient mice. *Cell Adh Migr* **12**, 5-18.

[40] Li H, Takeda Y, Niki H, Ogawa J, Kobayashi S, Kai N, Akasaka K, Asano M, Sudo K, Iwakura Y, Watanabe K (2003) Aberrant responses to acoustic stimuli in mice deficient for neural recognition molecule NB-2. *Eur J Neurosci* **17**, 929-936.

[41] Ashrafi S, Betley JN, Comer JD, Brenner-Morton S, Bar V, Shimoda Y, Watanabe K, Peles E, Jessell TM, Kaltschmidt JA (2014) Neuronal Ig/Caspr recognition promotes the formation of axoaxonic synapses in mouse spinal cord. *Neuron* **81**, 120-129.
[42] Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. *Science* **298**, 789-791.
[43] Baeriswyl T, Stoeckli ET (2008) Axonin-1/TAG-1 is required for pathfinding of granule cell axons in the developing cerebellum. *Neural Dev* **3**, 7.
[44] Wolman MA, Sittaramane VK, Essner JJ, Yost HJ, Chandrasekhar A, Halloran MC (2008) Transient axonal glycoprotein-1 (TAG-1) and laminin-alpha1 regulate dynamic growth cone behaviors and initial axon direction in vivo. *Neural Dev* **3**, 6.
[45] Peng YR, Tran NM, Krishnaswamy A, Kostadinov D, Martersteck EM, Sanes JR (2017) Satb1 Regulates Contactin 5 to Pattern Dendrites of a Mammalian Retinal Ganglion Cell. *Neuron* **95**, 869-883 e866.
[46] Poirier J (1994) Apolipoprotein E in animal models of CNS injury and in Alzheimer's disease. *Trends Neurosci* **17**, 525-530.
[47] Poirier J, Baccichet A, Dea D, Gauthier S (1993) Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. *Neuroscience* **55**, 81-90.
[48] Zarow C, Victoroff J (1998) Increased apolipoprotein E mRNA in the hippocampus in Alzheimer disease and in rats after entorhinal cortex lesioning. *Exp Neurol* **149**, 79-86.
[49] Champagne D, Rochford J, Poirier J (2005) Effect of apolipoprotein E deficiency on reactive sprouting in the dentate gyrus of the hippocampus following entorhinal cortex lesion: role of the astroglial response. *Exp Neurol* **194**, 31-42.
[50] White F, Nicoll JA, Roses AD, Horsburgh K (2001) Impaired neuronal plasticity in transgenic mice expressing human apolipoprotein E4 compared to E3 in a model of entorhinal cortex lesion. *Neurobiol Dis* **8**, 611-625.

Manuscript 3: Characterization of the protective CLU rs11136000 T variant and the clusterin protein throughout the spectrum of Alzheimer's disease

Marina Tedeschi Dauar[1,2,3,4], Cynthia Picard[1,2], Henrik Zetterberg[5,6,7,8,9,10], Ann Brinkmalm [6], Kaj Blennow [6,11,12,13], Sylvia Villeneuve[1,2,3,14], Judes Poirier*[1,2,3,14], for the PREVENT-AD Research Group.

1. Douglas Mental Health University Institute, 6875 Boulevard LaSalle, Montreal, QC, Canada, H4H 1R3
2. Centre for the Studies in the Prevention of Alzheimer's Disease, 6875 Lasalle boulevard, Montreal, QC, Canada, H4H 1R3
3. McGill University, 845 Sherbrooke St W, Montreal, Quebec, Canada, H3A 0G4
4. CAPES Foundation, Ministry of Education of Brazil, Brasília - DF 70040-020, Brazil
5. Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
6. Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden
7. Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK
8. UK Dementia Research Institute at UCL, London, UK
9. Hong Kong Center for Neurodegenerative Diseases, Clear Water Bay, Hong Kong, China
10. Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, USA
11. Inst. of Neuroscience and Physiology, University of Gothenburg, Mölndal, Sweden
12. Paris Brain Institute, ICM, Pitié-Salpêtrière Hospital, Sorbonne University, Paris, France
13. Neurodegenerative Disorder Research Center, Division of Life Sciences and Medicine, and Department of Neurology, Institute on Aging and Brain Disorders, University of Science and Technology of China and First Affiliated Hospital of USTC, Hefei, P.R. China
14. Department of Psychiatry, McGill University, 1033 Pine Ave West, Montreal, Quebec, Canada, H3A 1A1

* Correspondence to: Dr. Judes Poirier, C.Q., Deputy Director, Centre for the Studies in the Prevention of Alzheimer's disease, Douglas Mental Health University Institute, 6875 Lasalle, Montréal, H4H 1R3, Canada, Tel.: 514-761-6131 Ext. 6153, Fax.: 514-888-4094, judes.poirier@mcgill.ca.

Submitted for publication at Biochemical Biophysical Research Communication ®

1. Preface:

The GWAS performed in the QFP cohort identified twelve variants associated with AD. One of these variants was the rs11136000 T variant in the *CLU* gene. Although the *CLU* gene is a well-known genetic risk factor in the Caucasian population (34, 117) and the rs11136000 T variant has already been described as a protective variant (34, 135) the mechanism by which the *CLU* gene, its rs11136000 T polymorphism and the clusterin protein are involved in AD pathophysiology is not fully understood.

In this chapter we examine the role of the *CLU* rs11136000 T variant and the clusterin protein throughout the AD spectrum in different population cohorts to gain further insights into its role as a protective variant in AD

In this study, we investigate the association of the *CLU* rs11136000 T polymorphism and the clusterin protein with AD biomarkers, synaptic markers, disease progression and cognition in a cohort of cognitively unimpaired subjects at high risk for AD. Then, to explore the effect of this *CLU* variant and its protein in the late stages of the disease, we used autopsied brains from the QFP and the ROSMAP cohorts to assess the effect of the variant and the diagnosis of AD on gene expression and protein levels.

2. Abstract:

Introduction: The Clusterin (*CLU*) gene is one of the main genetic risk factors for Alzheimer's disease (AD) and the clusterin protein is associated with neuroprotection and cholesterol transport. However, the precise role of *CLU* and clusterin in AD is poorly understood. Our objective is to study the *CLU* protective variant rs11136000 T and the clusterin protein in different phases of the disease to better understand their role in the pathophysiology of AD.

Methods: Three different cohorts were used, encompassing the presymptomatic phase and the latest stage of AD. All subjects were genotyped for *APOE* and *CLU*. CSF clusterin, AD biomarkers, synaptic proteins, amyloid and tau PET were available for the cognitively unimpaired individuals. CLU mRNA and protein levels were measured in brain samples of AD and control subjects.

Results: Clusterin CSF levels were positively associated with CSF AD biomarkers, synaptic markers and tau deposition. There was no effect of the protective rs11136000 T on AD biomarkers, synaptic markers or clusterin protein levels at this early stage. In the autopsied cases, cortical clusterin mRNA and protein levels were increased in rs11136000 T carriers and in AD subjects, particularly in APOE-ε4 carriers.

Conclusion: The *CLU* rs11136000 T variant and the clusterin protein are associated with AD. Rs11136000 T likely decreases the risk of AD by increasing gene expression of its neuroprotective protein. The role of *CLU* rs11136000 T and clusterin are more prominent in the late stages of AD, particularly in APOE-ε4 carriers where it may act by restoring deficient cholesterol transport.

Keywords: Alzheimer's disease, Clusterin, *CLU*, apolipoproteins, *APOE*, APOE4, biomarkers, gene expression.

3. Introduction:

Clusterin (CLU), also known as apolipoprotein J, TRPM-2 and pADHC-9, is a glycoprotein widely expressed in the brain and in peripheral tissues (1, 2). It has a broad scope of function in normal physiology acting on cholesterol transport (3, 4), control of apoptosis (5, 6), developmentally regulated programmed cell death (7), protection against oxidative stress (8, 9) and as a molecular chaperone (10). Due to its ubiquitous presence and multiple functions, clusterin has been implicated in several different diseases (11-17), with a particular focus on Alzheimer's disease (AD) (2, 18).

Genome Wide Association Studies (GWAS) have actually identified a number of different variants in the *CLU* gene as associated with the risk/protection of AD (18-22), and *CLU* is now considered one of the most important genetic risk factors for this dementia (23). But while this association is well established in Caucasian populations (18, 21), it is less consistent in other ethnicities such as Asians (24-26), Hispanics (22, 27) and populations of African descent (22). The variant most often associated with AD is the rs11136000, with the T allele being protective against the disease (28).

Although the *CLU* gene is well accepted as a risk factor for AD, the precise role of its variants and of the clusterin protein in the pathophysiology of the disease is still poorly understood. Some studies have suggested that clusterin acts on Aß aggregation and clearance (29-31), while others have proposed that it plays a neuroprotective role by regulating neuroinflammation (32) and reducing oxidative stress (33). Clusterin is also considered as one of the main cholesterol transporters in the central nervous system (CNS), where it binds extracellular lipid complexes with APOE and APOA1 to form HDL-like particles that deliver cholesterol and phospholipids to

neuronal membranes expressing relevant cell-surface receptors (34). Previous research suggested that alterations in these apolipoproteins disrupt cholesterol transport and impairs the CNS's compensatory response to damage, which is highly detrimental in the context of a pathological process such as AD (35-38).

This research aims to study the *CLU* rs11136000 T variant and the clusterin protein in different phases of the AD spectrum. Our goal is to provide more insights into the role of this important genetic protective factor and its protein in the pathophysiology of AD before and after symptoms emergence.

4. Methods:

4.1 Study populations:

Analyses were performed with data from three different patient populations cohorts: the Pre-symptomatic Evaluation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) cohort, the Quebec Founder Population (QFP) cohort and the Religious Orders Study and Rush Memory and Aging Project (ROSMAP). All procedures were approved by the McGill University Faculty of Medicine and Douglas Hospital Research Centre Institutional Review Boards and complied with the ethical principles of the Declaration of Helsinki.

4.2 PREVENT-AD Cohort:

The PREVENT-AD cohort is composed of cognitively unimpaired individuals who have a first degree relative with AD and therefore are at a higher risk of developing this dementia (39). Over 370 participants are monitored annually with clinical and cognitive assessments, CSF and blood

biomarkers and neuroimaging modalities (structural and functional magnetic resonance imaging (MRI), and amyloid and tau positron emission tomography (PET) scans) (39). Data used in the preparation of this article were obtained from the PREVENT-AD program *(*https://douglas.research.mcgill.ca/stop-ad-centre*),* data release 7.0.

A complete listing of the PREVENT-AD Research Group can be found in the PREVENT-AD database:https://preventad.loris.ca/acknowledgements/acknowledgements.php?date=2024-04-01.

4.2.1 CSF:

Lumbar punctures were performed in a subset of volunteers in the morning following an overnight fast, with a Sprotte 24-gauge atraumatic needle as described in Tremblay-Mercier et al (2021) (40). CSF was centrifuged for 10 minutes at room temperature, cells and insoluble material were excluded, and aliquots were stored at –80°C. AD biomarkers (phosphorylated tau (p-tau)181, total tau (t-tau), and Aß42) were measured according to procedures developed by the BIOMARKAPD consortium, using validated Innotest enzyme-linked immunosorbent assay kits (P(181)-tau Cat.# 81581, T-tau Cat.# 81579, and Aβ42 Cat.# 81583) from Fujirebio (41). Synaptic markers were assessed in the CSF using immunoprecipitation followed by mass spectroscopy as previously described (42-45). Clusterin was quantified in the CSF using enzyme-linked immunosorbent assays (ELISA) (BioVendor R&D, Brno, Czech Republic Cat.# RD194034200R) according to manufacturer's instructions.

4.2.2 Positron emission tomography (PET) acquisition and processing:

PET scans were performed in PREVENT-AD subjects to measure brain amyloid and tau deposition. Aß level was measured by 18F-NAV4694 (Navidea Biopharmaceuticals, Dublin, OH, USA). Scans were acquired 40 to 70 minutes after injection and standardized uptake value ratios

(SUVRs) were obtained using the cerebellum as a reference region (46). Tau accumulation was quantified using Flortaucipir (18F-AV1451; Eli Lilly & Company, Indianapolis, IN, USA). Scans were acquired 80 to 100 minutes post injection and SUVRs were obtained using the inferior cerebellum gray matter as reference (46). Temporal Meta-ROI was calculated by averaging the brain regions that have the highest levels of tau-PET in AD (47). These regions are entorhinal cortex, parahippocampal, inferior temporal, middle temporal, fusiform gyri and amygdalae (47).

4.2.3 DNA extraction and genotyping:

DNA extraction from buffy coat samples was performed using the QIAsymphony DNA mini kit (Qiagen). CLU genotyping was performed by Omni2.5-8 BeadChip (Illumina). PLINK (http://pngu.mgh.harvard.edu/purcell/plink/) was used to filter sex mismatches, missingness at sample level (< 5%) and SNP level (< 5%), SNPs in Hardy–Weinberg disequilibrium (P>0.001) and assess sample heterozygosity. Only post-imputed SNPs with an info score>0.7 were kept. Genotype profiling of ApoE 112/158 single nucleotide polymorphisms (which determine the E2, E3, and E4 isoforms) was performed by PCR followed by pyrosequencing as previously described (35).

4.2.4 Alzheimer Progression Score (APS):

The Alzheimer Progression Score (APS) is a composite score that incorporates multimodal neuroimaging, neurosensory, cognitive, and CSF biomarkers in order to measure disease progression in the absence of visible cognitive deficits (due to the fact that PREVENT-AD subjects were cognitively unimpaired at enrollment). Scores are scaled as a standard normal distribution, with higher scores denoting increasing severity. The APS score is described in detail by Leoutsakos et al. (48).

4.2.5 The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS):

In the PREVENT-AD cohort, cognitive status is assessed and followed by RBANS scale. RBANS was developed with the purpose of detecting and characterizing early dementia in older adults as well as in younger individuals (49). RBANS tests assess five cognitive domains: immediate memory, visuospatial/constructional skills, attention, language, and delayed memory. Scores are calculated individually for each cognitive domain and then combined to provide a total score. The development and validation of the RBANS is described in detail by Randolph et al (1998) (49).

4.3 The Quebec Founder Population (QFP) Cohort:

The QFP cohort is a population isolate from eastern Canada that descends from the French settlers who founded Nouvelle France in the 17th and 18th centuries. This population is characterized by low genetic heterogeneity, large linkage disequilibrium blocks and low genetic noise which is a result of a founder effect, created by the migration and the isolated nature of the initial settlements (50). QFP human brain tissue was obtained from the Douglas Bell Canada Brain Bank. Histopathological diagnosis of AD was performed according to the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association) criteria (51).

4.3.1 DNA extraction and quality control:

DNA extraction and allele determination for *APOE* and *CLU* were performed following the same protocol described above for the PREVENT-AD cohort.

4.3.2 RNA extraction and quality control and microarray:

RNA was extracted from frontal cortex tissues using the Maxwell® 16 Tissue LEV Total RNA Purification Kit (Promega, WI, USA) on a Maxwell® 16 LEV Instrument (Promega, WI, USA). cDNA was obtained by reverse transcription on a Multigene thermocycler (Labnet International Inc.) using the high-capacity cDNA RT kit (Applied Biosystems, CA, USA) and 200ng of total RNA. Purity and integrity of extracted RNA samples were estimated using, respectively, the ratio of absorbance values at 260 nm and 280 nm evaluated on a Biotek Synergy H1 reader (Fisher Scientific, ON, Canada) and RNA integrity number (RIN) determined with a Bio-Rad's Experion instrument (Bio-Rad, CA, USA). Gene-level expressions of *CLU* was assessed with the Applied Biosystem Clariom D microarray (Applied Biosystem, USA) according to the manufacturer's protocols.

4.3.3 Clusterin protein levels in the frontal cortex:

Frontal cortex samples were homogenized mechanically with the Bead Ruptor 24 (Omni International, Tulsa, OK, USA) in ice-cold phosphate buffered saline with protease inhibitors. After 2 freeze-thaw cycles, homogenates were centrifuged at 4°C at 5000 rpm for 5 minutes and supernatants were frozen at -80°C until further use. Total protein concentrations were measured with the bicinchoninic acid assay (Thermo Fisher). Clusterin levels were assessed by ELISA (USA R&D Systems, Minneapolis, MN, USA, cat# DCLU00) according to manufacturer's instructions.

4.4 The ROSMAP cohort:

The ROSMAP cohort is composed of the Religious Orders Study (ROS) which includes nuns, priests, and brothers from across the United States and The Rush Memory and Aging Project (MAP) that includes lay people from the state of Illinois (52). Participants were initially

cognitively normal and were followed annually with neuropsychological evaluation and blood tests and consented to genotype and brain donation (52).

4.4.1 Cognitive assessment and diagnosis:

Cognitive assessment was performed annually using the Mini Mental State Evaluation (MMSE). Clinical diagnosis was performed based solely on clinical assessment, blinded to postmortem data. Participants were classified as: no cognitive impairment, Mild cognitive impairment (MCI) (one impaired domain) with no other cause of cognitive impairment, MCI (one impaired domain) with another cause of cognitive impairment, AD with no other cause of cognitive impairment (NINCDS Probable AD), AD with another cause of cognitive impairment (NINCDS possible AD) and other dementias.

4.4.2 Genotype:

Genotyping was performed using Affymetrix or the Illumina Omniquad express gene chips either on peripheral blood mononuclear cells or on frozen brain tissues. Imputation was performed by Sanger Imputation Service as described for PREVENT-AD and using the same quality control filters.

4.4.3 RNA-Sequencing data:

Consolidated RNA-Seq data from the dorsolateral prefrontal cortex is available at https://www.radc.rush.edu/home.htm. The Broad Institutes' Genomics Platform performed RNA-Seq library preparation using the strand specific dUTP method with poly-A selection. Sequencing was performed on the Illumina HiSeq. Quantile normalization method was applied to FPKM first, and combat package was used to remove potential batch effect.

4.4.4 CLU Protein measurements:

Protein levels were measured in the prefrontal cortex by a mass spectrometry-based protein quantification approach using isobaric multiplex tandem mass tags (TMT) as described previously by Ping et al. (53). Briefly, TMT labeling with synchronous precursor selection (SPS)-MS3 for reporter ion quantitation was used to achieve comprehensive global quantitation of 100 mg (wet tissue weight) pre-frontal cortex from healthy controls and AD cases. In total, 127,321 total unique peptides were identified from >1.5 million peptide spectral matches (PSMs), which mapped to 11,840 unique proteins groups, representing 10,230 gene symbols, that map to ≈65% of the protein coding genes in the brain. Here, we used P10909 which is the main full-length clusterin isoform.

4.5 Statistical methods:

The differences in sex and APOE-ε4 presence between diagnostic groups was assessed using chi-square tests. The difference in age between AD and controls for the QFP cohort was calculated using independent samples t-test. For the ROSMAP cohort, age was divided in four groups (<80, 80-84, 85-89, ≥90) since the precise age above 90 years old is not available.

Associations between clusterin level and other variants (CSF and PET biomarkers, synaptic proteins, RBANS and APS) were assessed by linear regression models corrected for age, sex and the presence of one or two allele of APOE-ε4. To assess the effect of either genotype or diagnosis on clusterin mRNA and protein levels ANOVA was used for normally distributed variants (corrected for age, sex and APOE-ε4 presence) and the Mann-Whitney U test for independent samples for variants that were not normally distributed. Significance level was considered at $p<0.05$.

5. RESULTS:

5.1 Demographics:

Table 5 summarizes the demographic characteristics of the cohorts used. In the QFP cohort there were no differences in the proportion of males and females or in age between diagnostic groups, but AD subjects were more likely to be APOE-ε4 positive (p = 0.012). In the ROSMAP cohort there was no difference in the proportion of males and females between diagnostic groups, but AD subjects were more likely to be older (>90 age group, $p=2.8x10^{-11}$) and APOE-ε4 carriers ($p=1.4x10^{-8}$).

Table 5: Demographics table

	PREVENT-AD	QFP			ROSMAP					
	n=117	AD n=56	CTL n=31	p		AD n=470	MCI n=251	CTL n=333	OTHERS n=20	p
Sex				0.5						0.305
Female	82 (70%)	24 (43%)	11 (35.5%)			325 (69%)	158 (63%)	216 (65%)	12 (60%)	
Male	35 (30%)	32 (57%)	20 (64.5%)			145 (31%)	93 (37%)	117 (35%)	8 (40%)	
APOE e4				0.012						$1.4x10^{-8}$
APOE e-	67 (57%)	24 (43%)	22 (71%)			302 (64%)	195 (78%)	277 (83%)	13 (65%)	
APOE e+	50 (43%)	32 (57%)	9 (29%)			168 (36%)	56 (22%)	56 (17%)	7 (35%)	
Age (years) mean ± SD	62.8 ± 5.3	80.71 ± 6.3	77.39 ± 11.4	0.14	**Age groups:**					$2.8x10^{-11}$
					<80	14 (3%)	16 (6%)	48 (14.4%)	4 (20%)	
					80-84	55 (12%)	42 (17%)	61 (18.3%)	3 (15%)	
					85-89	121 (26%)	69 (27.5%)	104 (31.2%)	5 (25%)	
					>90	280 (59%)	124 (49.5%)	120 (36.1%)	8 (40%)	

Abbreviations: APOE, apolipoprotein E; AD, Alzheimer's disease; MCI, Mild cognitive impairment; CTL, controls; SD, standard deviation; PREVENT-AD, Pre-symptomatic Evaluation of Experimental or Novel Treatments for Alzheimer's Disease; QFP, Quebec Founder Population; ROSMAP, Religious Orders Study Rush Memory and Aging Project.

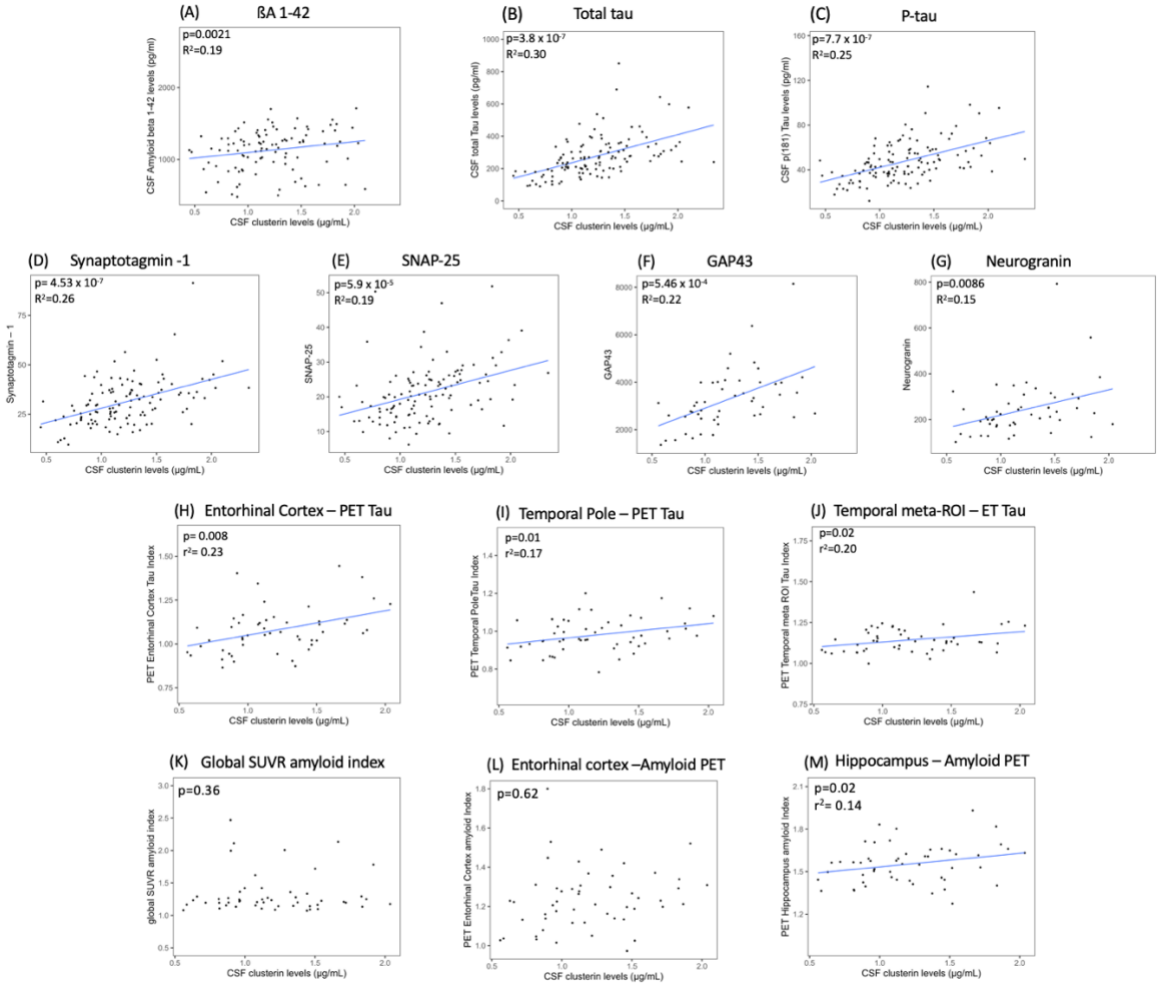
5.2 Associations between CSF Clusterin levels, CSF and PET AD biomarkers and synaptic proteins in the asymptomatic PREVENT-AD cohort.

CSF Clusterin levels were contrasted with the main CSF AD biomarkers, amyloid and tau deposition measured by PET and CSF synaptic proteins in cognitively unimpaired subjects at high risk for AD (Fig 19). Clusterin protein level was positively associated with Aß1-42 ($p=0.0021$, $r^2=0.19$), t-tau ($p=3.8 \times 10^{-7}$ $r^2=0.30$) and p-tau ($p=7.7 \times 10^{-7}$ $r^2=0.25$) in the CSF. We also observed positive correlation between clusterin and the synaptic markers synaptotagmin-1 ($p= 4.53 \times 10^{-7}$ $r^2=0.26$), SNAP-25 ($p=5.9 \times 10^{-5}$ $r^2=0.19$), GAP43 ($p=5.46 \times 10^{-4}$ $r^2=0.22$) and neurogranin ($p=0.0086$ $r^2=0.15$) in the CSF. CSF clusterin was positively correlated with tau deposition measured by PET in the entorhinal cortex ($p= 0.008$, $r^2= 0.23$), temporal pole ($p=0.01$, $r^2=0.17$) and the tau meta-ROI index ($p=0.02$, $r^2=0.20$). Correlations between CSF clusterin and amyloid deposition were only observed in the hippocampus ($p=0.02$, $r^2=0.14$). There were no correlations between CSF clusterin and amyloid deposition in the entorhinal cortex ($p=0.62$) or global SUVR amyloid index ($p=0.36$) in these asymptomatic "at-risk" PREVENT-AD subjects.

To evaluate the effect of APOE-ε4 on the correlations between CSF clusterin and AD biomarkers and synaptic proteins, we stratified the correlations between APOE-ε4 carriers and non-carriers (Fig. 20). For APOE-ε4 positive subjects, positive correlations were seen between CSF clusterin and: t-tau ($p= 0.01$ $r^2= 0.19$), p-tau ($p=0.003$, $r^2=0.21$), synaptotagmin-1 ($p=2.5 \times 10^{-3}$ $r^2=0.23$), PET temporal pole tau index ($p=0.02$, $r^2=0.32$) and PET temporal meta-ROI Tau index ($p=0.002$, $r^2=0.6$). For APOE-ε4 negative subjects, positive correlations were observed between CSF clusterin and: ßA1-42 ($p=0.005$, $r^2=0.12$), t-tau ($p=2.75 \times 10^{-6}$, $r^2=0.39$), p-tau ($p=1.5 \times 10^{-4}$ $r^2=0.23$), synaptotagmin-1 ($p=1.97 \times 10^{-4}$ $r^2=0.23$), SNAP-25 ($p=8.16 \times 10^{-5}$ $r^2=0.27$), GAP43

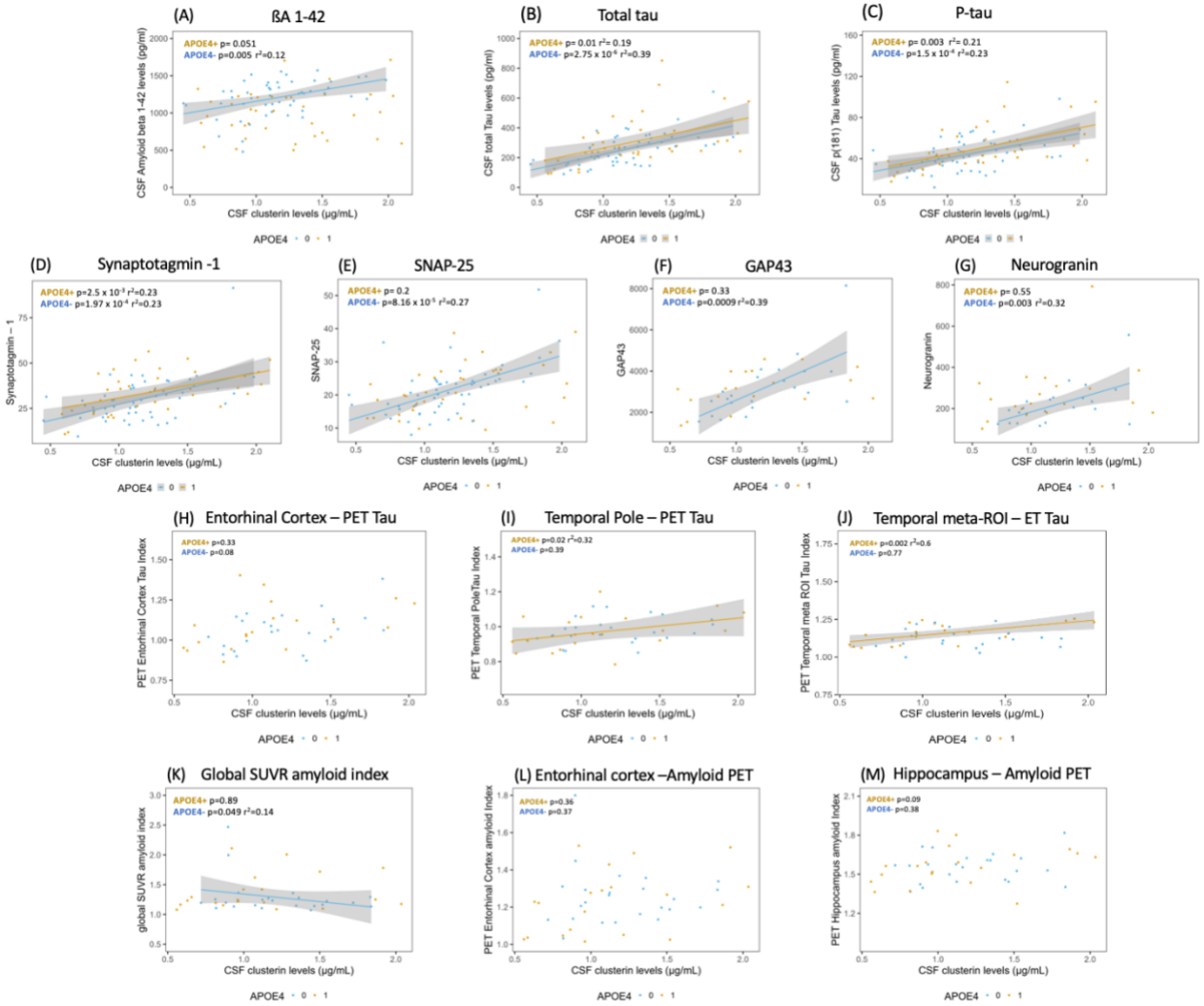
(p=0.0009 r^2=0.39) and neurogranin (p=0.003 r^2=0.32). CSF clusterin negatively correlated with global SUVR amyloid index (p=0.049 r^2=0.14).

Figure 19: Associations between CSF Clusterin and: CSF Biomarkers, PET Biomarkers and synaptic proteins in the PREVENT-AD cohort.



Clusterin protein was measured in the CSF using ELISA. CSF AD biomarkers Aß 1-42 (A, n=100), total tau (B, n=119) and p-tau (C, n=119) were measured by enzyme-linked immunosorbent assay according to the procedures from the BIOMARKAPD. The synaptic markers synaptotagmin-1 (D, n=115), SNAP-25 (E, n=117), GAP43 (F, n=49) and neurogranin (G, n=49) were quantified using selective reaction monitoring mass spectroscopy. Tau deposition was measured with flortaucipir PET: entorhinal cortex (H, n=52), temporal pole (I, n=52) and temporal meta-ROI (J, n=52). Amyloid was measured with [18F]NAV4694 PET: global SUVR amyloid index (K, n=52), entorhinal cortex (L, n=52) and hippocampus (M, n=52). Significant linear regressions are represented with a blue fitted line. R squares and p values are shown in the top left corners of each figure. Analyses were adjusted for age, sex, and APOE-ε4 presence.

Figure 20: Associations between CSF Clusterin and: CSF Biomarkers, PET Biomarkers and synaptic proteins in the PREVENT-AD cohort, divided by the presence and absence of the APOE-ε4 allele.

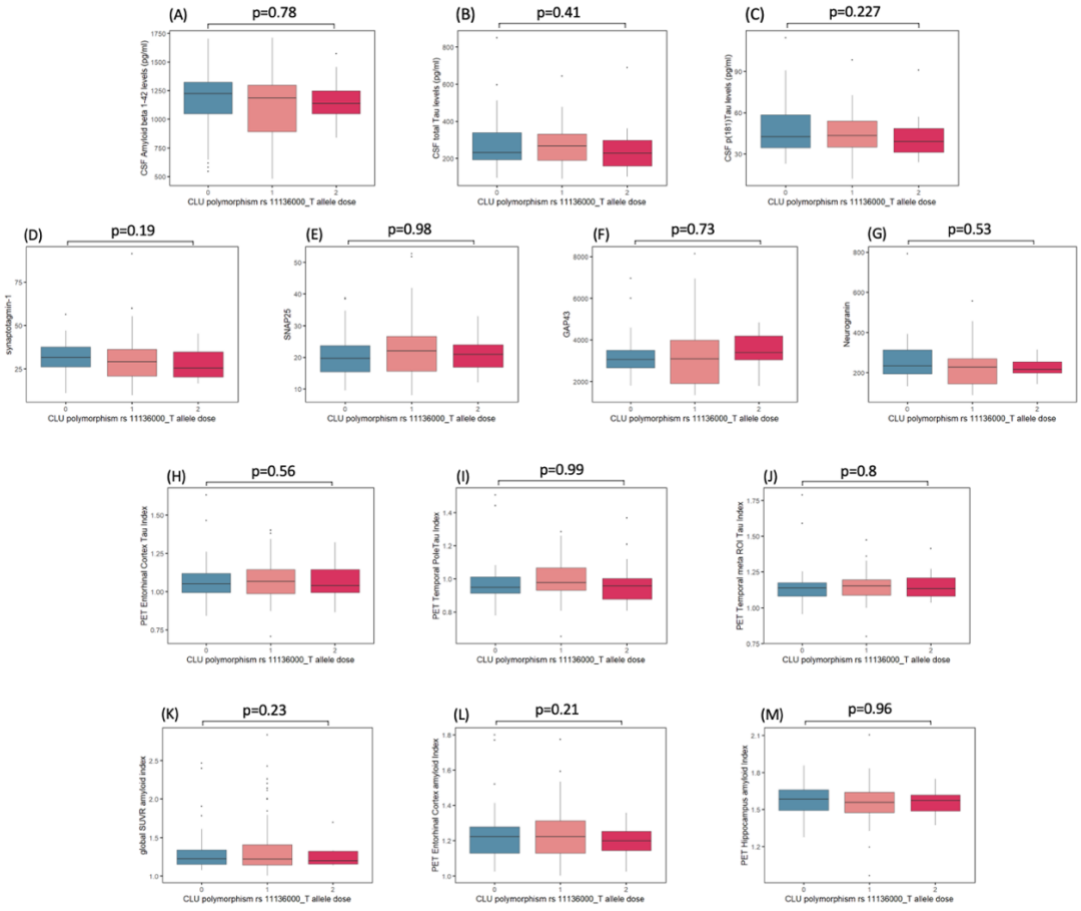


Clusterin protein was measured in the CSF using ELISA. CSF AD biomarkers Aß 1-42 (A, n: APOE-ε4- = 51, APOE-ε4+ = 38), total tau (B, n: APOE-ε4- = 53, APOE-ε4+ = 39) and p-tau (C, n: APOE-ε4- = 53, APOE-ε4+ = 39) were measured by enzyme-linked immunosorbent assay according to the procedures from the BIOMARKAPD. The synaptic markers synaptotagmin-1 (D, n: APOE-ε4- = 53, APOE-ε4+ = 36), SNAP-25 (E, n: APOE-ε4- = 52, APOE-ε4+ = 38), GAP43 (F, n: APOE-ε4- = 22, APOE-ε4+ = 19) and neurogranin (G, n: APOE-ε4- = 22, APOE-ε4+ = 19) were quantified using selective reaction monitoring mass spectroscopy. Tau deposition was measured with flortaucipir PET: entorhinal cortex (H, n: APOE-ε4- = 24, APOE-ε4+ = 20), temporal pole (I, n: APOE-ε4- = 24, APOE-ε4+ = 20) and temporal meta-ROI (J, n: APOE-ε4- = 24, APOE-ε4+ = 20). Amyloid index was measured with [18F]NAV4694 PET: global SUVR amyloid index (K, n: APOE-ε4- = 24, APOE-ε4+ = 20), entorhinal cortex (L, n: APOE-ε4- = 24, APOE-ε4+ = 20) and hippocampus (M, n: APOE-ε4- = 24, APOE-ε4+ = 20). Significant linear regressions are represented with a gray confidence region of the fitted line. R squares and p values are shown in the top left corners of each figure. Analyses were adjusted for age and sex.

5.3 Effect of CLU rs11136000 genotype on CSF Biomarkers, PET Biomarkers and synaptic proteins in the PREVENT-AD cohort.

In the cognitively unimpaired subjects of the PREVENT-AD cohort we did not see any effect of the *CLU* rs11136000 T genotype on CSF biomarkers, PET biomarkers or CSF synaptic proteins (Figure 21).

Figure 21: Effect of CLU rs 11136000 T genotype on CSF biomarkers, PET biomarkers and synaptic proteins.



Associations between *CLU* rs 11136000 T genotype and CSF Aß 1-42 (A, n=129), total tau (B, n=132), p-tau (C, n=132), synaptotagmin-1 (D, n=123), SNAP-25 (E, n=129), GAP43 (F, n=54) and neurogranin (G, n=53). Associations between *CLU* rs 11136000 T genotype and tau deposition in the entorhinal cortex (H, n=115), tau deposition in the temporal pole (I, n=115), temporal meta-ROI tau index (J, n=115), global SUVR amyloid index (K, n=115), entorhinal cortex amyloid deposition (L, n=115) and amyloid deposition in the hippocampus (M, n=115) measured by PET. Analyses were adjusted for age, sex, and APOE-ε4 presence.

5.4 Effect of time and genotype on CSF clusterin levels in PREVENT-AD:

We did not observe any change in CSF clusterin levels over time or as a function of *CLU* rs11136000 T allele dose response in the asymptomatic PREVENT-AD cohort (supplemental figure 1).

5.5 CLU genotype, protein levels and clinical assessments in the PREVENT-AD cohort.

Cognitive performance was evaluated using the RBANS scale and disease progression was estimated by the APS progression score (Supplemental figure 2). There was no effect of *CLU* rs11136000 T genotype on RBANS scores at baseline (C). Individuals who are homozygous for the rs11136000 T variant had APS scores 0.46 units higher than non-carriers at 24 months (A, p=0.016). We did not observe any correlation between CSF clusterin levels and RBANS (D) or APS scores (B).

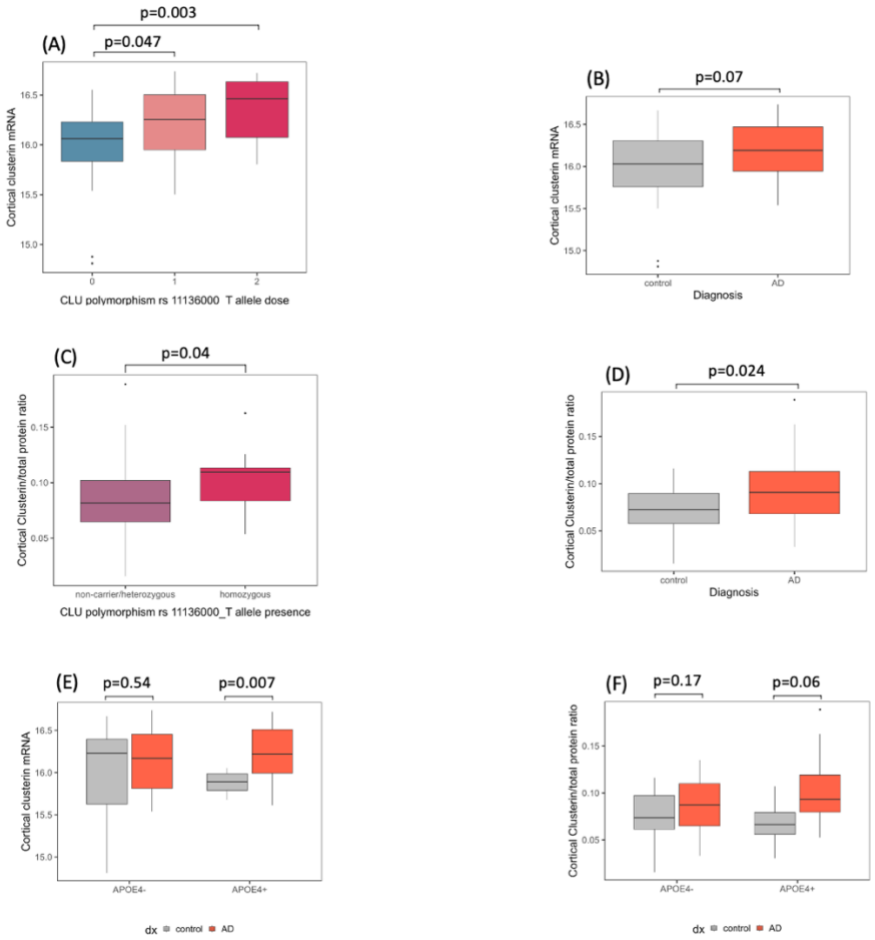
5.6 Effect of genotype and disease on gene expression and protein levels in the autopsied QFP cohort.

CLU mRNA and protein levels were examined in autopsy-confirmed AD subjects and controls from the QFP cohort. mRNA levels are increased in both rs11136000 T heterozygous (p=0.047) and homozygous (p=0.003) subjects compared to controls (Figure 22A). A trend towards elevated mRNA levels was observed in AD subjects compared to controls (p=0.07) (Figure 22B).

Protein levels were also increased in rs11136000 T homozygous subjects compared to a group of non-carriers and heterozygous (p=0.04, Figure 22C) and in AD patients compared to controls (p=0.024, Figure 22D).

Clusterin mRNA levels were found to be significantly higher in the APOE-ε4 positive AD subjects (p=0.007, Figure 22E), with a slight trend towards increased protein levels in the APOE-ε4-positive AD patients (p=0.06, Figure 22F).

Figure 22: Effect of CLU rs 11136000 T variant and AD on gene expression in the QFP cohort.

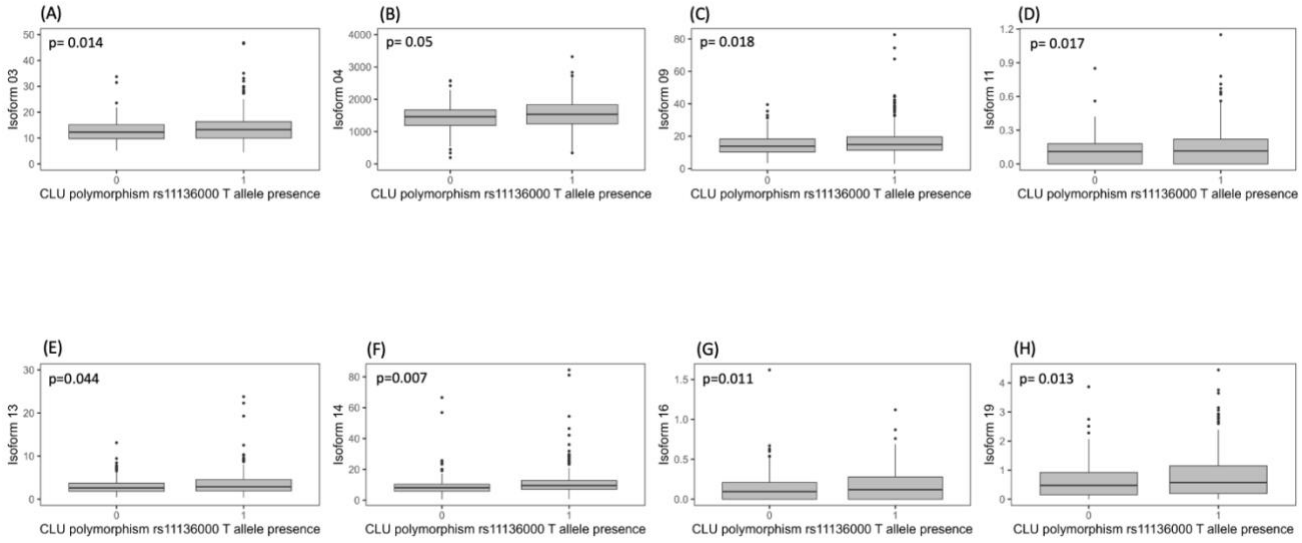


CLU mRNA was measured by microarray and clusterin protein was measured by ELISA in the frontal cortex of autopsied-confirmed AD (n=56) and control (n=31) subjects. Histopathological diagnosis of AD was performed according to the NINCDS-ADRDA criteria. *CLU* mRNA is increased according to genotype (A, heterozygous, p=0.047; homozygous, p=0.003). There was a trend towards increased *CLU* mRNA levels in AD subjects (B, p=0.07). Clusterin levels were increased in homozygous subjects compared to non-carriers and heterozygous (C, p=0.04) and in AD patients (D, p=0.024). When divided by APOE-ε4 allele presence, mRNA (E, p=0.007) and clusterin (p=0.06 – trend) levels were increased only in AD subjects who were APOE-ε4 positive. Analyses were adjusted for age, sex, and APOE-ε4 presence.

5.7 Effect of genotype and disease on CLU gene expression and protein levels in the ROSMAP cohort.

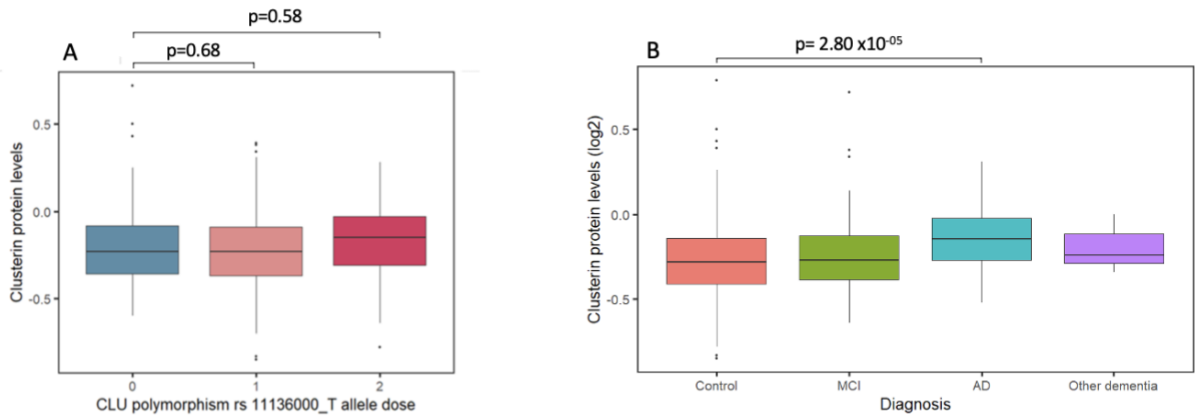
In the ROSMAP cohort, 8 mRNA isoforms were available (isoforms 3, 4, 9, 11, 13, 14, 16 and 19) for analysis. We observed increased mRNA levels in carriers of the rs11136000 T variant for all isoforms except isoform 4 (trend increase p=0.05) (Figure 23). No differences were observed in cortical clusterin levels in this cohort, regardless of the variant's presence (Figure 24A). Cortical clusterin levels were significantly increased in AD subjects compared to controls (p= 2.80 x10^{-05}), but not in MCI (trend only, p=0.08) or subject with other dementias (p=0.4) (Figure 24B).

Figure 23: Effect of CLU rs 11136000 T variant and AD on mRNA level in the ROSMAP cohort.



RNA sequencing on the ROSMAP cohort was performed on the Illumina HiSeq. Rs1136000 T carriers: n=302, rs1136000 T non-carriers: n=170. The presence of the rs11136000T allele was associated with increased levels of mRNA isoforms 03 (A, p=0.014), 09 (C, p=0.018), 11(D, p=0.017), 13(E, p=0.044), 14 (F, p=0.007), 16 (G, p=0.011) and 19 (H, p=0.013). There was a trend association between rs11136000T allele presence and increased levels of mRNA isoform 04 (B, p=0.05). Analyses were adjusted for age, sex, and APOE-ε4 presence.

Figure 24: Effect of CLU rs 11136000 T genotype and clinical diagnosis on clusterin levels



A) There was no effect of *CLU* rs 11136000 T genotype on clusterin levels (A, n=260). B) Clusterin levels were increased in AD (n=106, p= 2.80 x10^{-05}), but not in MCI (n=84, p=0.08) or other dementias (n=7, p=0.4) compared to controls (n=145). Analyses were adjusted for age, sex, and APOE-ε4 presence.

6. Discussion:

Clusterin is a glycoprotein expressed throughout most body tissues with the highest levels being detected in the brain, liver, and testicles (1, 54). In addition to this ubiquitous presence, it also participates in a wide range of physiological processes: clusterin has been shown to be a molecular chaperone (10), to regulate cell survival and apoptosis (5), to protect against oxidative stress (8, 55), to inhibit the complement cascade (32) and to participate in cholesterol transport and mobilization (3, 4). Therefore, it is not surprising that clusterin is involved in several different pathological processes with research suggesting that it promotes tumorigenesis and chemoresistance (11, 13), protects heart cells in different cardiovascular conditions (14, 15) and contributes to neuroprotection in neurological disorders (32, 56).

Clusterin is primarily a secreted protein but can occasionally be found intracellularly. While the pathways of synthesis and secretion of clusterin are known (57), the origins of intracellular clusterin are not clear. Current consensus is that intracellular clusterin is most likely to have exited the secretory pathway at some point or to have re-entered the cell after secretion (57). Different studies have proposed that it could stem from alternative splicing, impairments in trafficking or secretion, or reuptake of the secreted form (57). Although its precise origin is not clear, intracellular clusterin is thought to play an opposite role from the secreted form. Secreted clusterin has been shown to have mostly a protective role that promotes cell survival while intracellular clusterin is thought to induce apoptosis (58).

The presence of clusterin in the CNS and its increased expression in AD was first reported by May et al. 1988 (59) under the name of pADHC-9 using differential cDNA library screenings. Since then, its role in AD has been extensively investigated, and several GWAS confirmed that *CLU* variants are important risk factors of AD (18, 21, 22). The possible roles of clusterin in the pathophysiology of AD include inhibition of Aß aggregation (30, 60), regulation of Aß transport and clearance (29, 31), neuroprotection against oxidative stress (33) and cholesterol transport for membrane and synaptic remodeling (35, 38). However, the precise mechanism of action of the *CLU* gene, its variants and the clusterin protein in the disease are not completely established, particularly when considering the different phases of the disease.

We have assessed CSF clusterin level and its association with different AD biomarkers and synaptic proteins in cognitively unimpaired subjects at high risk for AD. We have found that CSF clusterin is strongly associated with CSF Aß, t-tau, p-tau, synaptic proteins and with tau deposition

measured by PET. However, in the preclinical phase of the disease we did not find change in CSF clusterin levels over time, and the rs11136000 T genotype was not associated with alterations in AD biomarkers, synaptic protein, cognitive measurements nor with CSF clusterin levels per se. These findings are in agreement with previous reports in the literature where CSF clusterin has been found to associate with AD biomarkers (61, 62) and synaptic proteins (63). Furthermore, CSF clusterin levels measured throughout the AD spectrum were found to be decreased in the early stages of the disease (62, 64) and increase in the presence of tau pathology and neurodegeneration (62, 65).

Our findings align with existing literature, confirming the role of clusterin in Alzheimer's disease and its connections to AD pathology and synaptic dysfunction. However, they also suggest that the role of clusterin changes throughout the disease spectrum. It is somewhat modest in the preclinical phase of the disease but becomes more significant in the late AD stages. Participants of the PREVENT-AD cohort, although at higher risk for AD, were recruited while still cognitively unimpaired, often over a decade younger than the age of disease onset in their first degree relative. Therefore, although AD pathology begins one to two decades before the onset of clinical symptoms, it is possible that the individuals in the PREVENT-AD cohort were still too early in the disease spectrum for any substantial influence of the *CLU* variant to be detected or changes in CSF clusterin concentrations to be seen.

Further investigations of rs11136000 T variant and clusterin protein were performed in the late stages of the disease using autopsy-confirmed AD and control subjects from two different post-mortem cohorts, QFP and ROSMAP. In both cohorts, we observed that the rs1136000 T variant is

associated with increased brain CLU mRNA levels in cortical areas. In the ROSMAP cohort, the rs11136000 T variant is associated with increased levels of all mRNA isoforms except isoform 4. Rs11136000 T variant is also associated with increased cortical clusterin protein levels in the QFP cohort. As reported before (2), cortical clusterin levels are significantly increased in AD patients compared to controls in both cohorts.

These changes found in the later symptomatic stages of the disease are quite relevant. Most evidence suggests that clusterin protein and the CLU rs11136000T variant play a protective role in AD (28, 66). However, the precise roles of the protein and of the variant have not been established. Here, we show that clusterin is elevated in AD in the final stages of the disease, and that this elevation likely occurs due to increases in gene expression as suggested before in a pilot study (67).

In the QFP cohort, stratification by APOE-ε4 presence shows that clusterin and *CLU* mRNA increases are restricted to the APOE-ε4 carriers, consistent with previous report in brain tissue (68). Clusterin and apolipoprotein E are the most important cholesterol transporters in the CNS. In the extracellular space, they interact with lipoproteins to produce HDL-like particles. These particles transport cholesterol and phospholipids crucial for neuronal membrane assembly, terminal and synaptic proliferation, and repair (38, 69). APOE-ε4, the most important genetic risk factor for AD, is associated with lower levels of apolipoprotein E in the brain and with less effective cholesterol transport (68, 70, 71). It has been shown that the gene expression of apolipoprotein E and clusterin increase in the hippocampus of mice one to two weeks after the initial insult in response to entorhinal cortex lesion and perforant path removal (35, 38, 69, 72).

The activation of this cascade during the early reinnervation phase, well passed the degenerative stage, suggests that the delayed response of both proteins is important in the process of terminal remodeling and neo-synaptogenesis following hippocampal deafferentation.

According to our working hypothesis, the elevated clusterin expression seen in APOE-ε4 positive individuals is a compensatory mechanism. This mechanism aims to compensate, at least in part, for decreased levels of apoE found in E4 carriers (68) with newly generated clusterin, restoring cholesterol transport and enabling neurons to better respond to the damaging effects of Alzheimer's disease.

The effects of *CLU* and *APOE* genotypes on clusterin levels show, from a biochemical point of view, how genetic interactions may influence the pathophysiology of AD. In fact, it has been described that *CLU* interacts not only with *APOE* but also with other genes such as *ABCA7* and *PICALM* to influence AD risk level and hippocampal neurodegeneration (73-75). These interactions are relevant because they could explain the differences in risk associated with genetic variants among different ethnic populations. This is particularly relevant for the *CLU* gene which is consistently shown to be a genetic risk factor for AD in Caucasian populations, but not in other ethnicities. For example, in Asian populations, studies have found an association with risk (28) and others have not (25), while in populations of African descent and Hispanics, most studies found no association between *CLU* variants and risk of AD (22, 28). Based on our literature review and findings, it appears that the discrepancies in risk can be attributed to the interplay between CLU and other genetic variants, which exhibit varying prevalence in different populations.

In conclusion, our study shows that the CLU rs 11136000 T variant is protective against AD likely due to increased gene expression and that role of this variant is more important in the later phases of the disease and in APOE-e4 carriers. Due to its importance in the pathophysiology of AD, elucidating the role of *CLU* variants and the clusterin protein could provide new pathways for diagnostic and treatment approaches. Going forward, it would be important to further investigate the neuroprotective functions of clusterin and the interactions between *CLU* variants and other genetic risk factors for AD.

7. References:

1. CLU clusterin [Homo sapiens (human)] - Gene - NCBI.
2. May PC, Lampert-Etchells M, Johnson SA, Poirier J, Masters JN, Finch CE. Dynamics of gene expression for a hippocampal glycoprotein elevated in Alzheimer's disease and in response to experimental lesions in rat. Neuron. 1990;5(6):831-9.
3. de Silva HV, Stuart WD, Duvic CR, Wetterau JR, Ray MJ, Ferguson DG, et al. A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. J Biol Chem. 1990;265(22):13240-7.
4. Jenne DE, Lowin B, Peitsch MC, Böttcher A, Schmitz G, Tschopp J. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. J Biol Chem. 1991;266(17):11030-6.
5. Kim N, Yoo JC, Han JY, Hwang EM, Kim YS, Jeong EY, et al. Human nuclear clusterin mediates apoptosis by interacting with Bcl-XL through C-terminal coiled coil domain. J Cell Physiol. 2012;227(3):1157-67.
6. Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, Wang CY. Clusterin inhibits apoptosis by interacting with activated Bax. Nat Cell Biol. 2005;7(9):909-15.
7. Buttyan R, Olsson CA, Pintar J, Chang C, Bandyk M, Ng PY, et al. Induction of the TRPM-2 gene in cells undergoing programmed death. Mol Cell Biol. 1989;9(8):3473-81.
8. Strocchi P, Smith MA, Perry G, Tamagno E, Danni O, Pession A, et al. Clusterin up-regulation following sub-lethal oxidative stress and lipid peroxidation in human neuroblastoma cells. Neurobiol Aging. 2006;27(11):1588-94.
9. Viard I, Wehrli P, Jornot L, Bullani R, Vechietti JL, Schifferli JA, et al. Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J Invest Dermatol. 1999;112(3):290-6.

10. Humphreys DT, Carver JA, Easterbrook-Smith SB, Wilson MR. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J Biol Chem. 1999;274(11):6875-81.
11. Wei L, Xue T, Wang J, Chen B, Lei Y, Huang Y, et al. Roles of clusterin in progression, chemoresistance and metastasis of human ovarian cancer. Int J Cancer. 2009;125(4):791-806.
12. Yom CK, Woo H-Y, Min SY, Kang SY, Kim HS. Clusterin overexpression and relapse-free survival in breast cancer. Anticancer Res. 2009;29(10):3909-12.
13. July LV, Akbari M, Zellweger T, Jones EC, Goldenberg SL, Gleave ME. Clusterin expression is significantly enhanced in prostate cancer cells following androgen withdrawal therapy. Prostate. 2002;50(3):179-88.
14. Ishikawa Y, Akasaka Y, Ishii T, Komiyama K, Masuda S, Asuwa N, et al. Distribution and synthesis of apolipoprotein J in the atherosclerotic aorta. Arterioscler Thromb Vasc Biol. 1998;18(4):665-72.
15. Liu G, Zhang H, Hao F, Hao J, Pan L, Zhao Q, et al. Clusterin Reduces Cold Ischemia-Reperfusion Injury in Heart Transplantation Through Regulation of NF-kB Signaling and Bax/Bcl-xL Expression. Cell Physiol Biochem. 2018;45(3):1003-12.
16. Ingram G, Loveless S, Howell OW, Hakobyan S, Dancey B, Harris CL, et al. Complement activation in multiple sclerosis plaques: an immunohistochemical analysis. Acta Neuropathol Commun. 2014;2:53.
17. Grewal RP, Morgan TE, Finch CE. C1qB and clusterin mRNA increase in association with neurodegeneration in sporadic amyotrophic lateral sclerosis. Neurosci Lett. 1999;271(1):65-7.
18. Lambert J-C, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, et al. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet. 2009;41(10):1094-9.
19. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, et al. Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet. 2011;43(5):436-41.
20. Bettens K, Brouwers N, Engelborghs S, Lambert JC, Rogaeva E, Vandenberghe R, et al. Both common variations and rare non-synonymous substitutions and small insertion/deletions in CLU are associated with increased Alzheimer risk. Mol Neurodegener. 2012;7:3.
21. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet. 2009;41(10):1088-93.
22. Jun G, Naj AC, Beecham GW, Wang LS, Buros J, Gallins PJ, et al. Meta-analysis confirms CR1, CLU, and PICALM as alzheimer disease risk loci and reveals interactions with APOE genotypes. Arch Neurol. 2010;67(12):1473-84.
23. ALZGENE - GENE OVERVIEW OF ALL PUBLISHED AD-ASSOCIATION STUDIES FOR CLU 2011 [updated 2011-04-11. Available from: http://www.alzgene.org/geneoverview.asp?geneID=323.

24. Yu JT, Li L, Zhu QX, Zhang Q, Zhang W, Wu ZC, et al. Implication of CLU gene polymorphisms in Chinese patients with Alzheimer's disease. Clin Chim Acta. 2010;411(19-20):1516-9.
25. Han Z, Qu J, Zhao J, Zou X. Analyzing 74,248 Samples Confirms the Association Between CLU rs11136000 Polymorphism and Alzheimer's Disease in Caucasian But Not Chinese population. Sci Rep. 2018;8(1):11062.
26. Zhang S, Li X, Ma G, Jiang Y, Liao M, Feng R, et al. CLU rs9331888 Polymorphism Contributes to Alzheimer's Disease Susceptibility in Caucasian But Not East Asian Populations. Mol Neurobiol. 2016;53(3):1446-51.
27. Lee JH, Cheng R, Barral S, Reitz C, Medrano M, Lantigua R, et al. Identification of novel loci for Alzheimer disease and replication of CLU, PICALM, and BIN1 in Caribbean Hispanic individuals. Arch Neurol. 2011;68(3):320-8.
28. Du W, Tan J, Xu W, Chen J, Wang L. Association between clusterin gene polymorphism rs11136000 and late-onset Alzheimer's disease susceptibility: A review and meta-analysis of case-control studies. Exp Ther Med. 2016;12(5):2915-27.
29. Nuutinen T, Huuskonen J, Suuronen T, Ojala J, Miettinen R, Salminen A. Amyloid-beta 1-42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes. Neurochem Int. 2007;50(3):540-7.
30. Narayan P, Orte A, Clarke RW, Bolognesi B, Hook S, Ganzinger KA, et al. The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1-40) peptide. Nat Struct Mol Biol. 2011;19(1):79-83.
31. Bell RD, Sagare AP, Friedman AE, Bedi GS, Holtzman DM, Deane R, et al. Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. J Cereb Blood Flow Metab. 2007;27(5):909-18.
32. McGeer PL, Kawamata T, Walker DG. Distribution of clusterin in Alzheimer brain tissue. Brain Res. 1992;579(2):337-41.
33. Perrotte M, Le Page A, Fournet M, Le Sayec M, Rassart É, Fulop T, et al. Blood-based redox-signature and their association to the cognitive scores in MCI and Alzheimer's disease patients. Free Radic Biol Med. 2019;130:499-511.
34. Danik M, Champagne D, Petit-Turcotte C, Beffert U, Poirier J. Brain lipoprotein metabolism and its relation to neurodegenerative disease. Crit Rev Neurobiol. 1999;13(4):357-407.
35. Dauar MT, Picard C, Labonté A, Breitner J, Rosa-Neto P, Villeneuve S, et al. Contactin 5 and Apolipoproteins Interplay in Alzheimer's Disease. J Alzheimers Dis. 2024;98(4):1361-75.
36. Boyles JK, Zoellner CD, Anderson LJ, Kosik LM, Pitas RE, Weisgraber KH, et al. A role for apolipoprotein E, apolipoprotein A-I, and low density lipoprotein receptors in cholesterol transport during regeneration and remyelination of the rat sciatic nerve. J Clin Invest. 1989;83(3):1015-31.
37. Poirier J, Minnich A, Davignon J. Apolipoprotein E, synaptic plasticity and Alzheimer's disease. Ann Med. 1995;27(6):663-70.
38. White F, Nicoll JA, Horsburgh K. Alterations in ApoE and ApoJ in relation to degeneration and regeneration in a mouse model of entorhinal cortex lesion. Exp Neurol. 2001;169(2):307-18.

39. Breitner JCS, Poirier J, Etienne PE, Leoutsakos JM. Rationale and Structure for a New Center for Studies on Prevention of Alzheimer's Disease (StoP-AD). J Prev Alzheimers Dis. 2016;3(4):236-42.
40. Tremblay-Mercier J, Madjar C, Das S, Pichet Binette A, Dyke SOM, Etienne P, et al. Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. Neuroimage Clin. 2021;31:102733.
41. Lleó A, Alcolea D, Martínez-Lage P, Scheltens P, Parnetti L, Poirier J, et al. Longitudinal cerebrospinal fluid biomarker trajectories along the Alzheimer's disease continuum in the BIOMARKAPD study. Alzheimers Dement. 2019;15(6):742-53.
42. Brinkmalm A, Brinkmalm G, Honer WG, Frölich L, Hausner L, Minthon L, et al. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. Mol Neurodegener. 2014;9:53.
43. Öhrfelt A, Brinkmalm A, Dumurgier J, Brinkmalm G, Hansson O, Zetterberg H, et al. The pre-synaptic vesicle protein synaptotagmin is a novel biomarker for Alzheimer's disease. Alzheimers Res Ther. 2016;8(1):41.
44. Portelius E, Olsson B, Höglund K, Cullen NC, Kvartsberg H, Andreasson U, et al. Cerebrospinal fluid neurogranin concentration in neurodegeneration: relation to clinical phenotypes and neuropathology. Acta Neuropathol. 2018;136(3):363-76.
45. Sandelius Å, Portelius E, Källén Å, Zetterberg H, Rot U, Olsson B, et al. Elevated CSF GAP-43 is Alzheimer's disease specific and associated with tau and amyloid pathology. Alzheimers Dement. 2019;15(1):55-64.
46. McSweeney M, Pichet Binette A, Meyer P-F, Gonneaud J, Bedetti C, Ozlen H, et al. Intermediate flortaucipir uptake is associated with Aβ-PET and CSF tau in asymptomatic adults. Neurology. 2020;94(11):e1190-e200.
47. Jack CR, Jr., Wiste HJ, Weigand SD, Therneau TM, Lowe VJ, Knopman DS, et al. Defining imaging biomarker cut points for brain aging and Alzheimer's disease. Alzheimers Dement. 2017;13(3):205-16.
48. Leoutsakos JM, Gross AL, Jones RN, Albert MS, Breitner JCS. 'Alzheimer's Progression Score': Development of a Biomarker Summary Outcome for AD Prevention Trials. J Prev Alzheimers Dis. 2016;3(4):229-35.
49. Randolph C, Tierney MC, Mohr E, Chase TN. The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS): preliminary clinical validity. J Clin Exp Neuropsychol. 1998;20(3):310-9.
50. Laberge AM, Michaud J, Richter A, Lemyre E, Lambert M, Brais B, et al. Population history and its impact on medical genetics in Quebec. Clin Genet. 2005;68(4):287-301.
51. Khachaturian ZS. Diagnosis of Alzheimer's disease. Arch Neurol. 1985;42(11):1097-105.
52. Bennett DA, Buchman AS, Boyle PA, Barnes LL, Wilson RS, Schneider JA. Religious Orders Study and Rush Memory and Aging Project. J Alzheimers Dis. 2018;64(s1):S161-s89.
53. Ping L, Duong DM, Yin L, Gearing M, Lah JJ, Levey AI, et al. Global quantitative analysis of the human brain proteome in Alzheimer's and Parkinson's Disease. Sci Data. 2018;5:180036.
54. Kamboh MI, Aston CE, Ferrell RE, Dekosky ST. Re: Genetic effect of alpha 1-antichymotrypsin on the risk of Alzheimer disease. Genomics. 1997;40(2):382-5.

55. López Malizia A, Merlotti A, Bonte PE, Sager M, Arribas De Sandoval Y, Goudot C, et al. Clusterin protects mature dendritic cells from reactive oxygen species mediated cell death. Oncoimmunology. 2024;13(1):2294564.
56. Sasaki K, Doh-ura K, Wakisaka Y, Iwaki T. Clusterin/apolipoprotein J is associated with cortical Lewy bodies: immunohistochemical study in cases with alpha-synucleinopathies. Acta Neuropathol. 2002;104(3):225-30.
57. Foster EM, Dangla-Valls A, Lovestone S, Ribe EM, Buckley NJ. Clusterin in Alzheimer's Disease: Mechanisms, Genetics, and Lessons From Other Pathologies. Front Neurosci. 2019;13:164.
58. Leskov KS, Araki S, Lavik JP, Gomez JA, Gama V, Gonos ES, et al. CRM1 protein-mediated regulation of nuclear clusterin (nCLU), an ionizing radiation-stimulated, Bax-dependent pro-death factor. J Biol Chem. 2011;286(46):40083-90.
59. MAY PC, LAMPERT-ETCHELLS MA, JOHNSON SA, POIRIER J, MILLAR SL, FINCH CE. CHARACTERIZATION OF pADHC-9: A POLY(A)RNA SEQUENCE OVEREXPRESSED IN ALZHEIMER'S DISEASE HIPPOCAHPUS. Alzheimer Disease & Associated Disorders. 1988;2(3):204.
60. Ghiso J, Matsubara E, Koudinov A, Choi-Miura NH, Tomita M, Wisniewski T, et al. The cerebrospinal-fluid soluble form of Alzheimer's amyloid beta is complexed to SP-40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. Biochem J. 1993;293 (Pt 1)(Pt 1):27-30.
61. Wang H, Ma LZ, Sheng ZH, Liu JY, Yuan WY, Guo F, et al. Association between cerebrospinal fluid clusterin and biomarkers of Alzheimer's disease pathology in mild cognitive impairment: a longitudinal cohort study. Front Aging Neurosci. 2023;15:1256389.
62. Tang L, Wang ZB, Ma LZ, Cao XP, Tan L, Tan MS. Dynamic changes of CSF clusterin levels across the Alzheimer's disease continuum. BMC Neurol. 2022;22(1):508.
63. Wang J, Zhang X, Zhu B, Fu P. Association of Clusterin Levels in Cerebrospinal Fluid with Synaptic Degeneration Across the Alzheimer's Disease Continuum. Neuropsychiatr Dis Treat. 2020;16:183-90.
64. Ko YA, Billheimer JT, Lyssenko NN, Kueider-Paisley A, Wolk DA, Arnold SE, et al. ApoJ/Clusterin concentrations are determinants of cerebrospinal fluid cholesterol efflux capacity and reduced levels are associated with Alzheimer's disease. Alzheimers Res Ther. 2022;14(1):194.
65. Nordengen K, Kirsebom BE, Richter G, Pålhaugen L, Gísladóttir B, Siafarikas N, et al. Longitudinal cerebrospinal fluid measurements show glial hypo- and hyperactivation in predementia Alzheimer's disease. J Neuroinflammation. 2023;20(1):298.
66. Lin YL, Chen SY, Lai LC, Chen JH, Yang SY, Huang YL, et al. Genetic polymorphisms of clusterin gene are associated with a decreased risk of Alzheimer's disease. Eur J Epidemiol. 2012.
67. Ling IF, Bhongsatiern J, Simpson JF, Fardo DW, Estus S. Genetics of clusterin isoform expression and Alzheimer's disease risk. PLoS One. 2012;7(4):e33923.
68. Bertrand P, Poirier J, Oda T, Finch CE, Pasinetti GM. Association of apolipoprotein E genotype with brain levels of apolipoprotein E and apolipoprotein J (clusterin) in Alzheimer disease. Brain Res Mol Brain Res. 1995;33(1):174-8.

69. Poirier J, Baccichet A, Dea D, Gauthier S. Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. Neuroscience. 1993;55(1):81-90.
70. Beffert U, Cohn JS, Petit-Turcotte C, Tremblay M, Aumont N, Ramassamy C, et al. Apolipoprotein E and beta-amyloid levels in the hippocampus and frontal cortex of Alzheimer's disease subjects are disease-related and apolipoprotein E genotype dependent. Brain Res. 1999;843(1-2):87-94.
71. Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, et al. Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. J Neurosci. 2008;28(45):11445-53.
72. Stone DJ, Rozovsky I, Morgan TE, Anderson CP, Lopez LM, Shick J, et al. Effects of age on gene expression during estrogen-induced synaptic sprouting in the female rat. Exp Neurol. 2000;165(1):46-57.
73. Yang X, Li J, Liu B, Li Y, Jiang T. Impact of PICALM and CLU on hippocampal degeneration. Hum Brain Mapp. 2016;37(7):2419-30.
74. Nazarian A, Cook B, Morado M, Kulminski AM. Interaction Analysis Reveals Complex Genetic Associations with Alzheimer's Disease in the CLU and ABCA7 Gene Regions. Genes (Basel). 2023;14(9).
75. An N, Fu Y, Shi J, Guo HN, Yang ZW, Li YC, et al. Synergistic Effects of APOE and CLU May Increase the Risk of Alzheimer's Disease: Acceleration of Atrophy in the Volumes and Shapes of the Hippocampus and Amygdala. J Alzheimers Dis. 2021;80(3):1311-27.

General Discussion

1. Summary of results:

In this thesis we investigated the rs1461684 G variant in the *CNTN5* gene and the rs11136000 T variant in the *CLU* gene in different phases of AD. These variants were identified as risk and protective factors (respectively) for AD therefore, understanding their role in the disease could help us better understand the pathophysiology of AD.

In manuscript 1 we describe, for the first time, the *CNTN5* rs1461684 G variant as a risk factor of AD. The role of this variant as a risk factor for AD was identified in the GWAS of the QFP cohort and confirmed in different population cohorts. In this chapter, we also show that the rs1461684 G variant is associated with decreased gene expression in the earlier phases of the disease and with faster disease progression in the presymptomatic phase, suggesting that its role is more prominent in the early disease stages. This assumption is supported by the fact that the CSF level of the contactin 5 protein was associated with AD biomarkers (in the CSF and PET) in cognitively unimpaired subjects at high risk for AD. CSF contactin 5 level also progressively increases in these presymptomatic subjects over time but is decreased in MCI and AD subjects.

In manuscript 2 we further investigate the role of this newly identified AD risk factor by looking into its associations with apolipoproteins which, similar to contactin 5, are also involved in processes of neuronal and synaptic formation and are also involved in AD. We show that CSF levels of contactin 5 are significantly associated only with apolipoproteins B, C, D and J, which

are the main cholesterol transporters in the brain. We also show that contactin 5 levels are associated with cholesterol levels both in the CSF and blood. More importantly we found that gene expression of contactin 5 (trend) and apolipoproteins B, C, D and J are changed in a mouse model of hippocampal deafferentation. These findings shown that these two classes of protein that have similarity in function are strongly associated in the early phases of AD and contribute to different phases of synaptic degeneration/reinnervation even in the absence of amyloid and tau pathology. These findings lead us to believe that both contactin 5 and apolipoproteins play a role in the brain response to pathological damage and that if their function is impaired it could lead to a lower ability of the brain to deal with the pathological harm caused by AD pathology.

In manuscript 3 we further investigate the role of the *CLU* rs11136000 T variant which is already established as a protective factor against AD (34, 135). In this work we show that although CSF clusterin is associated with several biomarkers and synaptic markers in cognitively unimpaired subjects at high risk for AD, the variant itself is not associated with cognitive scores, disease progression biomarkers or clusterin levels. On the other hand, in the later phase of the disease (autopsied brain of pathologically diagnosed AD and control subjects), the presence of the variant and the diagnosis of AD are associated with increased gene expression. Moreover, the increased gene expression is more pronounced in *APOE-ε4* carriers. These findings lead us to conclude that the *CLU* rs11136000 T protective variant has a more important role in the later disease phases and that its protective role is likely mediated by increases in gene expression. Additionally, the more pronounced increase in *APOE-ε4* carriers is likely a compensatory mechanism to improve cholesterol transport, since Apoe4 is a less effective cholesterol transporter.

2. Discussion

The sporadic form of AD is the most common form of dementia (144) and it is estimated to affect over 32 million people worldwide (145). Although significant progress has been made in the understanding of the pathophysiology of the disease, its diagnosis and treatment, a significant part of the underlying causes of AD are still unknown. The amyloid cascade hypothesis continues to be the dominant explanation for the pathological progression in AD. It states that Aß is the initial pathological trigger that causes neurofibrillary NFT formation/accumulation leading to all the other downstream events that will cause neurodegeneration (8, 9). However, different lines of evidence have challenged the amyloid cascade hypothesis in recent years and highlighted the importance of other pathological processes.

It has long been shown, by postmortem studies, that a significant proportion of individuals with Aß pathology compatible with a diagnosis of AD were cognitively unimpaired before death (146, 147). Additionally, Aß is known to reach a plateau early in the disease process (7, 148) and to not correlate well with cognitive decline in the dementia phase (7). On the other hand, it has been proposed, in more recent years, that tangle deposition is the primary event in AD pathological cascade in late onset AD (10). Several studies have found that tau pathology is present in cognitively normal subjects before amyloid is detected (149-152) and that tau deposition correlates better than Aß with cognitive symptoms, especially in the dementia phase (152, 153). Another interesting line of investigation has suggested that it is actually the interaction between Aß and tau, rather than their individual or consecutive effects that drives AD pathological decline (154). More importantly, recent pathological studies have shown that the AD pathological factors currently known correspond to only about one third of the variation of the cognitive decline in AD (155).

This indicates, not only that a significant part of the neuropathology is still unexplained, but it also highlights the complexity that underlies the pathophysiology of the disease and the links between the pathological hallmarks, the phenomenon of aging and the clinical manifestations.

In the study of the pathophysiology of AD, most research is focused on the pathological processes responsible for harming the brain. Research into the role of amyloid, tau and neuroinflammation for instance, center on how they lead to synaptic and neuronal injury/death and neurodegeneration. Significantly less research is dedicated to the processes that lead to increased vulnerability to the disease and to the factors that lead to an inability, in some individuals, to sustain and recover from a certain level of pathological harm without it leading to neurodegeneration. In a disease with the pathological and clinical complexity of AD, the ability of the brain to respond to pathological harm with compensatory changes to promote neuronal and synaptic regeneration and remodeling is an important process that demands more attention.

This is one of the reasons why our team has focused our core research program on the presymptomatic phase of the disease as it has become clear over recent years that late onset AD, in its asymptomatic phase, can last between 10 to 20 years as opposed to the 8-12 years that is so characteristic of symptomatic dementia phase. It takes twice as long to reach onset than it takes to die from AD. What is happening to the brain in the asymptomatic phase? Is it passively losing neurons or, does it try to fight the neurodegenerative process that is slowly compromising its neuronal network integrity?

The brain is the organ with the highest concentration of cholesterol and phospholipids, which are essential for the formation of neuronal membrane, synapses and myelin. Moreover, of the top 12 risk genes identified by GWAS in late onset AD, seven (*APOE, ApoJ* (*CLU*), *ABCA1, ABCA7*, *BIN1, PCALM, SORL1*) have been directly involved in lipid transport, binding, internalization and mobilization (99). *APOE-ε4*, the main genetic risk factor for the sporadic form of AD, is the most important lipid transporter in the brain, and a significant amount of evidence suggests that its role in the disease is associated with cholesterol and phospholipid transport. The presence of the *ε4* allele of the *APOE* gene leads to lower levels of apolipoprotein E and less efficient cholesterol transport (45-47), which was shown to lead to impaired ability of neurons to promote compensatory reinnervation, especially in the cholinergic system (80, 156).

Studies performed in a rodent model of hippocampal deafferentation following entorhinal cortex lesions have shown that *apoe* gene expression is markedly increased in the compensatory reinnervation phase (48-50, 157), suggesting that the *APOE* gene and its protein play an active role in the reinnervation process after neuronal damage, even in the absence of AD pathology. Conversely, the normal plastic response was shown to be completely abolished in ***apoe*** knockout animals, leading to both serious synaptic damage and cognitive deficits with aging (158, 159) and lesioning (160). This last piece of evidence further highlights the importance of apoE levels as opposed to pure genotypic variance.

Interestingly, *APOE-ε4* has been shown to play a similar plasticity-related role in other human neurological diseases besides AD. The presence of the *APOE-ε4* allele has been associated with worse cognitive performance and higher signs of neuronal injury in multiple sclerosis (161, 162)

and worse functional outcome following stroke (163) and traumatic brain injury (164, 165). A factor that all these conditions have in common is that they lead to brain damage unrelated to amyloid pathology and would require the brain to respond by promoting neuronal remodeling and repair for proper recovery. In all instances, the *APOE* genotype, with ε4 being the most severe, greatly affected both the recovery rate and its scope.

CLU is another major genetic risk factor for AD and one of the main cholesterol transporters working in tandem with *APOE* in the mature brain. As illustrated in figure 25, clusterin associates with ApoE and lipids to form HDL complexes responsible for distributing cholesterol and phospholipids to different cell types in the brain. In the second manuscript of the thesis, we show that *CLU* and *APOE* gene expression are simultaneously increased in the experimentally deafferented rodent hippocampus during the reinnervation phase in the lesioned mice. This finding, which is supported by previous literature (45-47, 154), suggests a role of both genes during the reinnervation phase, likely due to their ability to mobilize and deliver the necessary lipids required for neuronal reinnervation and compensatory synaptic remodeling. It's important to note that the development of new terminals and synapses during compensatory synaptogenesis relies heavily on cholesterol and phospholipids, which account for approximately 75% of the dry weight of these membrane structures (166).

In this very context, it is with little surprise that three of our QFP GWAS disease-associated genes (*APOE*, *CLU* (*APOJ*) and *CNTN5*) are actually dealing with lipid mobilization and distribution (*APOE*/*CLU*) as well as terminal remodeling (*CNTN5*) in response to neuronal damage throughout the Alzheimer's disease spectrum.

2.1 Terminal/Synaptic marker: *CNTN5*

In the first manuscript of this thesis we describe, for the first time, a *CNTN5* variant as associated with significant increased risk for AD. The contactin protein family has an important role on neurodevelopment through the regulation of neurite outgrowth, axon guidance, synaptogenesis, neuron-glia interactions and cell survival (109, 110). More importantly, contactins have been shown to act in response to neuronal injury and promote terminal proliferation (167). Contactin 5 is more specifically implicated in axonal arborization and synaptic formation (109). In presymptomatic AD, contactin 5 is strongly associated with pre-synaptic markers and phospho-tau production which, in animal models, serve as markers of terminal proliferation in response to experimental hippocampal deafferentation (168).

One possible interpretation of the results could thus be that contactin 5 is, in fact, recruited to action after neurodevelopment, during adulthood, to promote axonal and synaptic repair following neuronal damage in presymptomatic AD. In this context, the decreases in gene expression observed in *CNTN5* risk allele carriers may increase the risk of AD by impairing the CNS's ability to respond to underlying AD pathology.

Neuronal remodeling and terminal sprouting require not only the presence of plasticity-related proteins but also a significant amount of lipids such as cholesterol and phospholipids. This prompted us to further explore the role of *CNTN5* in association with clusterin (apoJ) and other apolipoproteins in the second manuscript. In a series of analyses, we show extensive correlations between CSF contactin 5 and the main apolipoproteins responsible for lipid transport in the brain but also with cholesterol. Neuronal contactin 5 is a lipophilic membrane protein that upon secretion

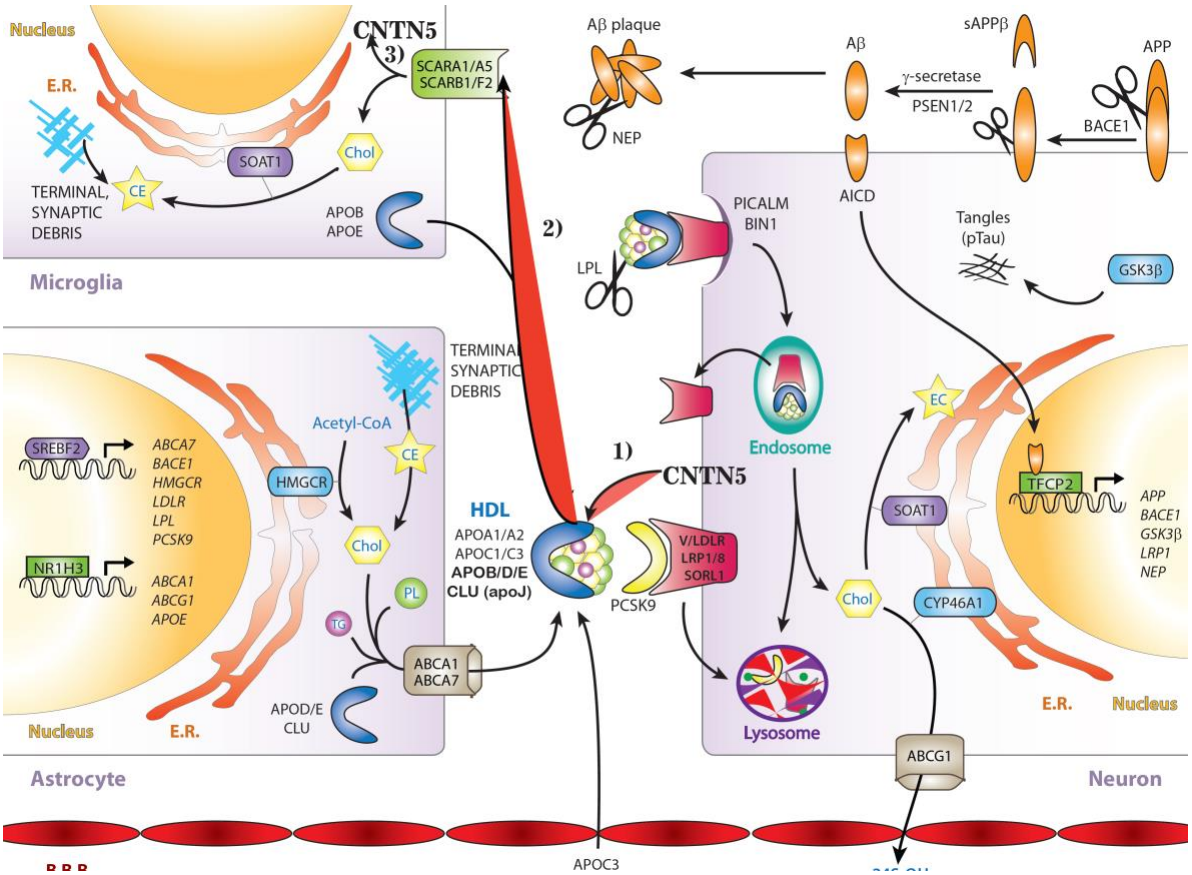
and/or synaptic pruning is taken up in the extracellular space by lipid-rich lipoprotein complexes in order to be transported to target cells (Figure 24) or, eliminated from the CNS via the blood brain barrier into the blood stream. In this hypothetic model, it is plausible that, following a neuronal or synaptic damage, neuronal contactin 5 is released and associates with CNS apolipoproteins to signal or trigger a compensatory response involving lipid mobilization and distribution to neurons undergoing remodeling (figure 24). In such a model, the so-called glial scavenging receptors could serve as the cell surface receptors mediating the internalization of the CNTN5/HDL complexes. Studies are underway in our laboratory to examine this possibility.

2.2 *CLU:* A Symptomatic Player with Little Presymptomatic Contribution

In the third and last manuscript we further investigate the role of *CLU (APOJ)* and its protective variant rs11136000 T throughout the disease spectrum. In contrast to our initial working hypothesis which assumed that a protective variant must have an impact in the presymptomatic phase of the disease, we found a rather completely different picture. Our results show that the presence of this variant or the diagnosis of definite AD are both associated with increased levels of gene expression in the end stage of the disease. More importantly, the gene expression of *CLU* is more pronounced in *APOE-ε4* carriers than non-carriers. The inverse relationship between apoE and ApoJ levels as the *ε4* allele dose increases in the AD subjects is not new. Our team did report a similar phenomenon in the hippocampus of AD cases (45). At that time, we proposed that the reduction of apoE in *ε4* carriers led to a compensatory increase of ApoJ designed to facilitate lipid transport throughout the brain in a situation of compromised efficiency. However, these original findings preceded the discovery of an Alzheimer specific protective variant in the *CLU* (*APOJ*) gene.

The current observation on the genetics of *CLU* leads us to conclude that the protective role of the rs11136000 T variant is mediated, at least in part, by increases in gene expression in the brain. The data also supports the important role of lipid transport in the brain since the increased CLU mRNA and protein levels are seen exclusively in *APOE-ε4* carriers. As proposed before, it is likely to serve as a compensatory mechanism to increase cholesterol mobilization and distribution in the presence of the *APOE-ε4* allele, which is characterized by ineffective cholesterol transport due to faster allele specific catabolism (105).

Figure 25: Apolipoprotein and cholesterol metabolism in the central nervous system



Possible molecular pathway for the release of CNTN5 during neuronal damage (1) and its binding to lipoproteins, lipids, APOE, and CLU (2), followed by migration towards glial cells expressing scavenging receptors. Internalization via the scavenging receptor (3) could lead to signal recognition and glial involvement in the compensatory response. Adapted from: Picard C, Nilsson N, Labonté A, Auld D, Rosa-Neto P; Alzheimer's Disease Neuroimaging Initiative; Ashton NJ, Zetterberg H, Blennow K, Breitner JCB, Villeneuve S, Poirier J; PREVENT-AD research group. Apolipoprotein B is a novel marker for early tau pathology in Alzheimer's disease. Alzheimers Dement. 2022 May;18(5):875-887(51).

2.3 The role of genetic research and the search for new genetic risk factors in AD.

As extensively presented in this work, the knowledge of underlying genetic risk factors has played an important role in the understanding of the pathophysiology of AD (and other diseases). However, identifying new genetic risk factors has become increasingly difficult even with GWAS that encompasses hundreds of thousands of patients, and the new variants identified contribute

very little to the risk level of the disease. In that context, more effective methods to identify the remaining genetic risk factors will be required in the future.

Most of the genetic risk factors identified so far are SNPs identified by microarray or PCR. However, other genetic mutations such as insertions, deletions or repeat expansions could also play a role in the risk of AD. The identification of such genetic changes requires more sophisticated technology such as whole genome sequencing which has been too expensive until recently. But with the increase affordability of such technologies, it would be interesting to also look into other types of genetic mutations, especially in coding regions, to assess their contribution for the risk of AD. Such an effort is currently underway in Europe and in the US, but it has met some significant roadblocks due to the shire quantity of data to be analyzed. The project is set to be completed by 2027 (https://www.nia.nih.gov/research/dn/alzheimers-disease-sequencing-project-consortia).

So far, most genetic studies (or scientific studies in general) are performed in Caucasian populations from Europe or North America. It is well-known that different genetic variants contribute to the risk of AD in different proportions in populations from different racial backgrounds. Even major risk factors such as *APOE-ε4* and *CLU* are known to increase the risk significantly in Caucasian people, but less so in populations with other racial background such as Asians, Hispanics and populations of African descent. This fact could be due to different reasons such as the effect of environmental risk factors or the interaction between genetic variants that exhibit varying prevalence in different populations. Therefore, it is of great importance to perform genetic studies in more diverse populations in order to uncover different risk factors and better understand how interactions affect their role in the disease.

Epidemiological data has consistently shown that AD is more prevalent in woman than in men (144), but the precise mechanism for this difference is not completely understood. The main factors shown to play a role in this difference in risk are: variation in the distribution of modifiable risk factors such as cardiovascular risk factor, education and work history (144); higher life expectancy in women (169); the role of sex hormones (170); and, of course, genetics (144). Genetic variants found both in sex and non-sex chromosomes have been shown to play different roles in the risk of AD in men and women. *APOE-ε4*, the most important genetic risk factor for AD, has been shown to have stronger association with AD risk (171) and CSF tau levels (172) in women compared to men (144). Increased age leads to higher rates of X or Y chromosomal loss in neuronal cells (173) and X and Y aneuploidy has been associated with increased risk of AD (173). Additionally, X -linked genes, some of which escape X-inactivation in women, have been shown to affect the risk AD (174). The precise mechanism by which genetic variants affect men and women differently is not completely understood and likely involves interactions with hormones and environmental and genetic risk factors. However, evidence is clear that genetic risk factors act differently according to sex, a factor that should be further investigated and should be taken into account in study designs for AD.

3. Study Limitations:

The most important limitation of this study is that most of the analysis were analysis of association, which, as we know, does not imply causation.

For the three studies investigating the two variants plus the *APOE* genotype, the results relating to the effects of the variants (rs1461684 G and rs 11136000 T), the genes (*CLU* and *CNTN5*) and the

proteins (clusterin and contactin 5) on biomarkers of the disease, clinical manifestations, gene expression and most other measures tested, consisted mostly of statistical associations. However, we believe that even though association does not imply causation, the fact that we obtained significant results that were consistent across different independent cohorts, in different phases of the disease and in line with the existing literature gives significant strength to our findings and support our conclusions. Additionally, these findings provide strong background and justification to follow-up experimental studies that can be far more complex and expensive.

We already discussed the issues of ethnicity and samples size before with our different cohorts. The focus on Caucasian populations (even in our clinical PREVENT-AD and autopsied QFP cohorts) is certainly the most important limitation of all. Some of the longitudinal cohorts we used in our different analyses such as the PREVENT-AD and the ROSMAP cohort have enrolled highly educated asymptomatic participants with master and Ph.D. level diplomas. Education was shown to significantly delay the onset of the disease and to cause a faster progression once symptoms emerge.

Conclusion

In this thesis we described a new genetic variant in the *CNTN5* gene as associated with increased risk of AD. Investigating the role of this variant in different population cohorts and in an animal model of hippocampal deafferentation, we were able to find that this new gene is active in the early stages of the disease affecting AD pathological markers such as phospho-Tau, likely through decreases in gene expression. We also investigated the role of a known protective variant in AD, the *CLU* rs11136000 T variant and concluded that this variant likely decreases the risk of AD through increases in gene expression, its impact being detected only in the symptomatic phase of the disease. Moreover, our findings suggest that the increases in clusterin gene expression work as a compensatory mechanism to compensate for the decreased efficiency in cholesterol transport in *APOE-ε4* carriers. Looking into the interactions between the two variants studied and their mechanism of action we propose that they likely play a role in the brain compensatory response to neuronal damage by facilitating neuronal remodeling and repair. Our studies lead us to believe that the compensatory responses to brain damage are an important process of AD pathophysiology that should be further explored, especially in the presymptomatic phase of the disease when tau and amyloid pathology are just beginning.

References

1. Alzheimer A, Stelzmann RA, Schnitzlein HN, Murtagh FR. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". Clin Anat. 1995;8(6):429-31.
2. Cipriani G, Dolciotti C, Picchi L, Bonuccelli U. Alzheimer and his disease: a brief history. Neurol Sci. 2011;32(2):275-9.
3. Müller U, Winter P, Graeber MB. A presenilin 1 mutation in the first case of Alzheimer's disease. Lancet Neurol. 2013;12(2):129-30.
4. Dahm R. Alzheimer's discovery. Curr Biol. 2006;16(21):R906-10.
5. Burns A, Byrne EJ, Maurer K. Alzheimer's disease. Lancet. 2002;360(9327):163-5.
6. Katzman R. Editorial: The prevalence and malignancy of Alzheimer disease. A major killer. Arch Neurol. 1976;33(4):217-8.
7. Jack CR, Knopman DS, Jagust WJ, Petersen RC, Weiner MW, Aisen PS, et al. Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. Lancet Neurol. 2013;12(2):207-16.
8. Hardy J, Allsop D. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends Pharmacol Sci. 1991;12(10):383-8.
9. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. Science. 1992;256(5054):184-5.
10. Arnsten AFT, Datta D, Del Tredici K, Braak H. Hypothesis: Tau pathology is an initiating factor in sporadic Alzheimer's disease. Alzheimers Dement. 2021;17(1):115-24.
11. Hyman BT, Phelps CH, Beach TG, Bigio EH, Cairns NJ, Carrillo MC, et al. National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease. Alzheimers Dement. 2012;8(1):1-13.
12. Group. F-NBW. BEST (Biomarkers, EndpointS, and other Tools) Resource [Internet]: Silver Spring (MD): Food and Drug Administration, Co-published by National Institutes of Health (US), Bethesda (MD); 2016 [updated November 29, 2021. Available from: https://www.ncbi.nlm.nih.gov/books/NBK326791/
13. Tyan SH, Shih AY, Walsh JJ, Maruyama H, Sarsoza F, Ku L, et al. Amyloid precursor protein (APP) regulates synaptic structure and function. Mol Cell Neurosci. 2012;51(1-2):43-52.
14. Chow VW, Mattson MP, Wong PC, Gleichmann M. An overview of APP processing enzymes and products. Neuromolecular Med. 2010;12(1):1-12.
15. Nguyen HL, Thi Minh Thu T, Truong PM, Lan PD, Man VH, Nguyen PH, et al. Aβ41 Aggregates More Like Aβ40 than Like Aβ42: In Silico and in Vitro Study. J Phys Chem B. 2016;120(30):7371-9.
16. Thal DR, Rub U, Orantes M, Braak H. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology. 2002;58(12):1791-800.
17. Mirra SS, Heyman A, McKeel D, Sumi SM, Crain BJ, Brownlee LM, et al. The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II.

Standardization of the neuropathologic assessment of Alzheimer's disease. Neurology. 1991;41(4):479-86.
18. Moncaster JA, Moir RD, Burton MA, Chadwick O, Minaeva O, Alvarez VE, et al. Alzheimer's disease amyloid-beta pathology in the lens of the eye. Exp Eye Res. 2022;221:108974.
19. Strozyk D, Blennow K, White LR, Launer LJ. CSF Abeta 42 levels correlate with amyloid-neuropathology in a population-based autopsy study. Neurology. 2003;60(4):652-6.
20. Janelidze S, Zetterberg H, Mattsson N, Palmqvist S, Vanderstichele H, Lindberg O, et al. CSF Aβ42/Aβ40 and Aβ42/Aβ38 ratios: better diagnostic markers of Alzheimer disease. Ann Clin Transl Neurol. 2016;3(3):154-65.
21. Lewczuk P, Matzen A, Blennow K, Parnetti L, Molinuevo JL, Eusebi P, et al. Cerebrospinal Fluid Aβ42/40 Corresponds Better than Aβ42 to Amyloid PET in Alzheimer's Disease. J Alzheimers Dis. 2017;55(2):813-22.
22. Fagan AM, Mintun MA, Mach RH, Lee SY, Dence CS, Shah AR, et al. Inverse relation between in vivo amyloid imaging load and cerebrospinal fluid Abeta42 in humans. Ann Neurol. 2006;59(3):512-9.
23. Hatashita S, Yamasaki H, Suzuki Y, Tanaka K, Wakebe D, Hayakawa H. [18F]Flutemetamol amyloid-beta PET imaging compared with [11C]PIB across the spectrum of Alzheimer's disease. Eur J Nucl Med Mol Imaging. 2014;41(2):290-300.
24. Rowe CC, Pejoska S, Mulligan RS, Jones G, Chan JG, Svensson S, et al. Head-to-head comparison of 11C-PiB and 18F-AZD4694 (NAV4694) for β-amyloid imaging in aging and dementia. J Nucl Med. 2013;54(6):880-6.
25. Medeiros R, Baglietto-Vargas D, LaFerla FM. The role of tau in Alzheimer's disease and related disorders. CNS Neurosci Ther. 2011;17(5):514-24.
26. Reddy PH. Abnormal tau, mitochondrial dysfunction, impaired axonal transport of mitochondria, and synaptic deprivation in Alzheimer's disease. Brain Res. 2011;1415:136-48.
27. Mamsa SSA, Meloni BP. Arginine and Arginine-Rich Peptides as Modulators of Protein Aggregation and Cytotoxicity Associated With Alzheimer's Disease. Front Mol Neurosci. 2021;14:759729.
28. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. Neurobiol Aging. 1995;16(3):271-8; discussion 8-84.
29. Jouanne M, Rault S, Voisin-Chiret AS. Tau protein aggregation in Alzheimer's disease: An attractive target for the development of novel therapeutic agents. Eur J Med Chem. 2017;139:153-67.
30. Jack CR, Jr., Andrews JS, Beach TG, Buracchio T, Dunn B, Graf A, et al. Revised criteria for diagnosis and staging of Alzheimer's disease: Alzheimer's Association Workgroup. Alzheimers Dement. 2024.
31. Schwarz AJ, Yu P, Miller BB, Shcherbinin S, Dickson J, Navitsky M, et al. Regional profiles of the candidate tau PET ligand 18F-AV-1451 recapitulate key features of Braak histopathological stages. Brain. 2016;139(Pt 5):1539-50.
32. Guerreiro R, Wojtas A, Bras J, Carrasquillo M, Rogaeva E, Majounie E, et al. TREM2 variants in Alzheimer's disease. N Engl J Med. 2013;368(2):117-27.

33. Naj AC, Jun G, Beecham GW, Wang LS, Vardarajan BN, Buros J, et al. Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. Nat Genet. 2011;43(5):436-41.
34. Lambert J-C, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, et al. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nature Genetics. 2009;41(10):1094-9.
35. Bis JC, Jian X, Kunkle BW, Chen Y, Hamilton-Nelson KL, Bush WS, et al. Whole exome sequencing study identifies novel rare and common Alzheimer's-Associated variants involved in immune response and transcriptional regulation. Mol Psychiatry. 2020;25(8):1859-75.
36. Hickman SE, Allison EK, El Khoury J. Microglial dysfunction and defective beta-amyloid clearance pathways in aging Alzheimer's disease mice. J Neurosci. 2008;28(33):8354-60.
37. Patel NS, Paris D, Mathura V, Quadros AN, Crawford FC, Mullan MJ. Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. J Neuroinflammation. 2005;2(1):9.
38. Blum-Degen D, Müller T, Kuhn W, Gerlach M, Przuntek H, Riederer P. Interleukin-1 beta and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. Neurosci Lett. 1995;202(1-2):17-20.
39. Vom Berg J, Prokop S, Miller KR, Obst J, Kälin RE, Lopategui-Cabezas I, et al. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. Nat Med. 2012;18(12):1812-9.
40. Shen Y, Yang L, Li R. What does complement do in Alzheimer's disease? Old molecules with new insights. Transl Neurodegener. 2013;2(1):21.
41. Miron J, Picard C, Frappier J, Dea D, Theroux L, Poirier J. TLR4 Gene Expression and Pro-Inflammatory Cytokines in Alzheimer's Disease and in Response to Hippocampal Deafferentation in Rodents. J Alzheimers Dis. 2018;63(4):1547-56.
42. Zhang J, Liu Q. Cholesterol metabolism and homeostasis in the brain. Protein Cell. 2015;6(4):254-64.
43. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. Lancet (London, England). 1993;342(8873):697-9.
44. Strittmatter WJ, Saunders AM, Schmechel D, Pericak-Vance M, Enghild J, Salvesen GS, et al. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. Proc Natl Acad Sci U S A. 1993;90(5):1977-81.
45. Bertrand P, Poirier J, Oda T, Finch CE, Pasinetti GM. Association of apolipoprotein E genotype with brain levels of apolipoprotein E and apolipoprotein J (clusterin) in Alzheimer disease. Brain Res Mol Brain Res. 1995;33(1):174-8.
46. Beffert U, Cohn JS, Petit-Turcotte C, Tremblay M, Aumont N, Ramassamy C, et al. Apolipoprotein E and beta-amyloid levels in the hippocampus and frontal cortex of Alzheimer's disease subjects are disease-related and apolipoprotein E genotype dependent. Brain Res. 1999;843(1-2):87-94.

47. Riddell DR, Zhou H, Atchison K, Warwick HK, Atkinson PJ, Jefferson J, et al. Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels. J Neurosci. 2008;28(45):11445-53.
48. Poirier J, Baccichet A, Dea D, Gauthier S. Cholesterol synthesis and lipoprotein reuptake during synaptic remodelling in hippocampus in adult rats. Neuroscience. 1993;55(1):81-90.
49. White F, Nicoll JA, Horsburgh K. Alterations in ApoE and ApoJ in relation to degeneration and regeneration in a mouse model of entorhinal cortex lesion. Exp Neurol. 2001;169(2):307-18.
50. Stone DJ, Rozovsky I, Morgan TE, Anderson CP, Lopez LM, Shick J, et al. Effects of age on gene expression during estrogen-induced synaptic sprouting in the female rat. Exp Neurol. 2000;165(1):46-57.
51. Picard C, Nilsson N, Labonte A, Auld D, Rosa-Neto P, Alzheimer's Disease Neuroimaging I, et al. Apolipoprotein B is a novel marker for early tau pathology in Alzheimer's disease. Alzheimers Dement. 2022;18(5):875-87.
52. Terrisse L, Poirier J, Bertrand P, Merched A, Visvikis S, Siest G, et al. Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. J Neurochem. 1998;71(4):1643-50.
53. Glockner F, Ohm TG. Hippocampal apolipoprotein D level depends on Braak stage and APOE genotype. Neuroscience. 2003;122(1):103-10.
54. Peña-Bautista C, Casas-Fernández E, Vento M, Baquero M, Cháfer-Pericás C. Stress and neurodegeneration. Clin Chim Acta. 2020;503:163-8.
55. DeKosky ST, Scheff SW. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. Ann Neurol. 1990;27(5):457-64.
56. Wilson RS, Leurgans SE, Boyle PA, Schneider JA, Bennett DA. Neurodegenerative basis of age-related cognitive decline. Neurology. 2010;75(12):1070-8.
57. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res. 1975;12(3):189-98.
58. Nasreddine ZS, Phillips NA, Bedirian V, Charbonneau S, Whitehead V, Collin I, et al. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. J Am Geriatr Soc. 2005;53(4):695-9.
59. Morris JC. Clinical dementia rating: a reliable and valid diagnostic and staging measure for dementia of the Alzheimer type. Int Psychogeriatr. 1997;9 Suppl 1:173-6; discussion 7-8.
60. Rosen WG, Mohs RC, Davis KL. A new rating scale for Alzheimer's disease. Am J Psychiatry. 1984;141(11):1356-64.
61. Randolph C, Tierney MC, Mohr E, Chase TN. The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS): preliminary clinical validity. J Clin Exp Neuropsychol. 1998;20(3):310-9.
62. Gauthier S, editor. Clinical Diagnosis and Management of Alzheimer’s Disease. Third ed. United Kingdon: Informa Healthcare; 2006.
63. Brunnstrom HR, Englund EM. Cause of death in patients with dementia disorders. Eur J Neurol. 2009;16(4):488-92.

64. Best J, Chapleau M, Rabinovici GD. Posterior cortical atrophy: clinical, neuroimaging, and neuropathological features. Expert Rev Neurother. 2023;23(3):227-36.
65. Polsinelli AJ, Apostolova LG. Atypical Alzheimer Disease Variants. Continuum (Minneap Minn). 2022;28(3):676-701.
66. Nabizadeh F, Pirahesh K, Aarabi MH, Wennberg A, Pini L. Behavioral and dysexecutive variant of Alzheimer's disease: Insights from structural and molecular imaging studies. Heliyon. 2024;10(8):e29420.
67. Armstrong MJ, Litvan I, Lang AE, Bak TH, Bhatia KP, Borroni B, et al. Criteria for the diagnosis of corticobasal degeneration. Neurology. 2013;80(5):496-503.
68. Lopez OL, McDade E, Riverol M, Becker JT. Evolution of the diagnostic criteria for degenerative and cognitive disorders. Curr Opin Neurol. 2011;24(6):532-41.
69. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. Neurology. 1984;34(7):939-44.
70. Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, Kokmen E. Mild cognitive impairment: clinical characterization and outcome. Arch Neurol. 1999;56(3):303-8.
71. Tifratene K, Robert P, Metelkina A, Pradier C, Dartigues JF. Progression of mild cognitive impairment to dementia due to AD in clinical settings. Neurology. 2015;85(4):331-8.
72. Mitchell AJ, Shiri-Feshki M. Rate of progression of mild cognitive impairment to dementia--meta-analysis of 41 robust inception cohort studies. Acta Psychiatr Scand. 2009;119(4):252-65.
73. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack CR, Jr., Kawas CH, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011;7(3):263-9.
74. Albert MS, DeKosky ST, Dickson D, Dubois B, Feldman HH, Fox NC, et al. The diagnosis of mild cognitive impairment due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011;7(3):270-9.
75. Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. Alzheimers Dement. 2011;7(3):280-92.
76. Jack CR, Jr., Bennett DA, Blennow K, Carrillo MC, Feldman HH, Frisoni GB, et al. A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. Neurology. 2016;87(5):539-47.
77. White P, Hiley CR, Goodhardt MJ, Carrasco LH, Keet JP, Williams IE, et al. Neocortical cholinergic neurons in elderly people. Lancet. 1977;1(8013):668-71.
78. Perry EK, Tomlinson BE, Blessed G, Bergmann K, Gibson PH, Perry RH. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. Br Med J. 1978;2(6150):1457-9.

79. Gauthier S. Advances in the pharmacotherapy of Alzheimer's disease. CMAJ. 2002;166(5):616-23.
80. Poirier J, Delisle MC, Quirion R, Aubert I, Farlow M, Lahiri D, et al. Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. Proc Natl Acad Sci U S A. 1995;92(26):12260-4.
81. Tang BC, Wang YT, Ren J. Basic information about memantine and its treatment of Alzheimer's disease and other clinical applications. Ibrain. 2023;9(3):340-8.
82. Budd Haeberlein S, Aisen PS, Barkhof F, Chalkias S, Chen T, Cohen S, et al. Two Randomized Phase 3 Studies of Aducanumab in Early Alzheimer's Disease. J Prev Alzheimers Dis. 2022;9(2):197-210.
83. Sims JR, Zimmer JA, Evans CD, Lu M, Ardayfio P, Sparks J, et al. Donanemab in Early Symptomatic Alzheimer Disease: The TRAILBLAZER-ALZ 2 Randomized Clinical Trial. JAMA. 2023;330(6):512-27.
84. van Dyck CH, Swanson CJ, Aisen P, Bateman RJ, Chen C, Gee M, et al. Lecanemab in Early Alzheimer's Disease. N Engl J Med. 2023;388(1):9-21.
85. Ogbonmide T, Rathore R, Rangrej SB, Hutchinson S, Lewis M, Ojilere S, et al. Gene Therapy for Spinal Muscular Atrophy (SMA): A Review of Current Challenges and Safety Considerations for Onasemnogene Abeparvovec (Zolgensma). Cureus. 2023;15(3):e36197.
86. Singh A, Irfan H, Fatima E, Nazir Z, Verma A, Akilimali A. Revolutionary breakthrough: FDA approves CASGEVY, the first CRISPR/Cas9 gene therapy for sickle cell disease. Ann Med Surg (Lond). 2024;86(8):4555-9.
87. Dougherty JA, Dougherty KM. Valoctocogene Roxaparvovec and Etranacogene Dezaparavovec: Novel Gene Therapies for Hemophilia A and B. Ann Pharmacother. 2024;58(8):834-48.
88. Storandt M, Balota DA, Aschenbrenner AJ, Morris JC. Clinical and psychological characteristics of the initial cohort of the Dominantly Inherited Alzheimer Network (DIAN). Neuropsychology. 2014;28(1):19-29.
89. Aguirre-Acevedo DC, Lopera F, Henao E, Tirado V, Muñoz C, Giraldo M, et al. Cognitive Decline in a Colombian Kindred With Autosomal Dominant Alzheimer Disease: A Retrospective Cohort Study. JAMA Neurol. 2016;73(4):431-8.
90. Campion D, Dumanchin C, Hannequin D, Dubois B, Belliard S, Puel M, et al. Early-onset autosomal dominant Alzheimer disease: prevalence, genetic heterogeneity, and mutation spectrum. American Journal of Human Genetics. 1999;65(3):664-70.
91. Gatz M, Reynolds CA, Fratiglioni L, Johansson B, Mortimer JA, Berg S, et al. Role of genes and environments for explaining Alzheimer disease. Arch Gen Psychiatry. 2006;63(2):168-74.
92. Ridge PG, Hoyt KB, Boehme K, Mukherjee S, Crane PK, Haines JL, et al. Assessment of the genetic variance of late-onset Alzheimer's disease. Neurobiol Aging. 2016;41:200.e13-.e20.
93. Ridge PG, Mukherjee S, Crane PK, Kauwe JSK, Alzheimer's Disease Genetics C. Alzheimer's disease: analyzing the missing heritability. PLoS One. 2013;8(11):e79771.
94. Goate A, Chartier-Harlin MC, Mullan M, Brown J, Crawford F, Fidani L, et al. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature. 1991;349(6311):704-6.

95. Ryan NS, Rossor MN. Correlating familial Alzheimer's disease gene mutations with clinical phenotype. Biomark Med. 2010;4(1):99-112.
96. Arboleda-Velasquez JF, Lopera F, O'Hare M, Delgado-Tirado S, Marino C, Chmielewska N, et al. Resistance to autosomal dominant Alzheimer's disease in an APOE3 Christchurch homozygote: a case report. Nat Med. 2019;25(11):1680-3.
97. Blennow K, de Leon MJ, Zetterberg H. Alzheimer's disease. Lancet (London, England). 2006;368(9533):387-403.
98. Livingston G, Huntley J, Liu KY, Costafreda SG, Selbæk G, Alladi S, et al. Dementia prevention, intervention, and care: 2024 report of the Lancet standing Commission. Lancet. 2024;404(10452):572-628.
99. Bellenguez C, Kucukali F, Jansen IE, Kleineidam L, Moreno-Grau S, Amin N, et al. New insights into the genetic etiology of Alzheimer's disease and related dementias. Nat Genet. 2022;54(4):412-36.
100. Strittmatter WJ, Roses AD. Apolipoprotein E and Alzheimer disease. Proc Natl Acad Sci U S A. 1995;92(11):4725-7.
101. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. Science. 1988;240(4852):622-30.
102. Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, et al. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. Science. 1993;261(5123):921-3.
103. Tang MX, Stern Y, Marder K, Bell K, Gurland B, Lantigua R, et al. The APOE-epsilon4 allele and the risk of Alzheimer disease among African Americans, whites, and Hispanics. JAMA. 1998;279(10):751-5.
104. Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, et al. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease. A meta-analysis. APOE and Alzheimer Disease Meta Analysis Consortium. JAMA. 1997;278(16):1349-56.
105. Gregg RE, Zech LA, Schaefer EJ, Brewer HB, Jr. Apolipoprotein E metabolism in normolipoproteinemic human subjects. J Lipid Res. 1984;25(11):1167-76.
106. Liu CC, Zhao N, Fu Y, Wang N, Linares C, Tsai CW, et al. ApoE4 Accelerates Early Seeding of Amyloid Pathology. Neuron. 2017;96(5):1024-32.e3.
107. Laberge AM, Michaud J, Richter A, Lemyre E, Lambert M, Brais B, et al. Population history and its impact on medical genetics in Quebec. Clin Genet. 2005;68(4):287-301.
108. Shifman S, Darvasi A. The value of isolated populations. Nature Genetics. 2001;28(4):309-10.
109. Oguro-Ando A, Zuko A, Kleijer KTE, Burbach JPH. A current view on contactin-4, -5, and -6: Implications in neurodevelopmental disorders. Mol Cell Neurosci. 2017;81:72-83.
110. Mohebiany AN, Harroch S, Bouyain S. New insights into the roles of the contactin cell adhesion molecules in neural development. Adv Neurobiol. 2014;8:165-94.
111. Mercati O, Danckaert A, Andre-Leroux G, Bellinzoni M, Gouder L, Watanabe K, et al. Contactin 4, -5 and -6 differentially regulate neuritogenesis while they display identical PTPRG binding sites. Biol Open. 2013;2(3):324-34.

112. Toyoshima M, Sakurai K, Shimazaki K, Takeda Y, Nakamoto M, Serizawa S, et al. Preferential localization of neural cell recognition molecule NB-2 in developing glutamatergic neurons in the rat auditory brainstem. J Comp Neurol. 2009;513(4):349-62.
113. Karuppan SJ, Vogt A, Fischer Z, Ladutska A, Swiastyn J, McGraw HF, et al. Members of the vertebrate contactin and amyloid precursor protein families interact through a conserved interface. J Biol Chem. 2022;298(2):101541.
114. van Daalen E, Kemner C, Verbeek NE, van der Zwaag B, Dijkhuizen T, Rump P, et al. Social Responsiveness Scale-aided analysis of the clinical impact of copy number variations in autism. Neurogenetics. 2011;12(4):315-23.
115. Nava C, Keren B, Mignot C, Rastetter A, Chantot-Bastaraud S, Faudet A, et al. Prospective diagnostic analysis of copy number variants using SNP microarrays in individuals with autism spectrum disorders. Eur J Hum Genet. 2014;22(1):71-8.
116. Lionel AC, Crosbie J, Barbosa N, Goodale T, Thiruvahindrapuram B, Rickaby J, et al. Rare copy number variation discovery and cross-disorder comparisons identify risk genes for ADHD. Sci Transl Med. 2011;3(95):95ra75.
117. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nature Genetics. 2009;41(10):1088-93.
118. ALZGENE - GENE OVERVIEW OF ALL PUBLISHED AD-ASSOCIATION STUDIES FOR CLU 2011 [updated 2011-04-11. Available from: http://www.alzgene.org/geneoverview.asp?geneID=323.
119. Zhang S, Li X, Ma G, Jiang Y, Liao M, Feng R, et al. CLU rs9331888 Polymorphism Contributes to Alzheimer's Disease Susceptibility in Caucasian But Not East Asian Populations. Mol Neurobiol. 2016;53(3):1446-51.
120. Korenberg JR, Argraves KM, Chen XN, Tran H, Strickland, DK, et al. Chromosomal localization of human genes for the LDL receptor family member glycoprotein 330 (LRP2) and its associated protein RAP (LRPAP1). Genomics. 1994;22(1):88-93.
121. de Silva HV, Stuart WD, Duvic CR, Wetterau JR, Ray MJ, Ferguson DG, et al. A 70-kDa apolipoprotein designated ApoJ is a marker for subclasses of human plasma high density lipoproteins. J Biol Chem. 1990;265(22):13240-7.
122. Jenne DE, Lowin B, Peitsch MC, Böttcher A, Schmitz G, Tschopp J. Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein A-I in human plasma. J Biol Chem. 1991;266(17):11030-6.
123. Kim N, Yoo JC, Han JY, Hwang EM, Kim YS, Jeong EY, et al. Human nuclear clusterin mediates apoptosis by interacting with Bcl-XL through C-terminal coiled coil domain. J Cell Physiol. 2012;227(3):1157-67.
124. Zhang H, Kim JK, Edwards CA, Xu Z, Taichman R, Wang CY. Clusterin inhibits apoptosis by interacting with activated Bax. Nat Cell Biol. 2005;7(9):909-15.
125. Strocchi P, Smith MA, Perry G, Tamagno E, Danni O, Pession A, et al. Clusterin up-regulation following sub-lethal oxidative stress and lipid peroxidation in human neuroblastoma cells. Neurobiol Aging. 2006;27(11):1588-94.
126. Viard I, Wehrli P, Jornot L, Bullani R, Vechietti JL, Schifferli JA, et al. Clusterin gene expression mediates resistance to apoptotic cell death induced by heat shock and oxidative stress. J Invest Dermatol. 1999;112(3):290-6.

127. Humphreys DT, Carver JA, Easterbrook-Smith SB, Wilson MR. Clusterin has chaperone-like activity similar to that of small heat shock proteins. J Biol Chem. 1999;274(11):6875-81.
128. Ishikawa Y, Akasaka Y, Ishii T, Komiyama K, Masuda S, Asuwa N, et al. Distribution and synthesis of apolipoprotein J in the atherosclerotic aorta. Arterioscler Thromb Vasc Biol. 1998;18(4):665-72.
129. Ingram G, Loveless S, Howell OW, Hakobyan S, Dancey B, Harris CL, et al. Complement activation in multiple sclerosis plaques: an immunohistochemical analysis. Acta Neuropathol Commun. 2014;2:53.
130. Grewal RP, Morgan TE, Finch CE. C1qB and clusterin mRNA increase in association with neurodegeneration in sporadic amyotrophic lateral sclerosis. Neurosci Lett. 1999;271(1):65-7.
131. Wei L, Xue T, Wang J, Chen B, Lei Y, Huang Y, et al. Roles of clusterin in progression, chemoresistance and metastasis of human ovarian cancer. Int J Cancer. 2009;125(4):791-806.
132. Yom CK, Woo H-Y, Min SY, Kang SY, Kim HS. Clusterin overexpression and relapse-free survival in breast cancer. Anticancer Res. 2009;29(10):3909-12.
133. July LV, Akbari M, Zellweger T, Jones EC, Goldenberg SL, Gleave ME. Clusterin expression is significantly enhanced in prostate cancer cells following androgen withdrawal therapy. Prostate. 2002;50(3):179-88.
134. Yu JT, Li L, Zhu QX, Zhang Q, Zhang W, Wu ZC, et al. Implication of CLU gene polymorphisms in Chinese patients with Alzheimer's disease. Clin Chim Acta. 2010;411(19-20):1516-9.
135. Du W, Tan J, Xu W, Chen J, Wang L. Association between clusterin gene polymorphism rs11136000 and late-onset Alzheimer's disease susceptibility: A review and meta-analysis of case-control studies. Exp Ther Med. 2016;12(5):2915-27.
136. Bettens K, Brouwers N, Engelborghs S, Lambert JC, Rogaeva E, Vandenberghe R, et al. Both common variations and rare non-synonymous substitutions and small insertion/deletions in CLU are associated with increased Alzheimer risk. Mol Neurodegener. 2012;7:3.
137. Han Z, Qu J, Zhao J, Zou X. Analyzing 74,248 Samples Confirms the Association Between CLU rs11136000 Polymorphism and Alzheimer's Disease in Caucasian But Not Chinese population. Sci Rep. 2018;8(1):11062.
138. Jun G, Naj AC, Beecham GW, Wang LS, Buros J, Gallins PJ, et al. Meta-analysis confirms CR1, CLU, and PICALM as alzheimer disease risk loci and reveals interactions with APOE genotypes. Arch Neurol. 2010;67(12):1473-84.
139. Narayan P, Orte A, Clarke RW, Bolognesi B, Hook S, Ganzinger KA, et al. The extracellular chaperone clusterin sequesters oligomeric forms of the amyloid-β(1-40) peptide. Nat Struct Mol Biol. 2011;19(1):79-83.
140. Nuutinen T, Huuskonen J, Suuronen T, Ojala J, Miettinen R, Salminen A. Amyloid-beta 1-42 induced endocytosis and clusterin/apoJ protein accumulation in cultured human astrocytes. Neurochem Int. 2007;50(3):540-7.

141. Bell RD, Sagare AP, Friedman AE, Bedi GS, Holtzman DM, Deane R, et al. Transport pathways for clearance of human Alzheimer's amyloid beta-peptide and apolipoproteins E and J in the mouse central nervous system. J Cereb Blood Flow Metab. 2007;27(5):909-18.
142. Perrotte M, Le Page A, Fournet M, Le Sayec M, Rassart É, Fulop T, et al. Blood-based redox-signature and their association to the cognitive scores in MCI and Alzheimer's disease patients. Free Radic Biol Med. 2019;130:499-511.
143. McGeer PL, Kawamata T, Walker DG. Distribution of clusterin in Alzheimer brain tissue. Brain Res. 1992;579(2):337-41.
144. 2024 Alzheimer's disease facts and figures. Alzheimers Dement. 2024;20(5):3708-821.
145. Gustavsson A, Norton N, Fast T, Frolich L, Georges J, Holzapfel D, et al. Global estimates on the number of persons across the Alzheimer's disease continuum. Alzheimers Dement. 2023;19(2):658-70.
146. Schmitt FA, Davis DG, Wekstein DR, Smith CD, Ashford JW, Markesbery WR. "Preclinical" AD revisited: neuropathology of cognitively normal older adults. Neurology. 2000;55(3):370-6.
147. Erten-Lyons D, Woltjer RL, Dodge H, Nixon R, Vorobik R, Calvert JF, et al. Factors associated with resistance to dementia despite high Alzheimer disease pathology. Neurology. 2009;72(4):354-60.
148. Wu L, Rowley J, Mohades S, Leuzy A, Dauar MT, Shin M, et al. Dissociation between brain amyloid deposition and metabolism in early mild cognitive impairment. PLoS One. 2012;7(10):e47905.
149. Price JL, Morris JC. Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. Ann Neurol. 1999;45(3):358-68.
150. Haroutunian V, Purohit DP, Perl DP, Marin D, Khan K, Lantz M, et al. Neurofibrillary tangles in nondemented elderly subjects and mild Alzheimer disease. Arch Neurol. 1999;56(6):713-8.
151. Bouras C, Hof PR, Morrison JH. Neurofibrillary tangle densities in the hippocampal formation in a non-demented population define subgroups of patients with differential early pathologic changes. Neurosci Lett. 1993;153(2):131-5.
152. Mitchell TW, Mufson EJ, Schneider JA, Cochran EJ, Nissanov J, Han LY, et al. Parahippocampal tau pathology in healthy aging, mild cognitive impairment, and early Alzheimer's disease. Ann Neurol. 2002;51(2):182-9.
153. Tanner JA, Rabinovici GD. Relationship Between Tau and Cognition in the Evolution of Alzheimer's Disease: New Insights from Tau PET. J Nucl Med. 2021;62(5):612-3.
154. Pascoal TA, Mathotaarachchi S, Mohades S, Benedet AL, Chung CO, Shin M, et al. Amyloid-β and hyperphosphorylated tau synergy drives metabolic decline in preclinical Alzheimer's disease. Mol Psychiatry. 2017;22(2):306-11.
155. Boyle PA, Wang T, Yu L, Wilson RS, Dawe R, Arfanakis K, et al. To what degree is late life cognitive decline driven by age-related neuropathologies? Brain. 2021;144(7):2166-75.
156. Arendt T, Schindler C, Bruckner MK, Eschrich K, Bigl V, Zedlick D, et al. Plastic neuronal remodeling is impaired in patients with Alzheimer's disease carrying apolipoprotein epsilon 4 allele. J Neurosci. 1997;17(2):516-29.

157. Zarow C, Victoroff J. Increased apolipoprotein E mRNA in the hippocampus in Alzheimer disease and in rats after entorhinal cortex lesioning. Exp Neurol. 1998;149(1):79-86.
158. Veinbergs I, Masliah E. Synaptic alterations in apolipoprotein E knockout mice. Neuroscience. 1999;91(1):401-3.
159. Fisher A, Brandeis R, Chapman S, Pittel Z, Michaelson DM. M1 muscarinic agonist treatment reverses cognitive and cholinergic impairments of apolipoprotein E-deficient mice. J Neurochem. 1998;70(5):1991-7.
160. Masliah E. MM, Alford M., Ge N., Mucke L. . Abnormal synaptic regeneration in hAPP695 transgenic and apoE knockout mice. In: Khalid Iqbal JAM, Bengt Winblad, Henry M. Wisniewski, , editor. Research Advances in Alzheimer's Disease and Related Disorders: John Wiley & Sons; 1995. p. 405–14.
161. McComb M, Parambi R, Browne RW, Bodziak ML, Jakimovski D, Bergsland N, et al. Apolipoproteins AI and E are associated with neuroaxonal injury to gray matter in multiple sclerosis. Mult Scler Relat Disord. 2020;45:102389.
162. Engel S, Graetz C, Salmen A, Muthuraman M, Toenges G, Ambrosius B, et al. Is APOE ε4 associated with cognitive performance in early MS? Neurol Neuroimmunol Neuroinflamm. 2020;7(4).
163. Rong X, Chen J, Pan D, Wang Y, Zhang C, Tang Y. Association between Apolipoprotein E genotype and functional outcome in acute ischemic stroke. Aging (Albany NY). 2023;15(1):108-18.
164. Giarratana AO, Zheng C, Reddi S, Teng SL, Berger D, Adler D, et al. APOE4 genetic polymorphism results in impaired recovery in a repeated mild traumatic brain injury model and treatment with Bryostatin-1 improves outcomes. Sci Rep. 2020;10(1):19919.
165. Atherton K, Han X, Chung J, Cherry JD, Baucom Z, Saltiel N, et al. Association of APOE Genotypes and Chronic Traumatic Encephalopathy. JAMA Neurol. 2022;79(8):787-96.
166. Westra M, Gutierrez Y, MacGillavry HD. Contribution of Membrane Lipids to Postsynaptic Protein Organization. Front Synaptic Neurosci. 2021;13:790773.
167. Cho H, Shimazaki K, Takeuchi K, Kobayashi S, Watanabe K, Oguro K, et al. Biphasic changes in F3/contactin expression in the gerbil hippocampus after transient ischemia. Exp Brain Res. 1998;122(2):227-34.
168. Blain JF, Sullivan PM, Poirier J. A deficit in astroglial organization causes the impaired reactive sprouting in human apolipoprotein E4 targeted replacement mice. Neurobiol Dis. 2006;21(3):505-14.
169. Seshadri S, Wolf PA, Beiser A, Au R, McNulty K, White R, et al. Lifetime risk of dementia and Alzheimer's disease. The impact of mortality on risk estimates in the Framingham Study. Neurology. 1997;49(6):1498-504.
170. Gilsanz P, Lee C, Corrada MM, Kawas CH, Quesenberry CP, Jr., Whitmer RA. Reproductive period and risk of dementia in a diverse cohort of health care members. Neurology. 2019;92(17):e2005-e14.
171. Altmann A, Tian L, Henderson VW, Greicius MD. Sex modifies the APOE-related risk of developing Alzheimer disease. Ann Neurol. 2014;75(4):563-73.

172. Hohman TJ, Dumitrescu L, Barnes LL, Thambisetty M, Beecham G, Kunkle B, et al. Sex-Specific Association of Apolipoprotein E With Cerebrospinal Fluid Levels of Tau. JAMA Neurol. 2018;75(8):989-98.
173. Guo L, Zhong MB, Zhang L, Zhang B, Cai D. Sex Differences in Alzheimer's Disease: Insights From the Multiomics Landscape. Biol Psychiatry. 2022;91(1):61-71.
174. Lopez-Lee C, Torres ERS, Carling G, Gan L. Mechanisms of sex differences in Alzheimer's disease. Neuron. 2024;112(8):1208-21.

Appendix

1. Supplemental Material Manuscript 1:

Supplemental Material 1:

The Religious Order Study and the Memory and Aging Project Datasets Used in this Study: *Cognitive Evaluation and Diagnosis:* Participants were assessed annually with the Mini Mental State Evaluation (MMSE). Clinical diagnosis was made based on clinical data alone and blinded to postmortem data. Participants were diagnosed as: no cognitive impairment, MCI (one impaired domain) with no other cause of cognitive impairment, MCI (one impaired domain) with another cause of cognitive impairment, AD with no other cause of cognitive impairment (NINCDS Probable AD), AD with another cause of cognitive impairment (NINCDS possible AD) and other dementias.

Genotyping: Genotype data were generated either from peripheral blood mononuclear cells or from frozen brain tissues using Affymetrix or the Illumina Omniquad express gene chips. Imputation was performed by Sanger Imputation Service as described above for PREVENT-AD, using the same quality control filters.

RNA-Sequencing data: The ROS-MAP study provides RNA-Seq data from the dorsolateral prefrontal cortex [28]. Consolidated data is available at https://www.radc.rush.edu/home.htm. The Broad Institutes' Genomics Platform performed RNA-Seq library preparation using the strand specific dUTP method with poly-A selection [29]. Sequencing was performed on the Illumina HiSeq. Quantile normalization method was applied to FPKM first and combat package was used to remove potential batch effect.

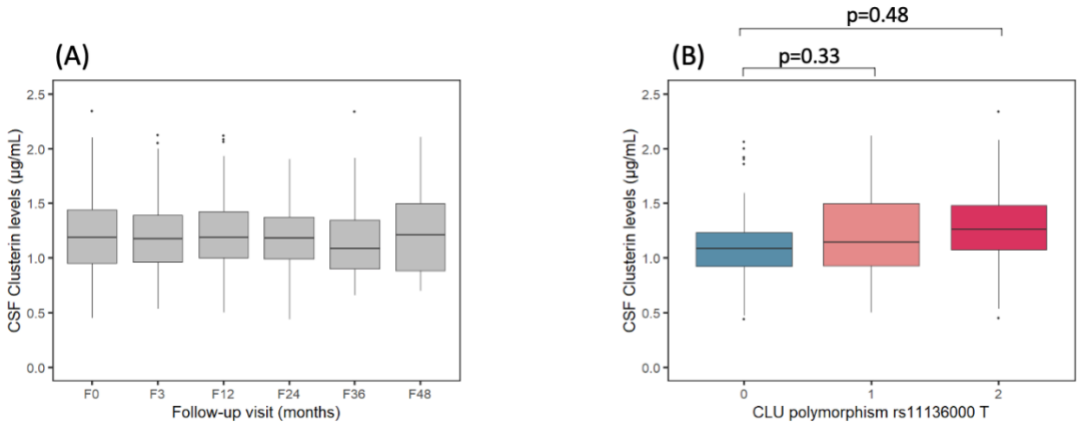
Supplementary Table 1: summary of results

	Contactin 5 CSF		Contactin 5 - brain		CNTN5 mRNA		Alzheimer Progression Score (APS)
	r^2	p	Full length	Isoform 3	Full length	Isoform 3	
Amyloid PET	0.1897	0.8983					
CSF Ab42	0.2591	0.00049					
CSF Aß40	0.134	0.00205					
PET Tau (entorhinal cortex)	0.2289	0.06482					
CSF Total Tau	0.4692	1.4E-11					
CSF p(181)Tau	0.5112	6.6E-12					
GAP43	0.5048	1.40E-06					
NEUROGRANIN	0.3726	0.00042					
SYNAPTOTAGMIN-1	0.4352	2.70E-05					
SNAP-25	0.4613	1.60E-05					
Cognitivelly unaffected Vs MCI		**p<0.0001**					
Cognitivelly unaffected Vs AD		**p<0.02**					
Braak stage			p>0.05	p>0.05	p=0.2	p=0.2	
Cerad stage					**p=0.066**	**p=0.027**	
CNTN5 Genotype G-positive Vs G-negative		p>0.05	p=0.07	p=0.10	p<0.003	p<0.005	p=0.01

Note: Green p values refer to the asymptomatic or early stage portion of the disease spectrum

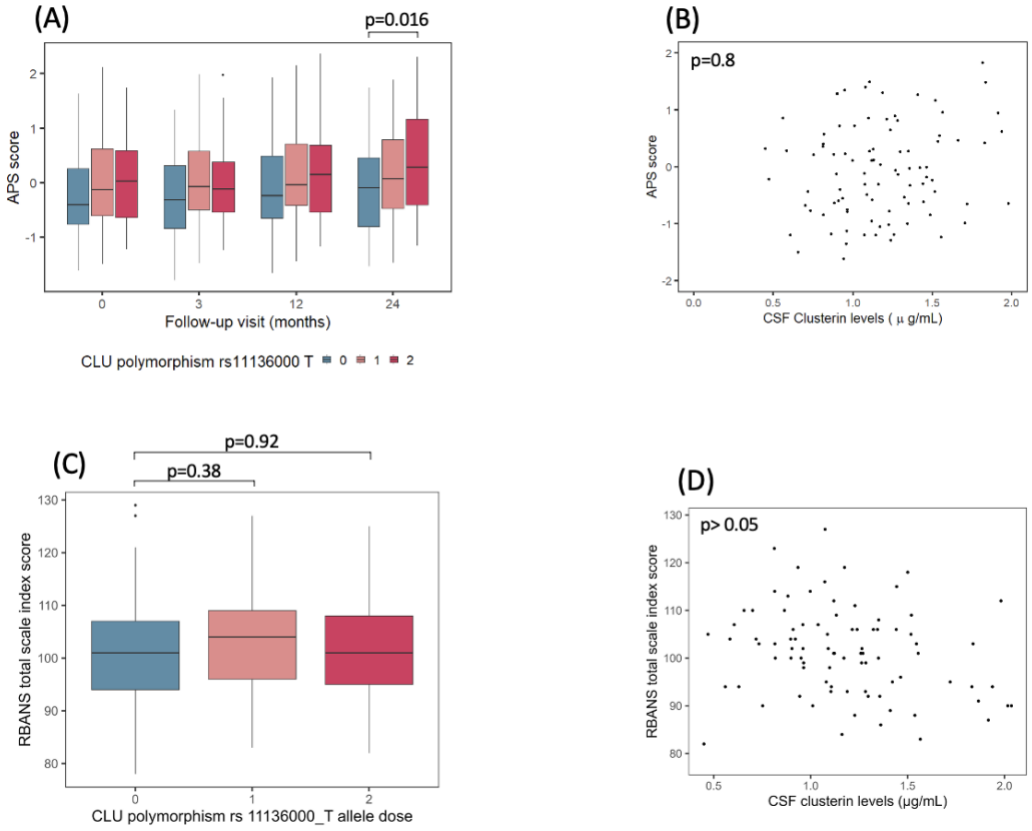
2. Supplemental Material Manuscript 3

Supplemental Figure 1: Effect of time and genotype on CSF Clusterin levels in the PREVENT-AD cohort.



A) There was no effect of time in CSF clusterin levels. B) There was no effect of *CLU* rs11136000 T genotype on clusterin levels at baseline (heterozygous, p=0.33; homozygous, p=0.48). Analyses were adjusted for age, sex, and APOE-ε4 presence.

Supplemental Figure 2: Associations between *CLU* rs 11136000 T genotype and CSF clusterin with cognition and disease progression.



A) There is a small increase in APS scores in subjects homozygous for the rs 11136000 T genotype on visit 24 (p=0.03); B) There was no association between APS scores and CSF clusterin levels at baseline (p=0.8); C) There was no effect of *CLU* rs11136000 T genotype on RBANS total index scores on baseline (heterozygous, p=0.38; homozygous, p=0.92). D) There was no association between RBANS total index scores and CSF clusterin levels at baseline (p>0.05). Analyses were adjusted for age, sex, and APOE-ε4 presence.