Insulin-like growth factor binding protein-2 and osteopontin in at-risk individuals with a parental history of Alzheimer's disease and in autopsy-confirmed Alzheimer brains

Marc James Quesnel

Supervisor: Dr. Judes Poirier

Integrated Program in Neuroscience

McGill University, Montréal

December 2023

A thesis submitted to McGill University in partial fulfilment of the requirements of the degree of MSc in Neuroscience

First published on December 6th 2024

Table Of Contents

Abstract

Introduction

Brain changes associated with Alzheimer's disease (AD) begin up to 20 years or more before the onset of symptoms. Unfortunately, current screening tools cannot identify pre-symptomatic individuals with complete accuracy. Furthermore, disease-modifying therapies for AD modestly slow down the progression of the disease in symptomatic individuals. Thus, it has been argued these therapies for AD are administered to patients too late in the disease. On the other hand, the decades-long pre-symptomatic period of AD provides an excellent window of opportunity to identify vulnerable individuals, unravel mechanism implicated in AD, and develop treatments to delay and/or prevent AD. Hence, we were prompted to search for potential protein candidates that could help identify pre-symptomatic individuals with more accuracy, and potentially serve as therapeutic targets.

Methods

We recruited pre-symptomatic individuals (60 + years old) with a parental or multi-sibling history of sporadic AD, who are at an increased risk of developing AD. We analyzed the cerebrospinal fluid (CSF) of these PREVENT-AD participants, which is believed to capture biochemical changes that occur in the brain. We studied two proteins in the CSF, notably, insulin like growth factor binding protein 2 (IGFBP2) and osteopontin (OPN), which are involved in insulin signaling and inflammation – two critical aspects of AD. We examined the relationship between these proteins and changes in cognition, AD biomarkers, and brain structure. To validate our findings, we analyzed data from an independent and well-characterized cohort (ADNI-1), consisting of cognitively unaffected elderly individuals and individuals with mild cognitive impairment (MCI). Finally, we examined IGFBP2 and OPN in a large sample of autopsy-confirmed AD and age-matched control brains from a unique population isolate from Eastern Canada (QFP cohort).

Results

Our results suggest IGFBP2 is upregulated in the brains of pre-symptomatic individuals from the PREVENT-AD cohort, and is associated with future decline in delayed memory and visuospatial

abilities. Furthermore, neuroimaging analyses revealed that IGFBP2 may be upregulated as a result of atrophy in several brain regions that are particularly vulnerable to early AD pathology, such as the entorhinal, piriform, inferior temporal and middle temporal regions. In the ADNI-1 cohort, CSF and plasma IGFBP2 were elevated in individuals with abnormal levels of CSF amyloid-beta 42 ($A\beta_{42}$) and CSF total-tau (CSF Aβ(+)/t-tau(+)). In addition, elevated plasma IGFBP2 was associated with a greater rate of conversion to AD. Finally, we observed a reduction in IGFBP2 mRNA levels in the frontal cortex of autopsy-confirmed AD brains from the QFP cohort. However, IGFBP2 protein levels did not differ between AD and control brains.

In our second study, we discovered OPN exhibits a bidirectional relationship with the stage of AD pathology in PREVENT-AD participants. Furthermore, positron emission tomography (PET) imaging revealed that OPN is associated with tau deposition in brain regions susceptible to early pathology, such as the entorhinal cortex, fusiform gyrus and lingual gyrus. Further neuroimaging analyses revealed that OPN is associated with reductions in cerebral ventricle volume. In the ADNI-1 cohort, CSF OPN was elevated in CSF Aβ(+)/t-tau(+) individuals, and was associated with an accelerated rate of conversion to AD. Finally, in the QFP cohort, OPN mRNA and protein levels were significantly elevated in the frontal cortex of autopsy-confirmed AD brains, compared to age-matched control brains.

Conclusion

IGFBP2 and OPN may be valuable biomarkers to identify pre-symptomatic individuals and facilitate screening for clinical trials for AD. Finally, the present work sheds light on the importance of insulin-related signaling and neuroinflammation in AD. Thus, we hope to spark interest in therapies targeting IGFBP2 and/or OPN during pre-symptomatic AD.

Résumé

Introduction

Les changements cérébraux associés à la maladie d'Alzheimer (MA) commencent jusqu'à 20 ans ou plus avant l'apparition des symptômes. Malheureusement, les outils de dépistage actuels ne peuvent pas identifier les individus présymptomatiques avec une certitude absolue. De plus, les traitements visant à modifier la progression de la MA ralentissent modestement la progression de la maladie chez les individus symptomatiques. Donc, il a été suggéré que ces traitements sont administrés aux patients trop tard dans la maladie. D'autre part, la période présymptomatique offre une excellente fenêtre d'opportunité pour identifier les individus les plus vulnérables, démêler les mécanismes moléculaires impliqués dans la MA, et développer des traitements visant à retarder et/ou prévenir la MA. Par conséquent, nous avons été incités à rechercher des protéines candidates qui pourraient aider à identifier les individus présymptomatiques avec plus de précision et potentiellement servir de cibles thérapeutiques.

Méthodes

Nous avons recruté des participants présymptomatiques (60 + ans) ayant un parent (ou plusieurs frères et sœurs) atteint(s) de la MA sporadique, qui montrent un risque accru de développer la MA. Nous avons analysé le liquide céphalo-rachidien (LCR) des participants de la cohorte PREVENT-AD, afin de détecter les changements biochimiques pertinents qui se produisent dans le cerveau. Nous avons étudié deux protéines dans le LCR, notamment, le facteur de liaison insulinique analogue de type 2 (IGFBP2) et osteopontine (OPN), qui sont impliquées dans la signalisation de l'insuline et l'inflammation cérébrale – deux aspects critiques de la MA. Nous avons examiné la relation qui existe entre ces protéines et les changements cognitifs, les biomarqueurs de la MA, et la structure tridimensionnelle cérébrale. Pour confirmer nos résultats, nous avons ensuite analysé les données d'une cohorte indépendante et bien caractérisée (la cohorte ADNI-1), composée d'individus non affectés sur le plan cognitif ainsi que d'individus atteints de troubles cognitifs légers. Enfin, nous avons examiné IGFBP2 et OPN dans un échantillon important de cerveaux Alzheimer dont la pathologie fut confirmée par autopsie et, de cerveaux témoins appariés pour

l'âge, qui proviennent d'un isolat populationnel génétique unique de l'Est du Canada (la cohorte QFP).

Résultats

Nos résultats suggèrent que IGFBP2 est augmentée dans le cerveau des individus présymptomatiques de la cohorte PREVENT-AD, et est associée à un déclin longitudinal de la mémoire et des capacités visuospatiales. De plus, des analyses de neuroimagerie ont révélé que IGFBP2 est fortement liée à l'atrophie de plusieurs régions cérébrales vulnérables à la pathologie précoce de la MA, telles que les régions corticales entorhinale, piriforme, temporale inferieure et temporale moyenne. Dans la cohorte ADNI-1, l'IGFBP2 était élevée dans le LCR et plasma des individus présentant des taux anormaux de beta-amyloïde 42 ($A\beta_{42}$) et de tau total (t-tau) dans le LCR (LCR Aβ(+)/t-tau(+)). De plus, l'IGFBP2 plasmatique était associée à un taux de conversion accéléré vers la MA. Enfin, utilisant des cerveaux autopsiés de la cohorte QFP, nous avons observé des réductions dans les niveaux d'ARNm de IGFBP2 dans le cortex frontal des patients Alzheimer. Cependant, les niveaux de protéine corticale de IGFBP2 ne différaient pas entre les cerveaux Alzheimer et ceux des témoins.

Dans notre deuxième étude, OPN présentait une relation bidirectionnelle avec les différents stades de la pathologie de la MA - chez les participants PREVENT-AD. En outre, l'imagerie par tomographie par émission de positrons a révélé que l'OPN est associée à un dépôt de la protéine tau dans les régions cérébrales sensibles à une pathologie précoce - telles que le cortex entorhinal, le gyrus fusiforme et le gyrus lingual. Des analyses subséquentes de neuroimagerie ont révélé que l'OPN est associée à une réduction du volume ventriculaire cérébral. Dans la cohorte ADNI-1, l'OPN était élevée dans le LCR chez les individus considérés positifs à la Aβ(+)/t-tau(+) dans le LCR. De plus, les niveaux élevés d'OPN étaient associés à un taux prononcé de conversion vers la MA. Enfin, dans la cohorte QFP, les niveaux d'ARNm et de protéines cérébrales de OPN étaient augmentés dans le cortex frontal des cerveaux Alzheimer comparés aux sujets témoins autopsiés.

Conclusion

IGFBP2 et OPN peuvent être considérées comme des marqueurs précieux permettant d'identifier les personnes présymptomatiques et peuvent faciliter le dépistage précoce des sujets à recruter pour les essais cliniques visant à ralentir ou même prévenir la MA. Enfin, le présent travail met en

lumière l'importance de la signalisation insulinique et de la neuroinflammation dans la MA. Nous espérons susciter l'intérêt de thérapies ciblant IGFBP2 et/ou OPN visant spécifiquement le stade présymptomatique de la MA.

Acknowledgments

First and foremost, I would like to thank my supervisor, Dr. Judes Poirier, for welcoming me in his lab during my undergraduate and graduate studies. I have learned a lot over the past two years, which will help me in my future endeavors. I would also like to thank you for your encouragement, financial support, and giving me the opportunity to attend and present my research at the Alzheimer's Association International Conferences over the past two years.

I am grateful to my advisory committee members, Dr. Lalit Srivastava and Dr. Ridha Joober, for their valuable suggestions. Furthermore, I would like to thank my program mentors Dr. Thomas Stroh and Dr. Mark Brandon. Finally, I would like to thank the Fonds de Recherche du Québec-Santé, the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada, for their financial support throughout my undergraduate and graduate studies.

I would like to acknowledge the PREVENT-AD participants, STOP-AD physicians, nurses, research assistants, students and affiliated members who have generously committed themselves to this important research project. To this end, I would like to acknowledge Cynthia Picard and Anne Labonté for their role in the collection of data acquired from the PREVENT-AD and QFP cohorts. Finally, I would like to thank Dr. Jose A. Correa and PhD Candidate in Biostatistics, Shuo (Mila) Sun, for their generosity in helping me with my statistical analyses.

Finally, I would like to thank my family. First, to my parents, Carolyn Toffi and James Quesnel, thank you for supporting me during my university studies, and encouraging me to challenge myself. To my brother, David Quesnel, thank you for encouraging me to pursue my hobbies - playing ice/ball hockey. I would also like to dedicate my thesis to my resilient grandmother, Patricia Martin-Toffi, and to my late grandparents, Elia Toffi, Harold Quesnel, Mary Quesnel (née Coleman), as well as my late aunt Kathryn Toffi, who have all played a big part in my life, and helped shape me into the person that I am today. Finally, I would like to thank my Shetland Sheepdog, Dusty Toffi, for cheering me up during challenging times.

In the end, I hope my work can help contribute to the prevention of AD and help all those affected by the disease.

Marc

Contribution to original knowledge

The first manuscript is an original article that has been submitted for publication to the journal *Brain* (impact factor of 14.5 in 2022). In this manuscript, we are the first group to examine the role of IGFBP2 in the earliest possible stage of sporadic AD (pre-symptomatic AD), as the existing literature has investigated the role of IGFBP2 in late-stage AD, which is when most brain changes have already occurred. More specifically, we measured CSF IGFBP2 levels over the course of five years, with the previous studies analyzing baseline measurements of IGFBP2. We discovered that CSF IGFBP2 is associated with longitudinal changes in cognition, with previous studies focusing on cross-sectional analyses. Similarly, we analyzed IGFBP2 levels across distinct stages of AD pathology, as recommended by recent biological frameworks. Finally, although one pilot study has examined IGFBP2 in the temporal cortex of the human AD brain, our group analyzed IGFBP2 in the frontal cortex of autopsy-confirmed AD brains and age-matched elderly control brains.

The second manuscript is an original article that has been submitted for publication to the journal *Molecular Neurodegeneration* (impact factor of 15.1 in 2022). Similar to the first manuscript, we are the first to examine the role of OPN during the pre-symptomatic stage of sporadic AD, as previous studies have investigated the role of OPN in pre-symptomatic familial AD (1% of total AD cases), and late-stage sporadic AD. Although OPN levels have been previously measured in the hippocampus and frontal cortex of the human AD brain (pilot studies), we analyzed OPN gene expression and protein levels in the frontal cortex of a large sample of autopsy-confirmed AD brains and age-matched elderly control brains from a unique population isolate.

Contribution of authors

Contribution to the overall thesis

Marc Quesnel: Preparation of the thesis.

Judes Poirier: Draft editing.

Chapter 1

Marc Quesnel: Literature review and writing of text.

Judes Poirier: Draft editing.

Chapter 2

Marc Quesnel: Study design, data collection, data analysis and writing of manuscript.

Judes Poirier: Study design and writing of manuscript.

Anne Labonté: Data collection.

Cynthia Picard: Data collection.

Henrik Zetterberg: Study design and data collection.

Kaj Blennow: Study design and data collection.

Sylvia Villeneuve: Study design and data collection.

PREVENT-AD Research Group: Data collection.

ADNI: Data collection.

Chapter 3

Marc Quesnel: Study design, data collection, data analysis and writing of manuscript.

Judes Poirier: Study design and writing of manuscript.

Anne Labonté: Data collection.

Cynthia Picard: Data collection.

Henrik Zetterberg: Study design and data collection.

Kaj Blennow: Study design and data collection.

Sylvia Villeneuve: Study design and data collection.

PREVENT-AD Research Group: Data collection.

ADNI: Data collection.

Chapter 4

Marc Quesnel: Writing of text.

Judes Poirier: Draft editing.

List of figures

Chapter 2

Chapter 3

List of tables

Chapter 2

Chapter 3

List of abbreviations

Aβ: Amyloid-beta

$A\beta_{42}$: Amyloid-beta 42

AD: Alzheimer's disease

ADNI: Alzheimer's Disease Neuroimaging Initiative

AICD: APP Intracellular domain

AKT: Protein Kinase B

ALCAM: Activated leukocyte cell adhesion molecule

ALS: Amyotrophic lateral sclerosis

ANCOVA: Analysis of covariance

APOE/APOE: Apolipoprotein E

APP/APP: Amyloid Precursor Protein

A/T/N: Amyloid/Tau/Neurodegeneration

AXL: Tyrosine-protein kinase receptor UFO

BACE1: Beta-site APP cleaving enzyme 1

BBB: Blood-brain barrier

BIOMARKAPD: Biomarkers for Alzheimer's and Parkinson's Disease

BLM: Bleomycin

BMI: Body mass index

BP: Blood pressure

CAIDE: Cardiovascular Risk Factors, Aging, and Incidence of Dementia

CA1: *Cornu Ammonis* area 1

CCNA: Canadian Consortium on Neurodegeneration in Aging

cDNA: Complementary DNA

CDR: Clinical Dementia Rating

CD44: Cluster of differentiation 44

CHI3L1: Chitinase-3-like protein 1

CI: Confidence interval

CJD: Creutzfeldt-Jakob disease

CNS: Central nervous system

CNTN1: Contactin-1

CSF: Cerebrospinal fluid

CSTB: Cystatin-B

CTF-α: C-terminal fragment-alpha

CTF-β: C-terminal fragment-beta

CTL: Control

CU: Cognitively unaffected

CV: Coefficient of variability

CXCL16: C-X-C motif chemokine 16

DNA: Deoxyribonucleic acid

DSM-V: Diagnostic and Statistical Manual of mental disorders, Fifth edition

EC: Entorhinal cortex

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

FDG: Fluorodeoxyglucose

fMRI: Functional magnetic resonance imaging

FRQS: Fonds de Recherche du Québec – Santé

FTD: Frontotemporal dementia

GAP43: Growth-associated protein 43

GSK3: Glycogen synthase kinase-3

GWAS: Genome-wide association study

HbA1c: Hemoglobin A1C

HR: Hazard ratio

HRT: Hormone replacement therapy

ICV: Intracerebroventricular

IFN-α: Interferon-alpha

IFN-γ: Interferon-gamma

IGF: Insulin-like growth factor

IGF-1/IGF-I: Insulin-like growth factor 1

IGF-2/IGF-II: Insulin-like growth factor 2

IGFBP: Insulin like growth factor binding protein

IGFBP2/IGFBP2: Insulin like growth factor binding protein 2

IGFBP5: Insulin like growth factor binding protein 5

IL-6RA: Interleukin-6 receptor subunit alpha

IL-10: Interleukin-10

IL-12: Interleukin-12

IL-17: Interleukin-17

IL-18BP : Interleukin-18-binding protein

IL-23: Interleukin-23

IL-27: Interleukin-27

KLK6: Kallikrein-6

KO: Knockout

LBD: Lewy body dementia

$\log_2$: Logarithm base 2

$\log_{10}$: Logarithm base 10

LP: Lumbar puncture

LTBR: Lymphotoxin-beta receptor

MAPK: Mitogen-activated protein kinase

MAPT: Microtubule-associated protein tau

MCI: Mild cognitive impairment

mmHg: Millimeters of mercury

MMP: Matrix metalloproteinase

MMP-2: Matrix metalloproteinase-2

MMP-3: Matrix metalloproteinase-3

MMP-7: Matrix metalloproteinase-7

MMP-9: Matrix metalloproteinase-9

MMSE: Mini-Mental State Examination

MoCA: Montréal Cognitive Assessment

MRI: Magnetic Resonance Imaging

mRNA: Messenger ribonucleic acid

NFT: Neurofibrillary tangle

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

NMDA: N-methyl-D-aspartate

NPX: Normalized Protein eXpression

NRGN: Neurogranin

NSAID: Nonsteroidal anti-inflammatory drug

OPN: Osteopontin

PD: Parkinson's disease

PEA: Proximity extension assay

PET: Positron Emission Tomography

PHF: Paired helical filaments

Phospho-tau: phosphorylated tau

PiB: Pittsburgh compound B

PI3K-AKT: Phosphoinositide 3-kinase-protein kinase B

PNS: Peripheral nervous system

PREVENT-AD: PRe-symptomatic EValuation of Experimental or Novel Treatments for Alzheimer's Disease

PSEN1/2: Presenilin 1, Presenilin 2

PSP: Progressive supranuclear palsy

P-tau: Phosphorylated tau

p_{181}-tau: Phosphorylated tau at residue 181

p_{231}-tau: Phosphorylated tau at residue 231

QFP: Québec Founder Population

qPCR: Quantitative polymerase chain reaction

RBANS: Repeatable Battery for the Assessment of Neuropsychological Status

RNA: Ribonucleic acid

ROI: Region of interest

Rpm: Revolutions per minute

SD: Standard deviation

SEM: Standard error of the mean

SHANK3: SH3 And Multiple Ankyrin Repeat Domains 3

SHPS1: Tyrosine-protein phosphatase non-receptor type substrate 1

SNAP: Suspected non-Alzheimer pathology

SNAP25: Synaptosomal-associated protein 25

SNARE: SNAP receptor

SPP1/SPP1: Secreted phosphoprotein 1

SUVR: Standardized uptake value ratio

SYT1: Synaptotagmin-1

TBI: Traumatic brain injury

TDP-43: TAR DNA-binding protein 23

TGF-β1: Transforming growth factor-beta 1

TNF-α: Tumor necrosis factor alpha

TNFRSF14: TNF receptor superfamily member 14

TREM2/TREM-2: Triggering receptor expressed on myeloid cells 2

t-tau: Total tau

UECL: Unilateral entorhinal cortex lesion

UPAR: Urokinase plasminogen activator surface receptor

VD: Vascular dementia

Introduction

Alzheimer's disease: a global health priority

In 2019, 57.4 million individuals worldwide were estimated to have dementia.[1] Due to increases in life expectancy and population growth, the number of adults living with dementia is expected to reach 152.8 million by 2050.[1] Furthermore, in 2019 alone, the global economic burden of Alzheimer's disease (AD) and related dementias was estimated to be $2.8 trillion.[2] By 2050, the cost of dementia care is projected to reach $16.9 trillion.[2] AD remains one of the most challenging medical mysteries of our generation, as it is the most common cause of dementia - accounting for 60-80% of all cases.[3] Unfortunately, a cure does not exist for AD, and disease-modifying therapies offer limited benefits, as they modestly slow down the progression of the disease.[4-6] Furthermore, these disease-modifying therapies are also associated with alarming rates of adverse events such as cerebral hemorrhages and cerebral edema.[4-6] Therefore, in order to evade the rise in AD cases and decrease the economic burden associated with AD, studying AD during the pre-symptomatic stage is imperative.[1,2,7] More specifically, the pre-symptomatic stage of AD offers an excellent window of opportunity to prevent, delay and/or slow down AD.[7] However, current screening methods cannot identify pre-symptomatic individuals with complete accuracy.[7] Thus, to this end, we were prompted to search for potential protein candidates that could help identify pre-symptomatic individuals with more accuracy, and potentially serve as therapeutic targets.

1. Literature review

1.1 Historical perspective: the case of Auguste Deter

In 1901, Auguste Deter, a 51-year-old woman living in Germany, was administered to the Frankfurt Psychiatric Hospital due to her symptoms that included difficulties in memory and language, disorientation, paranoia and auditory hallucinations.[8,9] However, at the time, Auguste's situation was unusual, as she was relatively young to be exhibiting signs of dementia.[8,9] Nevertheless, it was at this hospital that an intrigued psychiatrist and neuropathologist, Dr. Alois Alzheimer, observed Auguste Deter over the course of five years.[8,9] Following her death in April 1906, Dr. Alzheimer received permission to perform an autopsy on Auguste Deter's brain and identified two histological features in the cerebral cortex, most notably, extracellular amyloid-beta (Aβ) plaques and

intracellular neurofibrillary tangles (NFTs).[8,9] It was in November 1906 that Dr. Alzheimer presented the first known case of AD at the 37th Meeting of South-West German Psychiatrists in Tubingen.[8,9] However, it was not until 1910 that the term Alzheimer's disease was coined.[8,9] Since then, Dr. Alzheimer has been revered, for establishing the link between specific histopathological findings and clinical symptoms.

1.2 Clinical diagnosis of dementia and AD

In order to perform a clinical diagnosis of AD two steps are necessary. First, a diagnosis of dementia needs to be made using the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V).[10] According to the criteria, an individual must display significant cognitive impairments in at least one of the following: memory and learning, language, attention, executive function, social cognition and perceptual-motor function.[10] Most importantly, these cognitive deficits must be severe enough to interfere with activities of daily living.[10] However, if an individual displays a subtle decline in cognitive abilities that is unusual for their age but can still perform activities of daily living, a diagnosis of mild cognitive impairment (MCI) can be performed.[10] Thus, this intermediate stage, MCI, is often referred to as the prodromal phase of AD.[7] Several neuropsychological assessments such as the Mini-Mental State Examination (MMSE)[11] and Montreal Cognitive Assessment (MoCA)[12] can assist physicians in evaluating a patient's cognitive abilities and clinical stage.

Following a diagnosis of dementia, the NINCDS-ADRDA criteria can be used to make a diagnosis of probable AD.[13] Based on the presence or absence of specific cognitive deficits, physicians are able to exclude other causes of dementia.[13] Thus, in order to receive a diagnosis of probable AD, an individual must display a progressive decline in episodic memory and other cognitive functions.[13] Furthermore, the onset of symptoms must be insidious and occur between the ages of 40 and 90 (generally after the age of 65).[13] Finally, individuals must be free of disturbances of consciousness, systemic disorders or other brain diseases that could, on their own, explain the progressive deficits in memory and cognition.[13] However, it is important to recognize that the clinical symptoms of non-AD dementias such as vascular dementia (VD), frontotemporal dementia (FTD) and Lewy body dementia (LBD), often resemble the NINCDS-ADRDA criteria that are required for a probable diagnosis of AD.[14-16] Hence, the specificity of these diagnostic criteria can be as low as 44.3%.[17] In the end, a definitive diagnosis of AD can only be made at death following

histopathologic confirmation.[13] Nevertheless, recent advancements in positron emission tomography (PET) tracers that map amyloid and tau deposition in the living brain are currently improving diagnosis accuracy in memory clinics around the world, however, the cost of these imaging techniques remains a serious deterrent.[7]

1.3 Neuropathological hallmarks of AD

1.3.1 Amyloid-beta plaques

Upon post-mortem examination, amyloid-beta plaques must be present in the brain for a definite diagnosis of AD.[13] Amyloid plaques are extracellular aggregates that are composed of Aβ peptides, which are generated through the sequential cleavage of amyloid precursor protein (APP), a transmembrane protein that is located on the surface of neurons.[18] APP has been demonstrated to be implicated in numerous physiological processes, such as neurite outgrowth.[19] In the amyloidogenic pathway, APP is first cleaved by the beta-secretase enzyme (also known as beta site APP cleaving enzyme 1, BACE1).[18,20] This process results in a soluble peptide, soluble APPβ, as well as a membrane-bound fragment, CTF-β (C99).[18,20] Finally, CTF-β is cleaved by the gamma-secretase enzyme, which generates soluble Aβ peptides of various lengths, ranging from 36 to 43 amino acids, as well as a cytosolic peptide, APP intracellular domain (AICD).[18] In the end, it is the longer forms of Aβ, such as $A\beta_{42}$, that are more prone to aggregation in the brain, are more toxic, and are the major constituent of plaques.[21,22] During the aggregation process, Aβ peptides aggregate into oligomers and eventually, fibrils, which are the main component of plaques.[18] It is widely believed that intermediate Aβ species, notably Aβ oligomers, are more toxic than mature Aβ fibrils (although the surface of fibrils can accelerate the oligomerization process).[23,24] It is possible Aβ oligomers may exert their toxicity by binding and activating cell-surface receptors and forming pores in the cell membrane of neurons.[24]

However, it is important to consider that APP is also processed through a distinct, non-amyloidogenic pathway.[18,25] In this pathway, the alpha-secretase enzyme first cleaves APP in the middle of the Aβ peptide sequence, between amino acids 16 and 17 - thus preventing the production of Aβ.[18,25] This initial cleavage generates a soluble peptide, soluble APPα, as well as a membrane-bound fragment, CTF-α (C83).[18,25] The subsequent cleavage of CTF-α by gamma-secretase results in the secretion of P3, as well as the cytosolic peptide AICD.[18,25]

1.3.2 Neurofibrillary tangles

The second histological abnormality originally identified by Dr. Alzheimer is composed of the microtubule-stabilizing protein tau.[8,9] These critical cytoskeletal structures, microtubules, enable vital cargo such as mitochondria and synaptic vesicle precursors to be transported along axons to distal regions of the neuron.[26] Thus, when tau is abnormally ("hyper") phosphorylated, such as in AD and in other tauopathies, tau detaches from microtubules.[27,28] As a result, microtubules become unstable and disassemble, ultimately leading to impaired axonal transport.[27,28] Once tau proteins become hyperphosphorylated and detach from microtubules, they form intertwined oligomers known as paired helical filaments (PHFs).[27] Furthermore, when several PHFs aggregate, NFTs are formed.[27] In the early stages of AD, NFTs accumulate in the cell body of neurons, while they accumulate in neurites as "neuropil threads" as the disease progresses.[29] Eventually, NFTs escape from dying neurons and accumulate as extracellular "ghost NFTs".[30]

The distribution of NFTs throughout the course of the disease has been documented to follow a common pattern across individuals.[31] This pattern has been demonstrated through post-mortem brain tissue studies, and can be described using Braak stages.[31] In the earliest stage of the disease (Braak stage I), NFTs accumulate in the transentorhinal cortex.[31] As the disease progresses, NFTs emerge in the entorhinal cortex (Braak Stage II) and in limbic structures such as the hippocampus (Braak Stages III-IV).[31] In the final stages, the neocortex succumbs to tau deposition (Braak Stages V-VI).[31] In recent years, it has been argued that this stereotypical progression can be explained by a prion-like propagation of tau.[32] More specifically, it has been demonstrated that misfolded forms of tau can be secreted in exosomes in a trans-synaptic fashion, be taken up by nearby neurons, and serve as a template for native tau to misfold and aggregate.[33]

1.3.3 Neuronal and synaptic loss

The entorhinal cortex (EC) is the first region that is affected in AD. Indeed, in post-mortem brain tissue experiments, individuals with very mild AD exhibited 32% fewer EC neurons, compared to cognitively unaffected (CU) individuals.[34] In severe dementia cases, the number of layer II EC neurons was reduced by 90%.[34] As the EC serves as the main input to the hippocampus, this loss of EC neurons leads to a loss of synapses in the hippocampus.[35] Indeed, post-mortem studies of early AD brains have demonstrated that synaptophysin-immunoreactive presynaptic terminals are

reduced by 20% in the outer molecular layer of the dentate gyrus.[36] Furthermore, in definite AD, synaptophysin has been found to be reduced by 55% in the hippocampus.[37] Such a prominent loss of synapses in the hippocampus contributes to the early impairments in episodic memory that are observed in AD, as post-mortem hippocampal synaptophysin levels have been demonstrated to be positively correlated with antemortem performances on neuropsychological assessments in humans.[37]

Another key region that is affected early on in AD is the basal forebrain cholinergic network.[38] Indeed, it has been demonstrated that acetylcholine plays a pivotal role in memory processes.[38] For instance, the nucleus basalis of Meynert and the medial septum provide vital cholinergic inputs to the neocortex and hippocampus, respectively.[38] In the end, post-mortem brain tissue studies have demonstrated the selective vulnerability of these cholinergic neurons to early AD pathology.[38,39] More specifically, early studies found that individuals with AD exhibited 79% fewer neurons in the nucleus basalis of Meynert, compared to controls.[39]

1.3.4 Neuroinflammation

In addition to the traditional hallmarks typically associated with AD, chronic neuroinflammation is a cardinal feature of AD.[40] Indeed, in response to Aβ plaques and neuronal debris, microglia, the brain-resident immune cells, release pro-inflammatory cytokines that are toxic to healthy neurons.[40] In the end, this exacerbates the pathogenic processes that initially triggered the neuroinflammation.[40] Furthermore, several genetic risk factors for AD, such as variants in triggering receptor expressed on myeloid cells 2 (*TREM2)*, have been associated with inflammatory and immune-related processes.[41] Finally, in a prospective study, elevated circulating levels of inflammatory markers during midlife were associated with decreased hippocampal volume and reduced episodic memory, 24 years later.[42]

Although the use of non-steroidal anti-inflammatory drugs (NSAIDS) has been associated with a reduced prevalence of AD in relatively young elderly,[43] clinical trials conducted over the past few decades have failed to demonstrate positive outcomes.[44,45] More specifically, our group has previously demonstrated that the bidaily use of the NSAID naproxen sodium did not reduce the progression of AD in CU individuals with a parental history of AD, compared to placebo - over the course of 2 years.[46] Thus, our findings from the clinical trial suggest that the relationship

between neuroinflammation and AD is far more complex than previously anticipated. That is, it appears neuroinflammation can be beneficial or detrimental, depending on the stage of the disease. To support this hypothesis, we recently discovered that several immune-related proteins exhibit a bidirectional relationship with the stage of AD pathology.[47] These immune-related proteins were downregulated in response to early Aβ deposition and upregulated in response to cell death.[47]

In Chapter 3 of the present thesis, we aimed to elucidate the role of a key pro-inflammatory protein, namely, osteopontin (OPN).[48]

1.4 Biomarkers

1.4.1 Core AD CSF biomarkers

Since the cerebrospinal fluid (CSF) is in direct contact with the extracellular environment in the brain, it is a valuable tool for capturing biochemical changes that occur in the living brain.[49] Importantly, the core AD CSF biomarkers are highly concordant with PET imaging findings.[50] For instance, CSF biomarkers are capable of identifying early-stage AD with as much accuracy as amyloid PET scans.[50] Finally, it is much more practical to implement CSF biomarkers in the clinic, as they are far less costly than PET and magnetic resonance imaging (MRI) scans. For the purpose of this thesis, we will focus on the core AD CSF biomarkers, notably amyloid-beta 42 ($A\beta_{42}$), phosphorylated tau (p-tau) and total tau (t-tau), which are used in research and clinical settings.[49] Finally, we will examine emerging biomarkers of synaptic dysfunction and loss (synaptotagmin-1 (SYT1), synaptosomal-associated protein 25 (SNAP25), growth-associated protein 43 (GAP43) and neurogranin (NRGN)), which have only been used in research settings.[51]

1.4.1.1 $A\beta_{42}$

Post-mortem studies have demonstrated that CSF $A\beta_{42}$ is negatively correlated with Aβ deposition in the brain.[52,53] It is believed that as Aβ peptides accumulate in plaques in the brain, sequestered Aβ peptides become less available to enter the CSF.[49,52,53] Indeed, patients with AD exhibit a 32 to 71% reduction in CSF $A\beta_{42}$, compared to elderly controls.[49] Furthermore, CSF $A\beta_{42}$ is a powerful tool for differentiating AD from normal aging, as it possesses a sensitivity of 86% and specificity of 89%.[49] More specifically, CSF $A\beta_{42}$ is efficient in differentiating AD from depression, Parkinson's disease (PD) and progressive supranuclear palsy (PSP).[49] However, it is

important to acknowledge that mild-moderate reductions in CSF $A\beta_{42}$ have also been observed in other neurological disorders, where Aβ plaques are not present. For instance, reductions in CSF $A\beta_{42}$ have been found in FTD, LBD, VD, Creutzfeldt-Jakob disease (CJD), amyotrophic lateral sclerosis (ALS) and multiple system atrophy.[49]

1.4.1.2 P-tau

CSF p-tau is positively correlated with hyperphosphorylated tau and NFT levels in post-mortem brain tissues.[53] Thus, it is believed that CSF p-tau is representative of the phosphorylation state of tau in the brain.[49,53] Indeed, patients with AD exhibit a 200 to 348 % increase in CSF ptau, compared to elderly controls.[49] Similar to CSF $A\beta_{42}$, CSF p-tau is a powerful tool for differentiating AD from normal aging, as it possesses a sensitivity of 81% and specificity of 91%.[49] Furthermore, compared to CSF $A\beta_{42}$ and CSF t-tau, CSF p-tau possesses a greater ability to differentiate AD from other neurological disorders, such as depression, stroke, PD, VD, FTD, LBD, CJD and ALS.[49] Although tau possesses 85 possible phosphorylation sites,[54] the most widely used epitopes to measure CSF p-tau are p_{181}-tau[55] and p_{231}-tau.[56] Finally, in recent years, it has been demonstrated that plasma levels of p_{181}-tau, p_{217}-tau and p_{231}-tau possess impressive diagnostic potentials – thus offering a simple and less invasive alternative to lumbar punctures.[57]

1.4.1.3 T-tau

It is believed that CSF t-tau is a general marker of neuronal/axonal damage and neuronal death.[49] CSF t-tau is increased by 200 to 300% in patients with AD, relative to elderly controls.[49] CSF t-tau is a powerful tool to differentiate AD from normal aging, as CSF t-tau possesses a sensitivity of 81% and specificity of 91%.[49] Although CSF t-tau is not directly associated with the production of phosphorylated tau, it is highly correlated with CSF p-tau.[49] Furthermore, CSF t-tau is excellent in differentiating AD from patients with depression, PD and PSP.[49] However, CSF t-tau is not specific to AD, as CSF t-tau is significantly elevated in CJD, acute stroke, and to a lesser extent, in FTD and LBD (results are inconsistent for VD).[49]

Overall, the combination of CSF $A\beta_{42}$, p_{181}-tau and t-tau is effective in predicting the conversion from MCI to AD.[58] More specifically, individuals with MCI that displayed pathological concentrations for all three core AD CSF biomarkers had a significantly greater risk of developing

AD (hazard ratio (HR) = 19.8), compared to individuals with MCI that had normal levels of these markers.[58]

1.4.2 Synaptic biomarkers

It has been well-established that synaptic dysfunction and loss are critical aspects of AD symptomatology.[59,60] For instance, post-mortem brain tissue studies have found that hippocampal CA1 synapses are reduced by 55% in patients with mild AD, and by 18% in patients with MCI – compared to CU individuals.[59] Thus, studying synaptic biomarkers is of utmost importance in AD, as it is the loss of synapses that correlates most strongly with cognitive decline, rather than Aβ.[60]

1.4.2.1 SYT1

SYT1 is a presynaptic protein that is located in the membrane of synaptic vesicles.[51] SYT1 serves as a calcium ion sensor, which is needed for the exocytosis of synaptic vesicles, and therefore, neurotransmitter release.[51] It has been well-documented that SYT1 is elevated in the CSF of patients with MCI and AD, relative to CU elderly controls.[61] Furthermore, a study found that SYT1 was greater in the CSF of patients with MCI, compared to patients with AD, which suggests that SYT1 may be a valuable marker to identify synaptic dysfunction and loss before the onset of apparent symptoms.[51,61]

1.4.2.2 SNAP25

SNAP25 is a presynaptic protein that is located in the membrane of neurons.[51] More specifically, SNAP25 is a critical component of the SNARE complex, which regulates the fusion of synaptic vesicles with the presynaptic membrane.[51] Furthermore, SNAP25 has also been implicated in long-term potentiation and neurite outgrowth.[51] Numerous studies have found that SNAP25 is elevated in the CSF of patients with AD.[62] Thus, it has been suggested that SNAP25 may be able to predict disease progression, as a study has shown that baseline levels of CSF SNAP25 were increased in patients with MCI that eventually progressed to AD, compared to patients with stable MCI.[63] However, it is important to consider that increases in CSF SNAP25 have also been found in other neurodegenerative diseases such as PD and CJD.[51]

1.4.2.3 GAP43

GAP43 is a presynaptic protein that is located in growth cones and axon terminals during neuronal development and during the regeneration of axons.[51] Thus, GAP43 plays a pivotal role in axonal outgrowth, synaptic plasticity and memory.[51,64] Furthermore, when GAP43 is phosphorylated, it interacts with several synaptic proteins and promotes the recycling of synaptic vesicles.[51] In the human brain, GAP43 is highly expressed in the entorhinal cortex, hippocampus and neocortex.[51,64] Studies have found that GAP43 is elevated in the CSF of patients with AD relative to CU elderly controls.[64] However, a transient increase in CSF GAP43 has been observed in patients suffering from ischemic stroke.[51]

1.4.2.4 NRGN

NRGN is a postsynaptic protein that is located in dendritic spines, and is highly expressed in the hippocampus and in the neocortex.[51] NRGN interacts with calmodulin, thereby enhancing memory formation processes.[51] NRGN appears to have an excellent ability to predict disease progression, as baseline levels of CSF NRGN were elevated in individuals with MCI that progressed to AD, compared to individuals with stable MCI.[65,66] In the same study, elevated baseline NRGN was associated with a greater (future) rate of cognitive decline and hippocampal atrophy in MCI converters.[66] In addition, elevated CSF NRGN was associated with a pronounced rate of cortical glucose hypometabolism in patients with AD.[66] However, it is worth mentioning that increases in CSF NRGN have also been detected in CJD and in a subset of PD patients.[51] Nevertheless, out of the four synaptic markers, CSF NRGN is believed to be the most specific to AD.[51]

Overall, post-mortem brain tissue studies have demonstrated that SYT1, SNAP25, GAP43 and NRGN are decreased in the AD brain,[67-69] but are elevated in the CSF of patients with AD.[70] These synaptic changes can even be detected during the pre-symptomatic stage of the disease.[71] For instance, increases in CSF synaptic markers can also be seen in at-risk, CU participants with a parental history of AD.[71] Finally, with some exceptions, it is generally believed that CSF SYT1, SNAP25, GAP43 and NRGN are significantly elevated in AD compared to other neurological disorders (such as FTD, VD, LBD, PSP, primary progressive aphasia, etc).[70] Taken altogether, these findings highlight the importance of these synaptic proteins as disease-specific markers for pre-symptomatic AD, and suggest these proteins may play critical roles in differential diagnoses.

1.4.3 Neuroimaging

1.4.3.1 Aβ PET

^{11}C-Pittsburgh compound B (PiB) is the first and most widely used PET tracer for imaging (fibrillar) Aβ deposition in the brains of living participants.[72] Indeed, it has been shown that patients with AD display significant retentions of PiB in the frontal, parietal, and temporal cortices, and in the striatum.[72] Furthermore, PiB is an important tool for differentiating AD from other neurodegenerative diseases, such as FTD, which lack amyloid plaques.[73] In addition, it has been found that PiB can predict disease progression.[74] For instance, a study has shown that 82% of individuals with MCI that had positive PiB scans at baseline progressed to AD within 1-3 years.[74] Conversely, 7% of PiB negative individuals with MCI progressed to AD during follow-up.[74] However, it is important to recognize that PiB possesses a short half-life of approximately 20 minutes.[72,75] Therefore, to resolve this issue, another amyloid PET tracer, ^{18}F-florbetapir (^{18}F-AV-45), was developed. Indeed, florbetapir possesses a half-life of roughly 110 minutes.[75] In a study that conducted antemortem PET imaging and post-mortem brain autopsies within two years, the sensitivity and specificity of florbetapir to detect the presence of moderate to frequent plaques upon histological examination, were 92% and 100% respectively.[76] However, it is important to acknowledge that Aβ PET neuroimaging studies have demonstrated that up to 10-30% of cognitively unaffected elderly individuals exhibit significant Aβ plaque deposition but do not develop cognitive deficits or AD.[77]

1.4.3.2 Tau PET

^{18}F-AV-1451, also known as flortaucipir, is the most widely used radiotracer for imaging in vivo tau deposition.[78] More specifically, flortaucipir has a high affinity for PHFs.[78] Flortaucipir retention follows a spatiotemporal pattern that is similar to that seen in post-mortem Braak staging.[31] In fact, a recent study examined the correspondence between antemortem flortaucipir PET imaging and post-mortem immunohistochemical tau pathology.[79] Overall, the study found that antemortem flortaucipir uptake could predict post-mortem tau accumulation (of Braak stages V-VI) with a sensitivity of 92.3-100% and specificity of 52-92%.[79] Furthermore, antemortem flortaucipir uptake was shown to predict a high level of post-mortem AD neuropathological change

(defined by a sufficient presence of Aβ plaques) with a sensitivity of 94.7-100% and a specificity of 50-92.3%.[79]

1.4.3.3 FDG PET

Glucose metabolism in the brain can be measured in vivo through the PET tracer ^{18}F-fluorodeoxyglucose (FDG).[80] More specifically, FDG-PET is believed to reflect synaptic activity,[81] as antemortem FDG-PET measurements have been found to be positively corelated with post-mortem brain tissue levels of the pre-synaptic protein synaptophysin, in nonhuman primates.[82] Furthermore, in humans, the antemortem diagnosis of AD by FDG-PET could predict an autopsy-confirmed diagnosis of AD with a sensitivity of 93% and specificity of 63%.[83] In a similar fashion, a longitudinal study has demonstrated that individuals with MCI that converted to AD over at least 5 years exhibited reductions in glucose metabolism in the posterior cingulate gyrus, precuneus, fusiform gyrus, cuneus, parietal lobe and temporal lobe, compared to individuals with stable MCI.[84] Overall, in the context of AD, reductions in FDG are believed to reflect synaptic dysfunction and loss.[81] Interestingly, it is believed that reductions in FDG-PET occur early on in AD, prior to brain atrophy, as determined by structural MRI.[81]

1.4.3.4 Structural MRI

Structural MRI is a powerful tool for assessing the volume of certain brain regions as well as the thickness of the cerebral cortex.[81] More specifically, structural MRI is used to quantify cerebral atrophy, which reflects both synaptic and neuronal loss.[81] For instance, in a longitudinal study, participants with MCI that had low hippocampal volumes at baseline had a significantly greater risk of developing AD, over a mean period of 32.6 months.[85] However, it is important to recognize that reductions in hippocampal volume have also been associated with several other neurological diseases such as schizophrenia.[86] Nevertheless, several studies have attempted to identify the brans regions that exhibit notable cortical thinning in AD.[87,88] To this end, neuroimaging studies have demonstrated that cortical thickness is significantly reduced in the temporal and parietal lobes of patients with AD (and to a certain extent the frontal lobe), most notably, in the entorhinal, fusiform, parahippocampal, middle-temporal, inferior-temporal, temporal pole, precuneus, inferior parietal, superior parietal, inferior frontal and superior frontal regions.[87,88]

1.4.4 Temporal ordering

The sequence of pathological events in AD has been hypothesized based on the biomarkers described above. A model put forward by Jack Jr. proposes that AD-related brain changes occur decades before the onset of symptoms.[81,89] More specifically, the model argues that Aβ accumulation precedes the hyperphosphorylation and accumulation of tau.[81] Finally, it is believed that neurodegeneration (synaptic dysfunction, brain atrophy, etc) is the penultimate event that precedes the onset of cognitive decline.[81] To add complexity to the model, it is believed that changes in biofluid markers occur prior to changes in corresponding PET and MRI neuroimaging markers.[81] Overall, this sequence of pathological events has been further supported by data collected from individuals with pre-symptomatic familial AD.[89]

1.4.5 A/T/N criteria

A more recent model put forward by Jack Jr. argues for the use of a biological definition of AD.[90] More specifically, a new classification system referred to as the Amyloid/ Tau/ Neurodegeneration ("A/T/N") system has been proposed.[90] In this system, seven AD biomarkers acquired from a given individual are allocated into three binary categories that are related to the pathophysiological processes underlying AD.[90] More specifically, each individual is rated for the presence or absence of abnormal levels of Aβ, phosphorylated tau and neurodegeneration.[90] "A" refers to amyloid status as determined by CSF $A\beta_{42}$, or Aβ PET scans.[90] "T" refers to phosphorylated tau status as deduced by CSF p-tau or tau PET scans.[90] Finally, "N" refers to neurodegeneration status, as assessed by CSF t-tau, structural MRI or FDG PET scans.[90] Overall, in this A/T/N research framework, separating biomarkers of tau phosphorylation from markers of neurodegeneration is critical in differentiating AD from other causes of neurodegeneration and/or dementia.[90] Furthermore, one of the A/T/N system's greatest assets is its flexibility. For instance, the A/T/N criteria makes no assumptions about the temporal ordering of the pathology.[90] Furthermore, new biomarkers can be incorporated into one of the existing categories and/or the A/T/N system can be expanded to include new categories, which could quantify cerebrovascular disease, inflammation, etc.[90] In the end, alongside genetic and clinical information, the A/T/N system is a promising tool to screen for individuals for clinical trials, and facilitate the development of personalized therapies for AD.[90]

1.5 Etiology of AD

The complexity of AD can be attributed to several factors, which can be categorized as non-modifiable or modifiable.

1.5.1 Non-modifiable factors

1.5.1.1 Age

It is indisputable that age is the greatest risk factor for AD.[3] Indeed, in the United States, 5% of individuals aged 65-74, 13.1% of individuals aged 75-84, and 33.3% of individuals greater than 85 years of age are affected by dementia.[3]

1.5.1.2 Sex

It has been well-established that women are at a substantially greater risk of developing AD, as two thirds of patients with AD are women.[3] Indeed, it has been estimated that the lifetime risk of developing AD (at 45 years of age) is nearly 20% for women, and 10% for men.[3] Although women generally live longer than men, and age is the greatest risk factor for AD, other biological factors can likely explain this sex difference.[3] For instance, it is possible that post-menopausal women are at an increased risk of developing AD due to a lack of sex hormones, such as estrogen, which is believed to be protective against AD pathology.[91] Furthermore, it is possible that post-menopausal inflammation may also contribute to this discrepancy between women and men.[92] Although the use of hormone replacement therapy (HRT) has been associated with a 60% reduction in the risk of developing AD,[93] the use of HRT remains a highly debated topic in AD. For instance, certain studies have found that HRT was, in fact, associated with an increased risk of AD (HR = 1.24).[94] Nevertheless, it has also been hypothesized that the timing of HRT may partially account for these discrepancies.[95] For instance, it has been proposed that HRT may be protective during midlife shortly after menopause, and detrimental when taken during late-life.[95]

1.5.1.3 Race

In the United States, the prevalence of dementia in Blacks/African Americans above the age of 65 is 19.3%, 16.7% in Latinx/ Hispanics and 7.4% in Caucasians.[96] It has been suggested that these differences in prevalence may be partly due to socioeconomic and health disparities such as access

to quality health care, employment prospects and occupational safety.[3,96] However, it is important to recognize that well-established risk factors for AD, such as cardiovascular disease and diabetes, are prominent in Black and Hispanic populations.[3]

1.5.1.4 Genetics

1.5.1.4.1 Familial form of AD

Although it is rare (less than 1% of cases), an aggressive form of AD can be inherited in an autosomal dominant fashion due to mutations in certain genes.[97] Thus, in families affected by this familial form, nearly half of the descendants from each generation develop AD.[97] More specifically, genetic linkage analyses have identified causative mutations in *APP,* presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*).[97] These discoveries provided further support for the amyloid cascade hypothesis (Aβ is sole cause of AD), as these genes are tightly linked to the production of Aβ peptides.[97] Indeed, *APP* encodes for the transmembrane precursor of Aβ peptides, while the gene products of *PSEN1* and *PSEN2* form the catalytic subunits of the gamma-secretase enzyme, which is responsible for the final cleavage of CTF-β into Aβ and AICD peptides.[18] Nevertheless, the familial form of AD is associated with an earlier age of onset, with mutation carriers exhibiting symptoms before the age of 65, and in some cases, as early as 30 years of age.[97]

1.5.1.4.2 Sporadic form of AD

The vast majority of AD cases are sporadic, as they can be attributed to genetic and environmental risk factors.[97] The sporadic form is often associated with a late age of onset, typically beginning after the age of 65.[97] In individuals with a first-degree relative affected by AD, the lifetime risk of developing AD (by age 96) is 39%, which is two times greater than the general population.[98] Indeed, the inheritance of certain genetic risk factors, such as a single copy of apolipoprotein E (*APOE)* ε4, has been associated with a 3-fold greater risk of developing AD.[99] Furthermore, the inheritance of two copies of *APOE* ε4 can increase the risk of AD by up to 12-fold.[3] Conversely, the inheritance of *APOE* ε2 has been associated with a reduced risk of AD.[3] At the present time, genome-wide association (GWAS) studies have identified 75 loci associated with AD.[100] Interestingly, the identified genes are implicated in molecular pathways related to amyloid, tau, lipid metabolism, immunity and endocytosis.[100] However, it is important to note that several of the newly identified variants possess relatively small effect sizes and/or are rare occurring variants.[100]

1.5.2 Modifiable factors

According to *The Lancet* Commission report from 2020, nearly 40% of dementia cases could potentially be prevented or delayed if modifiable risk factors are addressed.[101] The following sections will examine these factors.

1.5.2.1 Education

Numerous studies suggest that a low educational attainment is associated with an increased risk of developing AD.[102] Indeed, it has been shown that individuals with less than 8 years of education are twice as likely to develop dementia, compared to those with a greater educational attainment.[102] Hence, it is believed that an increase in educational attainment may improve an individual's cognitive reserve.[103] More specifically, it has been proposed that individuals with a greater educational attainment may possess a greater ability to cope and compensate for the ongoing brain changes associated with AD.[103] Thus, it is possible that these individuals with a greater cognitive reserve may exhibit a delayed onset of symptoms, as they may be able to tolerate a greater amount of pathology, compared to individuals with a lower cognitive reserve.[103]

1.5.2.2 Cardiovascular factors

It is undeniable that a healthy cardiovascular system is essential for proper brain development, function and survival.[104] For instance, the brain accounts for nearly 2% of our body weight, however it relies on 20% of the body's oxygen, glucose and various other nutrients -which circulate in the blood.[104] The importance of the cardiovascular-brain relationship is highlighted by the fact that the occurrence of a stroke significantly increases the risk of dementia (HR = 1.60).[105]

1.5.2.2.1 Circulating cholesterol levels

It is important to recognize that several risk factors for cardiovascular disease are also risk factors for dementia. First and foremost, while *APOE ε4* is the greatest genetic risk factor for AD,[99] it is also a significant genetic risk factor for cardiovascular disease.[106] Furthermore, in a retrospective cohort study of 8845 people, elevated circulating cholesterol levels during midlife was associated with an increased risk of developing AD (HR = 1.42).[107] Interestingly, in a cross-sectional study, the use of cholesterol-lowering drugs in participants greater than 60 years of age was associated with a 60-73% reduction in the risk of developing AD, compared to the total patient population,

and to patients taking other cardiovascular-related medications.[108] However, it is important to acknowledge that several clinical trials have failed to demonstrate a clinical benefit of statins over placebo in AD patients.[109,110]

1.5.2.2.2 Blood pressure

In a 15-year longitudinal study, individuals that developed dementia between the ages of 79-85 exhibited elevated systolic and diastolic blood pressures at the age of 70, compared to individuals that did not progress to AD.[111] In fact, it has been established that hypertension during midlife is a significant risk factor for AD.[112] However, the association between blood pressure in late-life and risk of AD remains less clear.[112,113] Nevertheless, encouraging observational and interventional studies have demonstrated that long-term treatment of hypertension may reduce the risk of dementia (HR = 0.38-0.64)[114,115]

1.5.2.2.3 Obesity

A meta-analysis of 19 studies on 589 649 participants followed for up to 42 years explored the relationship between another cardiovascular disease risk factor, namely obesity, and dementia.[116] In this report, midlife obesity, as defined by a body mass index (BMI) ≥ 30, was associated with a 33% greater risk of developing dementia in late-life.[116] On the other hand, being overweight ($25 < BMI < 30$) during midlife was not associated with the risk of developing dementia.[116]

1.5.2.2.4 Diabetes

Diabetes is another pivotal factor that influences both brain and cardiovascular health.[117] For instance, a longitudinal study of 6370 elderly individuals revealed that type 2 diabetes was associated with a 90% increased risk of developing AD, over a mean follow-up of 2.1 years.[117] More specifically, it has been shown that elderly individuals with type 2 diabetes performed worse on cognitive assessments, and exhibited elevated hippocampal atrophy and cerebral infarcts compared to age-matched participants without type 2 diabetes.[118] Furthermore, several human post-mortem brain tissue studies have demonstrated that signaling molecules related to insulin and insulin-like growth factors (IGFs) are decreased in the Alzheimer brain.[119-122] Furthermore, the importance of insulin and IGFs has been emphasized in pilot clinical trials in which the administration of intranasal insulin improved memory, caregiver-rated functional abilities, and glucose metabolism in individuals with MCI or mild AD.[123,124] However, several studies have

reported that the benefits of intranasal insulin appear to be dependent on *APOE* genotype, with *APOE* ε4 carriers exhibiting poor responses to such therapies.[125] Nevertheless, intranasal insulin can bypass the blood-brain barrier (BBB) and reach the CSF within 10 minutes.[126] The administration of insulin and IGFs in this manner is critical, as it prevents unwanted side effects such as hypoglycemia and peripheral insulin resistance.[125] Taken altogether, these critical findings suggest that targeting the insulin-IGF system may offer a promising solution to delay, slow down and/or prevent AD. Thus, based on the crucial relationship between diabetes, insulin, IGFs and AD, in Chapter 2 of the present thesis we examined the role of an insulin-IGF related protein, namely insulin like growth factor binding protein 2 (IGFBP2).

1.5.2.3 Lifestyle factors

1.5.2.3.1 Diet

Although it is widely believed that diet plays a pivotal role in AD, it is challenging to identify individual components that may be protective against AD.[127] However, several meta-analyses of randomised controlled trials have revealed that the Mediterranean diet (frequent consumption of vegetables, legumes, fruits, nuts, cereals and olive oil; limited consumption of saturated lipids and meat) may offer modest benefits to global cognition in healthy older adults.[127]

1.5.2.3.2 Physical activity

It is possible physical activity may protect individuals from dementia by reducing obesity, diabetes and cardiovascular disease.[101] More specifically, physical activity may be protective to the brain by enhancing blood flow to the brain, increasing the levels of neurotrophic factors (such as brain-derived neurotrophic factor), and promoting neurogenesis in the dentate gyrus.[128] Ultimately, physical activity has been associated with increased hippocampal volume.[128] Indeed, it has been shown that moderate-to-vigorous physical activity in midlife reduced the risk of dementia (HR = 0.81) in 28 916 participants (aged 30-60 years) over the course of 25.2 years.[129] Furthermore, a meta-analysis of 19 observational studies of 404 840 participants (mean age of 45.5 years) followed for a mean of 14.9 years, found that the risk of AD (HR = 1.40) was greatest in individuals that were not physically active within the 10-year period before the clinical diagnosis of AD.[130] In the end, it is critical to acknowledge that while physical inactivity may contribute to the

development of AD, it is likely that physical inactivity may also be a manifestation of the prodromal dementia.[101]

1.5.2.3.3 Sleep

It has been suggested that sleep disturbance may influence the risk of dementia by affecting the glymphatic clearance of Aβ, inflammation and cardiovascular disease.[101] For instance, meta-analyses have found that sleep disturbances (insomnia, obstructive sleep apnea, poor sleep quality, etc) were associated with a 55% greater risk of developing AD.[131] However, it has been recently reported that the association between sleep and the risk of MCI or AD may follow a U-shape.[132] More specifically, it has been suggested that less than 5 hours and more than 10 hours of sleep are both associated with a greater risk of developing dementia (HR = 2.64 and 2.23, respectively).[132] Although these findings are inconsistent, it is possible that changes in sleep patterns may be caused by the underlying neurodegenerative processes, rather than only being a risk factor.[101]

1.5.2.3.4 Smoking and air pollution

Although dated studies have concluded that smoking was associated with a reduced risk of developing AD, it is likely that the true relationship was obscured due to smokers dying prematurely - before having the chance to potentially develop AD.[133] Thus, the general consensus remains that smoking increases the risk of developing AD, particularly during mid-late-life.[101,134] Indeed, a longitudinal study of 6870 individuals (aged 55 years and greater) found that smokers had a 2.3-fold increased risk of developing AD over a mean follow-up of 2.1 years, compared to non-smokers.[134] This association was most prominent in *APOE* ε4 non carriers (4.6-fold increase), and was not present in *APOE* ε4 carriers.[134] Finally, exposure to air pollutants such as nitrogen dioxide, fine particulate matter and carbon monoxide, has been also been associated with an increased risk of dementia, by a systematic review incorporating 13 longitudinal studies with a follow-up of 1-15 years.[101,135]

1.5.2.3.5 Alcohol

Excessive alcohol consumption is a well-known risk factor for AD.[101,136,137] For instance, a 5-year longitudinal study conducted in France examined over 31 million people admitted to hospitals and found that individuals with alcohol use disorders were at an increased risk of developing early-onset dementia (HR = 3.34).[136] Interestingly, a systematic review of 45 studies revealed that light

to moderate drinking was associated with a 28% reduction in the risk of developing dementia compared to no drinking.[137]

1.5.2.3.6 Social engagement and depression

It is widely accepted that social contact is a protective factor for AD.[101] Thus, it is believed that social contact may increase cognitive reserve or encourage beneficial behavior.[101] Furthermore, numerous studies suggest that less social engagement is associated with an increased risk of dementia.[101] For instance, a systematic review and meta-analysis incorporating 812 047 people reported that lifelong single and widowed people had a 42% and 20% greater risk of developing dementia, compared to married individuals, who are generally believed to have more interpersonal contact.[138] Besides social engagement, depression has been found to increase the risk of dementia.[101] For instance, a cohort study that followed more than 1.4 million Danish citizens for 41 years found that a diagnosis of depression during early-, middle- or late-life, significantly increased the risk of developing dementia (HR = 2.41).[139] Furthermore, in a study of 755 individuals that had both MCI and a history of depression, it was demonstrated that treatment with an antidepressant (citalopram) over the course of 4 years delayed the conversion to AD by about 3 years.[140] Taken altogether, rather than solely being risk factors, it is important to recognize that social isolation and depression may also be symptoms of the underlying dementia.[101]

1.5.2.3.7 Traumatic brain injury

A longitudinal study that followed 3 million Danish individuals (greater than 50 years of age) over 10 years (mean) discovered that traumatic brain injury (TBI) increased the risk of developing AD (HR = 1.16).[141] Strikingly, the risk for dementia was at its highest (HR = 4.06) in the 6 months after TBI, however the risk decreased (HR = 1.17) at more than 14 years since the injury.[141] These findings suggest that the risk of dementia may be greatest closer to the time of the TBI.[101,141,142] For instance, a meta-analysis found a 5.53 fold increased risk in individuals that experienced TBI within 10 years of AD onset, while individuals that experienced TBI greater than 10 years away from onset were at a 1.63 fold increased risk.[142] Furthermore, in the Danish study, the risk of dementia also increased with the number of TBIs, (one TBI, HR = 1.22; five or more TBI, HR = 2.83).[141] Taken altogether, it is possible that TBI may be associated with widespread phosphorylated tau pathology, as demonstrated in humans and mice.[101]

1.6 Current treatments

1.6.1 Symptomatic treatments

Although a cure does not currently exist for AD, traditional treatments aim to temporarily treat the symptoms.[143] However, they do not stop or slow down the progression of the disease.[143] For instance, acetylcholinesterase inhibitors (donepezil, rivastigmine, galantamine) are generally prescribed to patients to compensate for the selective vulnerability of cholinergic neurons to AD pathology.[38,39,143] More specifically, in an attempt to restore acetylcholine levels in the brain, these inhibitors interfere with the degradation of acetylcholine.[143] However, it is important to recognize that acetylcholinesterase inhibitors, such as donepezil, only offer clinical improvement from baseline for roughly 6-9 months.[144] Following this period, cognition appears to gradually decline in donepezil-treated individuals, however the decline appears to be less than that expected from no treatment.[144] Overall, the results suggest that donepezil may be a practical symptomatic treatment for AD, for nearly 4.9 years.[144] However, the limited benefits of acetylcholinesterase inhibitors appear to be dependent on *APOE* genotype, with *APOE* ε4 carriers exhibiting poor responses to such therapies.[145]

Memantine is another symptomatic treatment for AD.[143,146] Indeed, memantine is an NMDA receptor antagonist that is used to prevent an overactivation of NMDA receptors, which is associated with a toxic influx of calcium ions and ultimately, neuronal death.[146] Indeed, a study has found that 73% of patients with severe dementia that were treated with memantine over 12 weeks exhibited functional improvement.[147] Overall, memantine is often prescribed with an acetylcholinesterase inhibitor, as this combination of treatments offers greater improvements to cognition compared to the use of acetylcholinesterase inhibitors alone.[146,148]

1.6.2 Disease-modifying treatments

Although acetylcholinesterase inhibitors and memantine are symptomatic treatments, developing disease-modifying therapies remains the gold standard. To this end, several phase 3 clinical trials have tested the efficacy of beta-secretase[149] and gamma-secretase[150] inhibitors in patients with mild to moderate AD. However, these interventions did not halt or slow down the progression of the disease, and in some cases, even led to worse cognitive outcomes and more adverse events.[149,

[150] However, recent advancements have led to the development of promising therapies (aducanumab, lecanemab, donanemab) that remove Aβ plaques from the brains of patients with MCI or mild AD.[4-6] More specifically, several of these intravenous treatments involve the use of immunoglobulin G1 monoclonal antibodies that recognize distinct epitopes exposed on the surface of aggregated forms of Aβ (ranging from soluble oligomers to insoluble fibrils).[4-6] In the end, it is believed that these monoclonal antibody treatments enhance the phagocytosis of Aβ by microglia.[4-6] Indeed, several phase 3 clinical trials conducted over 18 months have demonstrated that aducanumab, lecanemab and donanemab slow down the progression of AD by 22-35% in patients with MCI or mild AD.[4-6]

Although this is a promising beginning to the era of disease-modifying therapies in AD, there are numerous limitations (which will also be discussed later on). First and foremost, it is possible that Aβ plaques may not exert a great influence on cognition on their own.[60] For instance, post-mortem neuropathological studies and Aβ PET neuroimaging studies have demonstrated that up to 10-30% of cognitively unaffected elderly individuals exhibit significant Aβ plaque deposition but do not develop AD.[77] An equally important observation is that the administration of another monoclonal antibody, namely solanezumab, did not offer any benefits to cognition compared to placebo, in two phase 3 clinical trials.[151,152] More specifically, in the initial trial, solanezumab failed in patients with mild-moderate AD,[151] while in the second trial, solanezumab failed in participants with preclinical AD.[152] The latter results are of critical importance, as they suggest that targeting Aβ alone is not enough, even during the pre-symptomatic stage of AD. Thus, developing therapies that promote the maintenance of healthy synapses might be the solution, as it is the loss of synapses that correlates most strongly with cognitive decline.[60] Therefore, it is evident that further research is urgently needed to elucidate mechanisms implicated in AD. Taken altogether, this may lead to the discovery of viable therapeutic targets which may provide substantial benefits to cognition when administered in combination therapies.

1.7 Rationale for thesis

As previously mentioned, the cardiovascular system is heavily implicated in AD, as numerous risk factors for cardiovascular disease are also risk factors for AD.[101,105-125] Furthermore, post-mortem brain tissue studies have demonstrated that cerebral atherosclerosis is prevalent among patients with AD.[153] Moreover, besides accumulating in-between neurons, Aβ has been shown to deposit

within the walls of the blood vessels in the brain, ultimately leading to cerebral amyloid angiopathy.[154] Thirdly, it has been demonstrated that patients with AD exhibit a 20% reduction in cerebral blood flow, compared to age-matched elderly individuals.[155] Finally, the breakdown of the BBB has been well-documented in AD; and this abnormality occurs as early as in the MCI stage.[156] Taken altogether, these observations suggest that the cardiovascular system and AD share an intricate relationship that certainly warrants further investigation. Finally, as previously alluded to, current disease-modifying therapies for AD only offer modest benefits to cognition compared to placebo.

Thus, in order to further elucidate the intricacies of the cardiovascular-AD relationship during the pre-symptomatic stage of AD, and to identify potential therapeutic candidates, we ran a cardiovascular panel assay on the CSF of asymptomatic, at-risk individuals with a parental or multi-sibling history of sporadic AD (PREVENT-AD cohort). Furthermore, we verified our observations using an independent and well-characterized cohort comprised of cognitively unaffected individuals as well as individuals with MCI (ADNI-1 cohort). Finally, we analyzed the final stage of the disease through autopsy-confirmed AD brains and age-matched control brains from a unique population isolate from Eastern Canada (QFP cohort).

1.8 Objectives

1.8.1 Manuscript 1

Given the critical relationship between type 2 diabetes, insulin-IGF resistance and AD, we examined an IGF-related protein from the cardiovascular panel, notably insulin like growth factor binding protein 2. For this manuscript we:

1- Investigated the relationship between IGFBP2 and AD biomarkers (AD CSF core biomarkers, synaptic biomarkers), longitudinal changes in cognition, cortical thickness and volumetric brain measurements in asymptomatic, at-risk PREVENT-AD participants.
2- Assessed the relationship between IGFBP2 and stage of AD pathology in cognitively unaffected individuals and individuals with MCI from the ADNI-1 cohort.
3- Analyzed the longitudinal relationship between IGFBP2 and risk of conversion to AD in ADNI-1 participants.

4- Contrasted IGFBP2 mRNA and protein levels in the frontal cortex of autopsy-confirmed AD brains and age-matched control brains from the QFP cohort.

1.8.2 Manuscript 2

Considering the pivotal role of neuroinflammation in AD, we further analyzed a pro-inflammatory protein from the cardiovascular panel, namely osteopontin. For this manuscript we:

1- Investigated the relationship between OPN and AD biomarkers (AD CSF core biomarkers, synaptic biomarkers), PET scans and volumetric brain measurements in asymptomatic, at-risk PREVENT-AD participants.
2- Assessed the relationship between OPN and stage of AD pathology in cognitively unaffected individuals and individuals with MCI from the ADNI-1 cohort.
3- Examined the longitudinal relationship between OPN and risk of conversion to AD in ADNI-1 participants.
4- Contrasted OPN mRNA and protein levels in the frontal cortex of autopsy-confirmed AD brains and control brains from the QFP cohort.

Submitted to *Brain*

2. Insulin-like growth factor binding protein-2 in at-risk adults and autopsy-confirmed Alzheimer brains

Marc James Quesnel,[1,2] Anne Labonté,[2,3] Cynthia Picard,[2,3] Henrik Zetterberg,[4,5,6,7,8,9] Kaj Blennow,[4,5] Ann Brinkmalm, [4,5] Sylvia Villeneuve[1,2,3] and Judes Poirier[1,2,3] for the Alzheimer's Disease Neuroimaging Initiative[*] and the PREVENT-AD Research Group[†]

2.1 Abstract

Insulin, insulin-like growth factors (IGF) and their receptors are highly expressed in the adult hippocampus. Thus, disturbances in the insulin-IGF signaling pathway may account for the selective vulnerability of the hippocampus to nascent Alzheimer's disease (AD) pathology**.** In the present study, we examined the predominant IGF-binding protein (IGFBP) in the cerebrospinal fluid (CSF) – IGFBP2.

CSF was collected from 109 asymptomatic members of the parental history-positive PREVENT-AD cohort. CSF levels of IGFBP2, core AD biomarkers and synaptic biomarkers were measured using proximity extension assay, ELISA and mass spectrometry. Cortical amyloid-beta (Aβ) and tau deposition were examined using ^{18}F-NAV4694 and flortaucipir. Cognitive assessments were performed up to 8 years of follow-up, using the Repeatable Battery for the Assessment of Neuropsychological Status. T1-weighted structural MRI scans were acquired, and neuroimaging analyses were performed on pre-specified temporal and parietal brain regions. Next, in an independent cohort, we allocated 241 dementia-free ADNI-1 participants into four stages of AD progression based on the biomarkers CSF $A\beta_{42}$ and total-tau (t-tau). In this analysis, differences in CSF and plasma IGFBP2 levels were examined across the pathological stages. Finally, IGFBP2 mRNA and protein levels were examined in the frontal cortex of 55 autopsy-confirmed AD and 31 control brains from the QFP cohort, a unique population isolate from Eastern Canada.

CSF IGFBP2 progressively increased over 5 years in asymptomatic PREVENT-AD participants. Baseline CSF IGFBP2 was positively correlated with CSF AD biomarkers and synaptic biomarkers, and was negatively correlated with longitudinal changes in delayed memory (*P* =

0.024) and visuospatial abilities (P = 0.019). CSF IGFBP2 was negatively correlated at a trend-level with entorhinal cortex volume (P = 0.082) and cortical thickness in the piriform (P = 0.039), inferior temporal (P = 0.008), middle temporal (P = 0.014) and precuneus (P = 0.033) regions. In ADNI-1, CSF (P = 0.009) and plasma (P = 0.001) IGFBP2 were significantly elevated in Stage 2 (CSF Aβ(+)/t-tau(+)). In survival analyses in ADNI-1, elevated plasma IGFBP2 was associated with a greater rate of AD conversion (HR = 1.62, P = 0.021). In the QFP cohort, IGFBP2 mRNA was reduced (P = 0.049), however IGFBP2 protein levels did not differ in the frontal cortex of autopsy-confirmed AD brains (P = 0.462).

Nascent AD pathology may induce an upregulation in IGFBP2, in asymptomatic individuals. CSF and plasma IGFBP2 may be valuable markers for identifying CSF Aβ(+)/t-tau(+) individuals and those with a greater risk of AD conversion.

Author affiliations:

1 McGill University, Montréal, Québec, Canada

2 Douglas Mental Health University Institute, Montréal, Québec, Canada

3 Centre for the Studies in the Prevention of Alzheimer's Disease, Douglas Mental Health University Institute, Montréal, Québec, Canada

4 Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

5 Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

6 Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK

7 UK Dementia Research Institute at UCL, London, UK

8 Hong Kong Center for Neurodegenerative Diseases, Hong Kong, China

9 Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, USA

*Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not

participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at:
http://adni.loni.usc.edu/wpcontent/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

†Data used in preparation of this article were obtained from the PRe-symptomatic EValuation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) program at the Centre for Studies on Prevention of Alzheimer's Disease (StoP-AD), Douglas Mental Health University Institute Research Centre (http://douglas.research.mcgill.ca/stop-ad-centre). A complete listing of the PREVENT-AD Research Group can be found at: https://preventad.loris.ca/acknowledgements/acknowledgements.php?date=2023-05-01.

Correspondence to: 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

Running title: Role of IGFBP2 Across the AD Spectrum

Keywords: Insulin-like growth factor-binding protein 2; Insulin-like growth factor; Alzheimer's disease; cerebrospinal fluid; post-mortem; RBANS

2.2 Introduction

By 2050, is it estimated that 152.8 million individuals worldwide will be affected by dementia.[1] Furthermore, the cost of dementia care is projected to reach $16.9 trillion in 2050.[2] Alzheimer's disease (AD) remains one of the most challenging medical mysteries, as it is the most common cause of dementia - accounting for 60-80% of all cases.[3] The neuropathological hallmarks of AD include the accumulation of extracellular amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles, as well as the loss of synapses and neurons.[4] These brain changes are believed to begin up to 20 years or more, before the onset of symptoms.[5]

It has been well established that insulin resistance and diabetes are risk factors for developing AD.[6,7] Indeed, impaired insulin and insulin-like growth factor (IGF) signalling plays a critical role in the pathogenesis of AD.[8-10] Post-mortem studies have demonstrated that insulin, IGFs as well

as their receptors and downstream signalling molecules, are decreased in the AD brain.[8-11] Furthermore, the insulin-IGF system has been shown to directly modulate Aβ degradation[12] and clearance,[13] phospho-tau production,[14,15] synaptic integrity[16] and neuronal survival.[17] It is also known that insulin, IGFs and their receptors are highly expressed in the hippocampus, relative to the frontal cortex in the human brain.[8] Overall, these findings suggest that impairments in insulin-IGF signaling may account for the selective vulnerability of the hippocampus to nascent AD pathology, and therefore, account for early impairments in episodic memory**.** Similarly, deficiencies in insulin-IGF signaling may contribute to the reductions in glucose metabolism that are seen in patients with AD and individuals at risk for AD.[18] The importance of insulin and IGFs has been emphasized in pilot clinical trials in which the administration of intranasal insulin improved memory, caregiver-rated functional abilities, and glucose metabolism in individuals with mild cognitive impairment (MCI) or mild AD.[19,20]

The insulin-IGF system encompasses a complex collection of proteins that play pivotal roles in glucose metabolism, neurogenesis, synaptogenesis and cell survival.[17,21,22] Insulin, IGF-I and IGF-II are the key proteins, which bind to their cell surface receptors.[22] The actions of IGF-I and IGF-II are modulated by six IGF-binding proteins (IGFBP), which can bind to IGFs with an equal or greater affinity than the IGF receptors.[23,24] Indeed, in the circulation, cerebrospinal fluid (CSF) and local tissues, most extracellular IGFs are bound to so-called IGFBPs, which prolong the half-life of IGFs.[22-24] For instance, it has been proposed that IGFBPs may prevent the degradation of IGFs during transport and mobilisation, and target IGFs to their receptors.[22-24] The latter may be achieved through IGFBPs binding to cell-surface proteoglycans[25] and integrins,[26] or proteolytic cleavage,[27] both of which reduce the binding affinity of IGFBPs for IGFs and promote IGF release.[22]

The main objective of the current study is to examine a less studied member of the IGF molecular cascade, IGFBP2, in both the pre-symptomatic and symptomatic stages of AD. Since IGFBP2 is the most abundant IGF-binding protein in the CSF,[28,29] we hypothesize IGFBP2 plays a critical role in the neurodegenerative process, most likely at the level of neuroprotection and resilience.[22] IGFBP2 is increased in the CSF and plasma of patients with a clinical diagnosis of AD,[30-35] and is associated with longitudinal atrophy in entorhinal, parahippocampal and inferior temporal regions.[36] Moreover, elevated circulating levels of IGFBP2 have been associated with an increased

risk of developing AD.[35,37,38] Finally, in a pilot study, IGFBP2 has been found to be decreased in the temporal cortex of AD patients.[9] These and other findings will be verified in the pre-symptomatic and symptomatic stages of the disease, as well as in autopsy-confirmed AD brains.

2.3 Materials and methods

2.3.1 PREVENT-AD cohort

2.3.1.1 Study participants

The PRe-symptomatic EValuation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) cohort consists of asymptomatic, "at-risk", individuals with a parental or multi-sibling history of sporadic Alzheimer's disease.[39] The majority of participants were over the age of 60, however individuals aged 55-59 years were included if they were within 15 years of the onset of their youngest-affected relative's symptoms. In order to confirm normal cognition, the Clinical Dementia Rating and Montreal Cognitive Assessment (MoCA) were used at the study eligibility visit. 386 active PREVENT-AD participants have been followed longitudinally, as annual visits include cognitive assessments, neurosensory tests, blood and (for a subset of individuals) CSF collections, structural and functional magnetic resonance imaging (MRI), as well as positron emission tomography (PET) scans. Each participant and their study partner provided written informed consent. 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. A detailed description of the PREVENT-AD cohort is available elsewhere.[39]

2.3.1.2 Cerebrospinal fluid measurements

A Sprotte 24-gauge atraumatic needle was used to perform lumbar punctures (LP) in PREVENT-AD participants, following an overnight fast. In order to exclude cells and insoluble material, CSF samples were centrifuged (~2000g) within 4 hours, for 10 minutes at room temperature. Finally, the CSF samples were aliquoted (0.5 mL) into polypropylene cryotubes and stored at -80°C.

CSF IGFBP2 levels were measured in a subset of PREVENT-AD participants (n = 109) using the Olink Cardiovascular III panel (Uppsala, Sweden), which employs proximity extension assay

(PEA) technology. Olink measurements are expressed in arbitrary Normalized Protein eXpression units (NPX), which are on a $\log_2$ scale.

CSF AD biomarkers $A\beta_{42}$, phosphorylated tau (p_{181}-tau) and total tau (t-tau) were measured in a subset (n = 101) of PREVENT-AD participants, using the validated Innotest enzyme-linked immunosorbent assay (ELISA) kit (Fujirebio, Ghent, Belgium) following the standardized protocols established by the BIOMARKAPD consortium ($A\beta_{42}$ Cat.# 81583, p_{181}-tau Cat.# 81581 and t-tau Cat.# 81579).

Of the 109 PREVENT-AD participants that had CSF IGFBP2 measurements, 106 individuals had the synaptic proteins SNAP25 and SYT1 assayed. CSF SNAP25 and SYT1 were immunoprecipitated and their concentrations were determined by mass spectrometry, as previously described.[40,41,42] Mass spectrometry results are expressed in arbitrary units.[43] As previously reported, CSF levels of GAP43 and NRGN were assessed by validated ELISAs in a subset of PREVENT-AD individuals (n = 46).[44,45]

2.3.1.3 Neuroimaging acquisition and processing

In vivo cortical Aβ and phosphorylated tau pathologies were determined using positron emission tomography (PET) tracers ^{18}F-NAV4694 (Navidea Biopharmaceuticals, Dublin, OH, USA) and flortaucipir (^{18}F-AV1451; Eli Lilly & Company, Indianapolis, IN, USA), in a subset of PREVENT-AD participants that also had CSF IGFBP2 measurements (n = 46, n = 49 respectively). Aβ and Tau PET scans were performed 40 to 70 minutes and 80 to 100 minutes post-injection, respectively. A 3T Siemens Trio scanner was used to acquire T1-weighted structural MRI scans at the Douglas Mental Health University Institute (Montreal). A Siemens standard 12 or 32-channel coil was used (Siemens Medical Solutions, Erlangen). FreeSurfer 5.3 was used to process the MRI scans, and the Desikan-Killiany atlas was used for parcellation. The preprocessing pipeline for PET images has previously been described.[46] Briefly, standardized uptake value ratios (SUVRs) were generated by dividing the signal in the regions of interest (ROI) by the signal in the reference region. Thus, cerebellar grey matter was used as a reference region for ^{18}F-NAV4694, whilst the inferior cerebellar grey matter was used for flortaucipir. A global cortical ROI was computed to evaluate Aβ deposition, whilst tau deposition was assessed by averaging flortaucipir SUVRs in the entorhinal cortex and lingual gyri. The imaging processing pipeline CIVET 1.1.12

was used to estimate cortical thickness from T1-weighted images (n =104).[47] Brain volumes were computed using a volumetric pipeline that has been previously described.[48]

2.3.1.4 *APOE* genotyping

The QIAsymphony apparatus and DNA Blood Mini QIA Kit were used to isolate DNA from 200 ul whole blood (Qiagen, Valencia, CA, USA). The standard QIASymphony isolation program was used following the manufacturer's instructions. The PyroMark Q96 pyrosequencer (Qiagen, Toronto, Ontario, Canada) was used to determine *APOE* genotype in PREVENT-AD. qPCR was used to amplify DNA, with primers rs429358 amplification forward 5'-ACGGCTGTCCAAGGAGCTG-3', rs429358 amplification reverse biotinylated 5'-CACCTCGCCGCGGTACTG-3', rs429358 sequencing 5'CGGACATGGAGGACG-3', rs7412 amplification forward 5'-CTCCGCGATGCCGATGAC-3', rs7412 amplification reverse biotinylated 5'-CCCCGGCCTGGTACACTG-3' and rs7412 sequencing 5'-CGA TGACCTGCAGAAG-3'.

2.3.1.5 Cognitive testing

At annual visits, the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) was used to assess the cognitive performance of PREVENT-AD participants. The RBANS possesses an excellent sensitivity in differentiating normal cognition from MCI.[49] Five cognitive domains are evaluated, which include immediate memory, delayed memory, attention, language and visuospatial abilities.[49] A total summary score is included as well. Each participant's score is standardized by their age, such that a score of 100 represents the expected cognitive performance for a given age.[49] In order to reduce practice effects in longitudinal assessment, the RBANS was available in four equivalent versions. Furthermore, the battery was administered in English or French depending on the participants' preferred language. Cognitive measurements are available for up to 8 years of follow-up.

2.3.2 ADNI-1 cohort

2.3.2.1 Study participants

Led by Principal Investigator Michael W. Weiner, MD, the primary objective of the Alzheimer's Disease Neuroimaging Initiative (ADNI) has been to detect the earliest changes associated with

AD, and to track the progression of AD pathology. Given our interest in the earliest possible stages of AD, we restricted our primary analyses to 241 ADNI-1 participants with CSF data available from 92 cognitively unaffected (CU) individuals and 149 individuals with MCI. For analyses involving plasma samples, we restricted our analyses to 58 CU individuals and 396 individuals with MCI that had available data. Two individuals with ambiguous diagnoses were excluded from analyses.

2.3.2.2 Cerebrospinal fluid measurements

LPs were performed with a 20- or 24- gauge spinal needle, following an overnight fast. CSF samples were frozen within 1 hour after collection and shipped on dry ice to the ADNI Biomarker Core laboratory. Following thawing (1h) with gentle mixing at room temperature, the samples were aliquoted (0.5 mL) into polypropylene vials and stored at -80°C. CSF AD biomarkers $A\beta_{42}$, p_{181}-tau and t-tau were measured in ADNI-1 samples using the INNO-BIA AlzBio3 immunoassay kits (Fujirebio, Ghent, Belgium) and the xMap Luminex platform (Austin, Texas, USA).

CSF levels of 159 inflammatory, metabolic and lipid analytes, including IGFBP2 had been assessed with the Human Discovery Map panel, a multiplex immunoassay panel developed by Rules Based Medicine (RBM). In the case of IGFBP2, 8 (imputed) samples exceeded the detectable analyte concentration range and were omitted from subsequent analyses. Finally, in supplementary analyses, we further analyzed multiple reaction monitoring mass spectrometry (MRM-MS) measurements of CSF IGFBP2.

2.3.2.3 Pathological staging of participants

Following the recent emergence of biological frameworks for defining AD,[50] we used baseline CSF $A\beta_{42}$ and CSF t-tau measurements to stage 90 CU individuals and 145 individuals with MCI. We applied the recommended CSF $A\beta_{42}$ and CSF t-tau thresholds of <192 pg/mL and >93 pg/mL, respectively.[51] These cutoff values have been generated from autopsy-based AD CSF samples and have been reported to detect mild AD and predict the conversion from MCI to AD.[51] Two individuals with biomarker measurements equivalent to the threshold values were removed.

ADNI-1 participants were assigned to Stage 0, Aβ(-)/t-tau(-), if they had normal levels of CSF $A\beta_{42}$ and CSF t-tau. Participants in Stage 1, Aβ(+)/t-tau(-), exhibited early amyloid pathology, as

reflected by reduced levels of CSF $A\beta_{42}$. However, individuals in Stage 1 did not display significant levels of neuronal loss, as reflected by low levels of CSF t-tau. In Stage 2, Aβ(+)/t-tau(+), participants exhibited low levels of CSF $A\beta_{42}$ and elevated levels of CSF t-tau. Finally, the Suspected Non-Alzheimer Pathology (SNAP) group, Aβ(-)/t-tau(+), exhibited normal levels of CSF $A\beta_{42}$ and elevated levels of CSF t-tau, thus suggesting other causes of neurodegeneration and/or dementia.

2.3.2.4 Plasma measurements

At the baseline visit, plasma samples were drawn following an overnight fast. 192 analytes that have been reported to be altered in cancer, cardiovascular disease, metabolic disorders, inflammation and AD were analyzed with the Human Discovery Map panel, a multiplex immunoassay panel developed on the Luminex xMAP platform by RBM. In order to meet model assumptions, plasma IGFBP2 levels were $\log_{10}$ transformed by the ADNI investigators.

2.3.2.5 *APOE* genotyping

The ABI 7900 real-time thermo-cycler (Applied Biosystems, Foster City, CA) was used to determine the *APOE* genotype of ADNI participants. TaqMan qPCR was applied to DNA prepared from EDTA whole blood.

2.3.3 QFP cohort

2.3.3.1 Study participants

The Quebec Founder Population (QFP) is composed of the descendants of a few thousand French settlers that colonized Nouvelle France in the 17th and 18th centuries.[52] The migration and the isolated nature of settlements created a founder effect, which resulted in a population with less genetic heterogeneity.[52] Genealogical information for this population, for almost four centuries, is available in the BALSAC database. In the present study, we analyzed the brains of 55 autopsy-confirmed AD cases and 31 autopsy-confirmed elderly controls, which were obtained from the Douglas-Bell Canada Brain Bank. According to medical record reviews, neuropsychological examinations and caregiver interviews, there was no evidence of memory problems, neurological or neuropsychiatric diseases in the elderly control group. Furthermore, controls only exhibited

neuropathology that is associated with healthy aging (plaque and tangle densities <10/mm^3 and < 20/mm^3 in at least one hippocampal and neocortical section). AD cases had to fulfill the histopathological NINCDS-ADRDA criteria for definite AD.[53] This study was conformed to the Code of Ethics of the World Medical Association and was approved by the Ethics Board of the Douglas Mental Health University Institute. This study complied with the ethical principles of the Declaration of Helsinki. Each participant provided written informed consent.

2.3.3.2 IGFBP2 gene expression in the frontal cortex

In the frontal cortex of 31 autopsy-confirmed controls and 55 autopsy-confirmed AD cases, transcriptome-wide gene expression was measured using the human Clariom D Assay, by Génome Québec. Briefly, the Nanodrop Spectrophotometer ND-1000 was used to measure total RNA (NanoDrop Technologies, Inc.). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer. 10 ng of total RNA was used to synthesize sense-strand cDNA. The GeneChip WT Terminal Labeling Kit was used to fragment and label single-stranded cDNA, following the manufacture's instructions. 5 µg cDNA was hybridized on the GeneChip cartridge array and incubated at 45°C for 17 hours in the GeneChip Hybridization Oven 640, at 60 rpm. The microarrays were washed in the GeneChip Fluidics Station 450, using the GeneChip Hybridization, Wash, and Stain Kit, according to the manufacture's instructions. Finally, microarrays were scanned in a GeneChip Scanner 3000. IGFBP2 mRNA levels are presented on a $\log_2$ scale.

2.3.3.3 IGFBP2 protein levels in the frontal cortex

Of the 86 brains that had IGFBP2 mRNA levels measured, 78 brains (n = 25 Controls, n = 53 AD) had IGFBP2 protein levels measured. Frontal cortex brain samples were placed in pre-filled tubes containing 2.8 mm ceramic beads (Omni International, GA, USA). One tablet of protease inhibitor was dissolved in 50 mL of cold phosphate-buffered saline. 1 mL of protease inhibitor solution was added to each tube. The Bead Ruptor 24 (Omni International, Kennesaw, GA, USA) was used to mechanically homogenize brain samples. The Bead Ruptor 24 was set twice at 5.65m/s for 30 seconds, with a 15 second pause between runs. Following homogenization, the samples were stored overnight at -20°C. In order to break the cell membranes, two freeze-thaw cycles were performed. Finally, the homogenates were centrifuged for 5 minutes at 5000 rpm and 4°C. The supernatant was collected and stored for future use at -80°C.

Frontal cortex IGFBP2 protein levels were measured using a commercially available enzyme-linked immunosorbent (ELISA) kit (Cat.# OKEH00084, Aviva Systems Biology, CA, USA). Protocols were performed according to the manufacturers' instructions and results were obtained using the BioTek Synergy H1 microplate reader (Winooski, Vermont, USA). Sample replicates had a coefficient of variability (CV) of less than 20%. Finally, in order to normalize IGFBP2 protein levels, total protein concentration was measured using a commercially available bicinchoninic acid assay developed by Pierce (Cat.# 23225). Finally, normalized IGFBP2 protein ratios were $\log_2$ transformed in order to meet model assumptions.

2.3.3.4 *APOE* genotyping

DNA was extracted from brain tissue with the DNeasy Tissue Kit (Qiagen). As previously described in PREVENT-AD,[39] the PyroMark Q96 pyrosequencer was used to determine *APOE* genotype.

2.3.4 Statistical analyses

Annual changes in CSF IGFBP2 levels were assessed in a subset of PREVENT-AD participants (n = 27). Each participant's trajectory was analyzed using a linear mixed model with a random intercept and slope. Age, sex and *APOE* ε4 carrier status – adjusted linear regression models were used to examine the associations between baseline CSF IGFBP2 and baseline measurements of CSF AD biomarkers ($A\beta_{42}$, p_{181}-tau, t-tau), CSF synaptic proteins (SYT1, SNAP25, GAP43, NRGN) as well as PET and structural (volumetric, cortical thickness) neuroimaging data. For each PREVENT-AD participant that was followed for 5-8 years (n = 89), an estimated cognitive performance trajectory slope was calculated for each of the five cognitive domains of the RBANS. Thus, linear regression models adjusted for age, sex, *APOE* ε4 carrier status and years of education were used to examine the relationship between rate of change in cognition and baseline CSF IGFBP2 levels. Across all analyses, IGFBP2 was assigned as a dependent variable, except for the association with CSF $A\beta_{42}$. Finally, given the critical role of the insulin-IGF system in diverse metabolic processes, we further controlled for clinical covariates such as body mass index (BMI), systolic blood pressure and hemoglobin A1C levels (HbA1c), in supplementary analyses. However, the results of these analyses were similar to those of the original model. Therefore, the following results are presented according to the original model.

In the ADNI-1 cohort, ANCOVA was used to assess the relationship between baseline CSF and baseline plasma IGFBP2 with pathological stage as determined by CSF $A\beta_{42}$ and CSF t-Tau positivity.[50,51] To correct for multiple planned comparisons between pathological stages, statistical significance was considered at $P \leq 0.01$. Finally, Cox proportional hazards models examined the association between baseline plasma IGFBP2 levels and rate of conversion to AD. CU participants and individuals with MCI were followed from the baseline visit to the time of diagnosis (of AD), or to the time the participant was last confirmed to be free of AD. Cox models were adjusted for age, gender and *APOE* ε4 carrier status.

In the QFP cohort, the relationship between frontal cortex mRNA, protein levels, and diagnosis was evaluated with ANCOVA adjusted for age at death, sex, *APOE* ε4 carrier status and post-mortem delay. Statistical significance was considered at $P \leq 0.05$. R^2 values are presented as adjusted R^2. All Analyses were two-tailed, and performed in SPSS 23 (IBM) and JMP Pro 16 (SAS).

2.3.5 Data availability

Data pertaining to the PREVENT-AD cohort can be downloaded from data release 6.0 at https://openpreventad.loris.ca/. CSF, plasma, genetic and clinical data from the ADNI-1 cohort were downloaded from the ADNI website (http://adni.loni.usc.edu/). Data collected from the QFP cohort are not publicly available, however the data are available from the corresponding author upon reasonable request.

2.4 Results

2.4.1 Demographics

Table 1 summarizes the demographic characteristics of the three cohorts that were used to analyze the role of IGFBP2 in the CSF of asymptomatic (PREVENT-AD) and symptomatic individuals (ADNI-1), as well as in the frontal cortex of autopsy-confirmed, age-matched control and AD cases (QFP).

2.4.2 PREVENT-AD cohort

2.4.2.1 CSF IGFBP2 increases annually and is associated with CSF and PET biomarkers in asymptomatic AD

In a subset of CU PREVENT-AD participants that had longitudinal CSF IGFBP2 measurements available (n = 27), a random intercept and random slope linear mixed model revealed that CSF IGFBP2 levels progressively increase over the course of five years (β = 0.132, P = 0.005, Fig. 1).

In PREVENT-AD participants (n = 101) that had CSF AD pathological biomarker measurements, baseline CSF IGFBP2 levels were positively correlated with CSF $A\beta_{42}$ (R^2 = 0.055, β = 91.232, P = 0.023, Fig. 2A), CSF p_{181}-tau (R^2 = 0.162, β = 0.014, P = 4.50 x 10^{-5}, Fig. 2B) and CSF t-tau (R^2 = 0.121, β = 0.001, P = 0.001, Fig. 2C). In a subset of PREVENT-AD participants that underwent Aβ and tau PET scans (n = 46 and n = 49), CSF IGFBP2 was not associated with global cortical Aβ deposition (P = 0.540, Fig. 2D). However, CSF IGFBP2 was positively correlated (trend-level) with tau deposition in the entorhinal cortex (R^2 = 0.027, β = 1.498, P = 0.082, Fig. 2E) and lingual gyrus (R^2 = 0.034, β = 2.226, P = 0.067, Supplementary Fig. 1).

Finally, CSF IGFBP2 was positively associated with synaptic proteins in the CSF, including SNAP25 (trend-level, R^2 = 0.051, β = 0.014, P = 0.076, Fig. 2F), SYT1 (R^2 = 0.060, β = 0.008, P = 0.043, Fig. 2G), GAP43 (R^2 = 0.243, β = 3.06 x 10^{-4}, P = 2.52 x 10^{-4}, Fig. 2H), and NRGN (trend-level, R^2 = 0.033, β = 0.002, P = 0.063, Fig. 2I).

2.4.2.2 CSF IGFBP2 is linked to changes in delayed memory and visuospatial abilities in asymptomatic AD

Upon computing RBANS cognitive performance trajectory slopes estimated over the course of 5 to 8 years in a subset of PREVENT-AD participants (n = 89), baseline CSF IGFBP2 levels were negatively correlated with estimated rates of change in delayed memory scores (R^2 = 0.072, β = -0.098, P = 0.024, Fig. 3). Furthermore, baseline CSF IGFBP2 levels were negatively correlated with rates of change in visuospatial constructional abilities (R^2 = 0.077, β = -0.082, P = 0.019, Fig. 3). However, baseline IGFBP2 levels were not associated with changes in immediate memory (P = 0.191), language (P = 0.332) or attention (P = 0.679) (data not shown).

2.4.2.3 CSF IGFBP2 is associated with cortical atrophy in AD-specific brain regions in asymptomatic AD

We analyzed baseline structural neuroimaging data collected from a subset of PREVENT-AD individuals (n = 104) in a cross-sectional fashion. Four individuals were omitted from analyses due to failed quality control regarding subject-specific stereotaxic registration and/or brain masking. After adjusting for total intracranial volume, baseline CSF IGFBP2 was negatively correlated with entorhinal cortex volumes in the left hemisphere at a trend-level (R^2 = 0.117, β = -39.720, P = 0.082, Fig. 4A). However, CSF IGFBP2 was not associated with entorhinal cortex volume in the right hemisphere (P = 0.962).

Next, we analyzed baseline cortical thickness in pre-specified temporal and parietal brain regions that are vulnerable to early AD pathology. Baseline CSF IGFBP2 was found to be negatively correlated with cortical thickness in the piriform cortex (trend, left hemisphere: R^2 = 0.121, β = -0.628, P = 0.064; right hemisphere: R^2 = 0.128, β = -0.709, P = 0.039, Fig. 4B), inferior temporal gyrus (left hemisphere: R^2 = 0.153, β = -1.253, P = 0.008; right hemisphere: P = 0.315, Fig. 4C), middle temporal gyrus (left hemisphere: R^2 = 0.144, β = -1.369, P = 0.014; right hemisphere: R^2 = 0.116, β = -0.959, P = 0.090, Fig. 4D), and precuneus (left hemisphere: P = 0.123; right hemisphere: R^2 = 0.131, β = -1.353, P = 0.033, Supplementary Fig. 2).

2.4.2.4 Changes in CSF IGFBP2 are specific to the CNS in asymptomatic AD

To examine possible blood-brain barrier (BBB) dysfunction and possible peripheral vascular contributions to CSF IGFBP2 levels, we measured microprotein levels, red blood cell count and white blood cell count in the CSF. We did not find any associations between these vascular factors and CSF IGFBP2 (Supplementary Fig. 3); consistent with a relatively intact BBB in asymptomatic PREVENT-AD participants.[54]

2.4.3 ADNI-1 cohort

2.4.3.1 CSF and plasma IGFBP2 concentrations are elevated in CSF Aβ(+)/t-tau(+) individuals

89 CU individuals and 144 individuals with MCI from ADNI were staged as amyloid and/or tau positive according to recommended CSF $A\beta_{42}$ and CSF t-tau thresholds of 192 pg/mL and 93 pg/mL, respectively (Fig. 5A).[50,51] The results from the CSF multiplex immunoassay (Fig. 5B) revealed that baseline CSF IGFBP2 levels did not differ between Stages 0 (n = 80) Aβ(-)/t-tau(-) and Stage 1 (n = 68) Aβ(+)/t-tau(-), P = 0.541. However, CSF IGFBP2 was significantly elevated at Stage 2 (n = 77) Aβ(+)/t-tau(+) relative to Stage 0 (P = 0.009) and Stage 1 (P = 0.001). Finally, CSF IGFBP2 was significantly increased in SNAP (n = 8) Aβ(-)/t-tau(+), relative to Stage 0 (P = 0.010).

To reproduce our findings, we performed replication analyses using CSF mass spectrometry data acquired from a subset of the same ADNI participants. These supplementary analyses (Supplementary Fig. 4) revealed that the CSF IGFBP2 peptide HGLYNLK was significantly reduced at Stage 1 (n = 62) relative to Stage 0 (n = 75), P = 0.005. However, this reduction at Stage 1 was not statistically significant for the CSF IGFBP2 peptide LIQGAPTIR (P = 0.051), at a significance level of $P < 0.01$. Although a similar reduction at Stage 1 was observed with the multiplex immunoassay data, it was not statistically significant. However, similar to the multiplex immunoassay data, both IGFBP2 peptides were significantly elevated at Stage 2 (n = 72) relative to Stage 1, $P = 1.50 \times 10^{-5}$ and $P = 2.21 \times 10^{-4}$. Likewise, both IGFBP2 peptides were markedly increased in SNAP (n = 9) relative to Stage 0, $P = 4.90 \times 10^{-5}$ and P = 0.002, consistent with the immunoassay results.

Finally, we staged 58 CU individuals and 196 individuals with MCI from ADNI, that had both baseline plasma IGFBP2 measurements and CSF $A\beta_{42}$ and CSF t-tau measurements (Fig. 5C). Baseline plasma IGFBP2 levels were significantly elevated in Stage 2 (n = 84) relative to Stage 0 (n = 98), P = 0.001, but not to Stage 1 (n = 60), P = 0.041 – at a significance threshold of $P < 0.01$. Finally, plasma IGFBP2 did not differ between Stage 0 and Stage 1 (P = 0.208), or between Stage 0 and SNAP (n = 12), P = 0.874.

2.4.3.2 Elevated plasma IGFBP2 is associated with a faster rate of conversion to AD

In the primary analysis for conversion to AD in ADNI, we established baseline plasma IGFBP2 threshold values at the 25th percentile (≤1.83251, first quartile, log_{10} transformed) and above the 75th percentile (≥2.09342, fourth quartile, log_{10} transformed). A total of 226 individuals that were either CU or had MCI were included in these analyses. Of these dementia-free participants, 107 individuals eventually met the clinical criteria for a diagnosis of AD (mean follow-up, 3.8 years; range, 0.5-16.5 years). Cox proportional hazards models revealed that individuals with plasma IGFBP2 values greater than the 75th percentile exhibited a faster rate of conversion to AD, than individuals with plasma IGFBP2 values less than the 25th percentile (Hazard ratio (HR) 1.616, 95% CI 1.074-2.430, P = 0.021, Fig. 6).

In the secondary analysis, baseline IGFBP2 plasma levels were kept as continuous, and 439 ADNI-1 dementia-free participants with plasma IGFBP2 measurements were included. Of these individuals, 214 were eventually diagnosed with AD (mean follow-up, 3.6 years; range, 0.5-16.5 years). Similar to the first model, elevated plasma IGFBP2 was associated with a greater rate of conversion to AD (HR 1.857, 95% CI 1.054-3.270, P = 0.032).

2.4.4 QFP cohort

2.4.4.1 Despite reductions in IGFBP2 mRNA, protein levels do not differ in the frontal cortex of AD brains

IGFBP2 gene expression was assessed by DNA microarray in the QFP cohort, and demonstrated to be significantly reduced in the frontal cortex of autopsy-confirmed AD brains (n = 55), compared to elderly controls (n = 31) (P = 0.049, Fig. 7A). However, as demonstrated through ELISA, IGFBP2 protein levels did not differ in the frontal cortex of AD cases (n = 53) after controlling for total protein levels, relative to controls (n = 25, P = 0.462, Fig. 7B).

2.5 Discussion

It has been well established that impaired insulin-IGF signalling plays a critical role in AD.[8-10] Furthermore, insulin and IGF proteins are highly expressed in the hippocampus.[8] Therefore, it is

possible that impairments in insulin-IGF signaling may account for the selective vulnerability of the hippocampal formation to nascent AD pathology**.** Hence, targeting the insulin-IGF system may offer a promising solution to delay, slow down and/or prevent AD, either alone or in combination therapies**.** However, in order to develop these therapies, unraveling the molecular intricacies of the insulin-IGF system is necessary**.** To this end, we investigated the role of the most abundant IGF-binding protein in the CSF, IGFBP2,[28,29] during the earliest possible asymptomatic stage of AD, in "at-risk", parental history-positive PREVENT-AD participants.

We observed a positive relationship between IGFBP2 and CSF $A\beta_{42}$ (Fig. 2A), which is consistent with the proposed IGF-mediated clearance of Aβ.[12,13] This notion is in agreement with the finding that current Aβ-lowering therapies induce an increase in CSF $A\beta_{42}$ in participants with MCI or mild AD.[55] Furthermore, our data suggest IGFBP2 may be upregulated as a result of early neuronal loss in the asymptomatic stage of the disease (Fig. 2C); which is consistent with the extensive literature regarding the upregulation of IGFs and IGFBPs following several rodent models of brain damage and recovery.[56-63] Furthermore, the administration of des-IGF-I, an analogue of IGF-1 with a low affinity for IGFBPs, failed to attenuate neuronal cell death in mice with experimental hypoxic ischemic injuries.[64] However, the administration of IGFBP-compatible IGF-I significantly reduced the observed neuronal loss.[64]

Our findings also suggest that IGFBP2 may be modulated by early CSF p_{181}-tau production (Fig. 2B) and deposition in asymptomatic individuals. PET imaging analyses revealed that IGFBP2 is positively associated (at a trend-level) with significant tau deposition in the entorhinal cortex (Fig. 2E) and lingual gyrus (Supplementary Fig. 1), features that are typically associated with early Braak Stages 2-3.[65] These findings are certainly consistent with the fact that insulin and IGFs normally inhibit GSK3 activity and phospho-tau production in human neurons, through the PI3K-AKT signaling pathway.[14,15] For instance, the pharmacological inhibition of GSK3 has been linked to increases in IGF-I in the rodent brain,[66] whereas conditional transgenic mice overexpressing GSK3 in the cortex and hippocampus display increased tau phosphorylation in AD relevant epitopes,[67] degeneration of the dentate gyrus[68] and spatial memory impairments.[69] Finally, mice overexpressing IGFBP2 display an increase in AKT activity, a GSK3 inhibitor, in the brain.[70] Thus, it is tempting to postulate that IGF regulation of GSK3 activity in turn modulates IGFBP2

production via a phospho-tau mediated process that ensures some form of local autoregulation and/or resilience.

Given the prominent loss of synapses in AD, we examined the relationship between IGFBP2 and synaptic proteins in the CSF, namely SNAP25 (Fig. 2F), SYT1 (Fig. 2G), GAP43 (Fig. 2H) and NRGN (Fig. 2I).[40-45] The present study's results suggest that synaptic dysfunction and loss may trigger an increase in IGFBP2 synthesis and secretion. This finding is in agreement with the upregulation and regenerative abilities of IGFs to grow axons during the development of the CNS and regrow axons during repair following injury to the CNS and/or PNS.[21,71-76]

Considering the relationship between CSF synaptic markers and CSF IGFBP2, we were interested in examining the relationship between changes in cognition and baseline CSF IGFBP2 in PREVENT-AD. We have demonstrated that CSF IGFBP2 levels are associated with cognitive decline in RBANS delayed memory and visuospatial abilities over a 5-8-year period, in a subset of PREVENT-AD participants (Fig. 3). This finding is consistent with a report that plasma levels of IGFBP2 were negatively correlated with episodic memory performances in participants from the ADNI cohort.[77] Thus, given the critical role of the hippocampus in the consolidation of declarative memory and in spatial processing, our data provide compelling evidence that IGFBP2 plays a pivotal role in the integrity of the hippocampal formation, which displays elevated expression levels of insulin, IGFs and their receptors – compared to other brain regions, such as the frontal cortex.[8] Indeed, plasma IGFBP2 levels have been demonstrated to be negatively correlated with hippocampal volumes in amyloid-negative individuals from the ADNI cohort.[77] Consistent with this view, IGFBP2 has been demonstrated to be expressed by neurons and astrocytes in the hippocampus during development and following CNS injury.[57-59,62,78,79] Furthermore, the relationship between IGFBP2, delayed memory and hippocampal structure is consistent with the finding that the administration of IGFBP2 has been shown to increase the number of dendritic spines in the dentate gyrus of rodent models of post-traumatic stress disorder.[80] In a similar fashion, in cell culture experiments, antibodies targeted against IGFBP2 have been demonstrated to inhibit neurogenesis – a phenomenon that occurs in the dentate gyrus.[81] Moreover, *IGFBP2* knockout mice exhibit deficits in long-term potentiation as well as impaired performances on the Morris water maze, which heavily relies on the hippocampus.[79] Finally, the administration of an IGFBP2-derived peptide has been demonstrated to rescue deficits in synaptic plasticity,

memory and learning in a mouse model of *SHANK3*-mediated post-synaptic deficits.[82] Overall, these findings suggest that IGFBP2 is tightly linked to neuroprotection and hippocampal-mediated cognitive abilities.

Given the relationship between IGFBP2 and cognitive abilities, we were prompted to examine whether baseline levels of CSF IGFBP2 were associated with anatomical changes in the brain. Interestingly, in asymptomatic PREVENT-AD participants, IGFBP2 was associated with atrophy in the left entorhinal cortex, at a trend-level (Fig. 4A).This is a critical finding, as the entorhinal cortex is the first region that is affected in AD.[65,83,84] Furthermore, IGFBP2 was associated with cortical thinning in several pre-specified temporal and parietal brain regions that are known to be affected early on in AD, such as the piriform cortex (Fig. 4B), inferior (Fig. 4C) and middle temporal gyri (Fig. 4D), and precuneus (Supplementary Fig. 2).[85,86] Overall, the structural neuroimaging results are in agreement with previous reports of IGFBP2 being associated with atrophy in AD-associated brain regions.[36,87,88] Furthermore, our neuroimaging results are in line with our CSF results, and provide further evidence that IGFBP2 may be upregulated in response to synaptic and neuronal loss in vulnerable brain regions.

Finally, in the PREVENT-AD cohort, changes in CSF IGFBP2 appear to be specific to changes in the CNS, as we do not suspect any peripheral vascular contribution in these asymptomatic individuals (Supplementary Fig. 3).[54]

To independently validate our observations, we further analyzed data from a well-characterized cohort of CU individuals and individuals with MCI from ADNI-1 (Fig. 5A). Our results suggest that elevated CSF (Fig. 5B, Supplementary Fig. 4) and plasma IGFBP2 (Fig. 5C) may be valuable biomarkers of CSF Aβ(+)/t-tau(+) individuals and thus, facilitate screening for suitable patients for clinical trials.[50,51] Furthermore, upon conducting survival analyses we found that elevated circulating IGFBP2 levels were associated with a pronounced rate of conversion to AD (Fig. 6). Overall, our results are consistent with the existing literature that plasma IGFBP2 has been associated with a greater risk of developing AD.[35,37,38] Finally, in contrast to the PREVENT-AD cohort, CSF IGFBP2 immunoassay measurements have been previously found to correlate with plasma IGFBP2 levels in the ADNI cohort – which incorporates individuals with a disrupted BBB.[77]

Finally, given the regional-specificity of the insulin-IGF system, we further examined IGFBP2 levels in the human brain.[8] IGFBP2 mRNA levels were reduced in the frontal cortex of autopsy-confirmed AD brains from the QFP cohort (Fig. 7A). However, cortical IGFBP2 protein levels did not differ between AD brains and elderly controls (Fig. 7B). Thus, our results provide further evidence that support the (hippocampal) regional specificity of the insulin-IGF system, potentially including the IGFBP2 protein.[8] However, due to the scarcity of medial temporal lobe tissues, we were unable to confirm the regional specificity of the IGFBP2 protein in the QFP cohort. Nevertheless, our results differ from those of a pilot study, that found IGFBP2 was decreased in the temporal cortex of AD brains.[9] It is possible our results differ since we measured cytoplasmic and extracellular IGFBP2, whilst the pilot study measured membrane-bound IGFBP2.[9] Nevertheless, temporal cortex IGFBP2 levels were negatively correlated with senile plaque levels – suggesting a role for IGFBP2 in neuroprotection and resilience.[9] Similarly, although IGFBP2 has been demonstrated to contain a nuclear localization signal and regulate the expression of several genes such as vascular endothelial growth factor, we were unable to differentiate between IGF-dependent and IGF-independent IGFBP2.[89]

Overall, our data suggest that IGFBP2 may play a critical role in neuroprotection during the asymptomatic stage of AD. Indeed, there is compelling evidence that IGFBP2 is involved in resilience, as several neuroprotective genes have been demonstrated to be upregulated by the (soluble) α-secretase cleaved fragment of amyloid precursor protein (sAPPα), amongst them, *IGFBP2* and *IGF2*.[90] It is possible that an increase in binding of IGFBP2 to IGFs may attenuate IGF degradation and/or promote the targeting of IGFs to their receptors, thus enhancing synaptic and terminal resilience in face of the emerging neurodegenerative process. The receptor targeting hypothesis is notably supported by evidence that transgenic mice overexpressing IGFBP2 lacking a proteoglycan-binding domain exhibit severe reductions in synaptic markers and hippocampal weight.[91] Finally, in the context of the insulin-IGF system, it will be exciting to follow the results of ongoing phase 3 clinical trials involving the administration of semaglutide (Ozempic), a glucagon-like peptide-1 receptor agonist that is clinically approved for the treatment of type 2 diabetes.[92] Considering the role of semaglutide in stimulating insulin secretion and signalling,[92] exploring therapeutic strategies that enhance IGF signalling in the brain,[93] perhaps through IGFBP2, may also warrant further investigation in asymptomatic AD.

2.6 Acknowledgements

The authors would like to thank Dr. Naguib Mechawar at the Douglas Institute/Bell Canada Brain Bank for providing human brain tissues from the Quebec Founder Population. We also wish to thank Mrs. Jennifer Tremblay-Mercier, Marie-Elyse Lafaille-Magnan and Melissa Savard as well as Drs. Pedro Rosa-Neto, Daniel Auld and David Lafontaine for their technical expertise.

2.7 Funding

JP is supported by the Fonds de recherche du Québec-Santé (FRQS), the Canadian Institutes of Health Research (CIHR #PJT 153287) and the J.L. Levesque Foundation. SV is supported by a Canada Research Chair and a Canada Fund for Innovation grant, the FRQS, the CIHR, Brain Canada, McGill University and the Alzheimer's Association. HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2022-01018 and #2019-02397), the European Union's Horizon Europe research and innovation programme under grant agreement No 101053962, Swedish State Support for Clinical Research (#ALFGBG-71320), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), the AD Strategic Fund and the Alzheimer's Association (#ADSF-21-831376-C, #ADSF-21-831381-C, and #ADSF-21-831377-C), the Bluefield Project, the Olav Thon Foundation, the Erling-Persson Family Foundation, Stiftelsen för Gamla Tjänarinnor, Hjärnfonden, Sweden (#FO2022-0270), the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE), the European Union Joint Programme – Neurodegenerative Disease Research (JPND2021-00694), the National Institute for Health and Care Research University College London Hospitals Biomedical Research Centre, and the UK Dementia Research Institute at UCL (UKDRI-1003). KB is supported by the Swedish Research Council (#2017-00915 and #2022-00732), the Swedish Alzheimer Foundation (#AF-930351, #AF-939721 and #AF-968270), Hjärnfonden, Sweden (#FO2017-0243 and #ALZ2022-0006), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986 and #ALFGBG-965240), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), the Alzheimer's Association 2021 Zenith Award (ZEN-21-848495), and the Alzheimer's Association 2022-2025 Grant (SG-23-1038904 QC). MJQ is supported by the FRQS.

PREVENT-AD was launched in 2011 as a $13.5 million, 7-year public-private partnership using funds provided by McGill University, FRQS, an unrestricted research grant from Pfizer Canada, the Levesque Foundation, the Douglas Hospital Research Centre and Foundation, the Government of Canada, and the Canada Fund for Innovation. Private sector contributions are facilitated by the Development Office of the McGill University Faculty of Medicine and by the Douglas Hospital Research Centre Foundation (http://www.douglas.qc.ca/).

Data collection and sharing for this project was funded by the ADNI (National Institutes of Health Grant U01 AG024904) and DOD ADNI (Department of Defense award number W81XWH-12-2-0012). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: AbbVie, Alzheimer's Association; Alzheimer's Drug Discovery Foundation; Araclon Biotech; BioClinica, Inc.; Biogen; Bristol-Myers Squibb Company; CereSpir, Inc.; Cogstate; Eisai Inc.; Elan Pharmaceuticals, Inc.; Eli Lilly and Company; EuroImmun; F. Hoffmann-La Roche Ltd and its affiliated company Genentech, Inc.; Fujirebio; GE Healthcare; IXICO Ltd.;Janssen Alzheimer Immunotherapy Research & Development, LLC.; Johnson & Johnson Pharmaceutical Research & Development LLC.; Lumosity; Lundbeck; Merck & Co., Inc.;Meso Scale Diagnostics, LLC.; NeuroRx Research; Neurotrack Technologies; Novartis Pharmaceuticals Corporation; Pfizer Inc.; Piramal Imaging; Servier; Takeda Pharmaceutical Company; and Transition Therapeutics. The CIHR is providing funds to support ADNI clinical sites in Canada. Private sector contributions are facilitated by the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Therapeutic Research Institute at the University of Southern California. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of Southern California.

2.8. Competing interests

JP serves as a scientific advisor to the Alzheimer Society of France. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND,

Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). KB has served as a consultant and at advisory boards for Acumen, ALZPath, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

2.9 Supplementary material

Supplementary material is available at *Brain* online.

2.10 References

1. GBD 2019 Dementia Forecasting Collaborators. Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. *Lancet Public Health*. 2022;7(2):e105-e125.
2. Nandi A, Counts N, Chen S, et al. Global and regional projections of the economic burden of Alzheimer's disease and related dementias from 2019 to 2050: A value of statistical life approach. *EClinicalMedicine*. 2022;51:101580.
3. Alzheimer's Association. 2022 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2022;18(4):700-789.
4. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspect Med*. 2011;1(1):a006189.
5. Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119-128.
6. Arvanitakis Z, Wilson RS, Bienias JL, Evans DA, Bennett DA. Diabetes mellitus and risk of Alzheimer disease and decline in cognitive function. *Arch Neurol*. 2004;61(5):661-666.

7. Arnold SE, Arvanitakis Z, Macauley-Rambach SL, et al. Brain insulin resistance in type 2 diabetes and Alzheimer disease: concepts and conundrums. *Nat Rev Neurol*. 2018;14(3):168-181.
8. Steen E, Terry BM, J Rivera E, et al. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease–is this type 3 diabetes?. *J Alzheimers Dis*. 2005;7(1):63-80.
9. Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging*. 2010;31(2):224-243.
10. Talbot K, Wang HY, Kazi H, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest*. 2012;122(4):1316-1338.
11. Kar S, Poirier J, Guevara J, et al. Cellular distribution of insulin-like growth factor-II/mannose-6-phosphate receptor in normal human brain and its alteration in Alzheimer's disease pathology. *Neurobiol Aging*. 2006;27(2):199-210.
12. Farris W, Mansourian S, Chang Y, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid β-protein, and the β-amyloid precursor protein intracellular domain in vivo. *Proc Natl Acad Sci U S A*. 2003;100(7):4162-4167.
13. Carro E, Trejo JL, Gomez-Isla T, LeRoith D, Torres-Aleman I. Serum insulin-like growth factor I regulates brain amyloid-β levels. *Nat Med*. 2002;8(12):1390-1397.
14. Hong M, Lee VM. Insulin and insulin-like growth factor-1 regulate tau phosphorylation in cultured human neurons. *J Biol Chem*. 1997;272(31):19547-19553.
15. Schubert M, Brazil DP, Burks DJ, et al. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. *J Neurosci*. 2003;23(18):7084-7092.
16. De Felice FG, Vieira MN, Bomfim TR, et al. Protection of synapses against Alzheimer's-linked toxins: insulin signaling prevents the pathogenic binding of Aβ oligomers. *Proc Natl Acad Sci U S A*. 2009;106(6):1971-1976.
17. Doré S, Kar S, Quirion R. Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. *Proc Natl Acad Sci U S A*. 1997;94(9):4772-4777.

18. Willette AA, Bendlin BB, Starks EJ, et al. Association of Insulin Resistance With Cerebral Glucose Uptake in Late Middle-Aged Adults at Risk for Alzheimer Disease. *JAMA Neurol*. 2015;72(9):1013-1020.
19. Reger MA, Watson GS, Green PS, et al. Intranasal insulin improves cognition and modulates beta-amyloid in early AD. *Neurology*. 2008;70(6):440-448.
20. Craft S, Baker LD, Montine TJ, et al. Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. *Arch Neurol*. 2012;69(1):29-38.
21. O'Kusky JR, Ye P, D'Ercole AJ. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J Neurosci*. 2000;20(22):8435-8442.
22. Russo VC, Gluckman PD, Feldman EL, Werther GA. The insulin-like growth factor system and its pleiotropic functions in brain. *Endocr Rev*. 2005;26(7):916-43.
23. Allard JB, Duan C. IGF-binding proteins: why do they exist and why are there so many?. *Front Endocrinol (Lausanne).* 2018;9:117.
24. Hwa V, Oh Y, Rosenfeld RG. The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocr Rev*. 1999;20(6):761-787.
25. Russo VC, Bach LA, Fosang AJ, Baker NL, Werther GA. Insulin-like growth factor binding protein-2 binds to cell surface proteoglycans in the rat brain olfactory bulb. *Endocrinology*. 1997;138(11):4858-4867.
26. Schütt BS, Langkamp M, Rauschnabel U, Ranke MB, Elmlinger MW. Integrin-mediated action of insulin-like growth factor binding protein-2 in tumor cells. *J Mol Endocrinol*. 2004;32(3):859-868.
27. Russo VC, Rekaris G, Baker NL, Bach LA, Werther GA. Basic fibroblast growth factor induces proteolysis of secreted and cell membrane-associated insulin-like growth factor binding protein-2 in human neuroblastoma cells. *Endocrinology*. 1999;140(7):3082-3090.
28. Roghani M, Lassarre C, Zapf J, Povoa G, Binoux M. Two insulin-like growth factor (IGF)-binding proteins are responsible for the selective affinity for IGF-II of cerebrospinal fluid binding proteins. *J Clin Endocrinol Metab*. 1991;73(3):658-666.

29. Ocrant I, Fay CT, Parmelee JT. Characterization of insulin-like growth factor binding proteins produced in the rat central nervous system. *Endocrinology*. 1990;127(3):1260-1267.
30. Tham A, Nordberg A, Grissom FE, Carlsson-Skwirut C, Viitanen M, Sara VR. Insulin-like growth factors and insulin-like growth factor binding proteins in cerebrospinal fluid and serum of patients with dementia of the Alzheimer type. *J Neural Transm Park Dis Dement Sect*.1993;5(3):165-176.
31. Salehi Z, Mashayekhi F, Naji M. Insulin like growth factor-1 and insulin like growth factor binding proteins in the cerebrospinal fluid and serum from patients with Alzheimer's disease. *Biofactors*. 2008;33(2):99-106.
32. Hertze J, Nägga K, Minthon L, Hansson O. Changes in cerebrospinal fluid and blood plasma levels of IGF-II and its binding proteins in Alzheimer's disease: an observational study. *BMC Neurol*. 2014;14:64.
33. Åberg D, Johansson P, Isgaard J, et al. Increased cerebrospinal fluid level of insulin-like growth factor-II in male patients with Alzheimer's disease. *J Alzheimers Dis*. 2015;48(3):637-646.
34. O'Bryant SE, Xiao G, Barber R, et al. A serum protein-based algorithm for the detection of Alzheimer disease. *Arch Neurol*. 2010;67(9):1077-1081.
35. Doecke JD, Laws SM, Faux NG, et al. Blood-based protein biomarkers for diagnosis of Alzheimer disease. *Arch Neurol*. 2012;69(10):1318-1325.
36. Bonham LW, Geier EG, Steele NZ, et al. Insulin-like growth factor binding protein 2 is associated with biomarkers of Alzheimer's disease pathology and shows differential expression in transgenic mice. *Front Neurosci*. 2018;12:476.
37. McGrath ER, Himali JJ, Levy D, et al. Circulating IGFBP-2: a novel biomarker for incident dementia. *Ann Clin Transl Neurol*. 2019;6(9):1659-1670.
38. Rocha de Paula M, Gómez Ravetti M, Berretta R, Moscato P. Differences in abundances of cell-signalling proteins in blood reveal novel biomarkers for early detection of clinical Alzheimer's disease. *PLoS One*. 2011;6(3):e17481.
39. Tremblay-Mercier J, Madjar C, Das S, et al. Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. *Neuroimage Clin*. 2021;31:102733.

40. Brinkmalm A, Brinkmalm G, Honer WG, et al. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Mol Neurodegener*. 2014;9:53.
41. Öhrfelt A, Brinkmalm A, Dumurgier J, et al. The pre-synaptic vesicle protein synaptotagmin is a novel biomarker for Alzheimer's disease. *Alzheimers Res Ther*. 2016;8(1):41.
42. Tible M, Sandelius Å, Höglund K, et al. Dissection of synaptic pathways through the CSF biomarkers for predicting Alzheimer disease. *Neurology*. 2020;95(8):e953-e961.
43. Xu C, Sellgren CM, Fatouros-Bergman H, et al. CSF levels of synaptosomal-associated protein 25 and synaptotagmin-1 in first-episode psychosis subjects. *IBRO Rep*. 2020;8:136-142.
44. Sandelius Å, Portelius E, Källén Å, 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.
45. Portelius E, Olsson B, Höglund K, et al. Cerebrospinal fluid neurogranin concentration in neurodegeneration: relation to clinical phenotypes and neuropathology. *Acta Neuropathol*. 2018;136(3):363-376.
46. McSweeney M, Binette AP, Meyer PF, et al. Intermediate flortaucipir uptake is associated with Aβ-PET and CSF tau in asymptomatic adults. *Neurology*. 2020;94(11):e1190-1200
47. Zijdenbos AP, Forghani R, Evans AC. Automatic "pipeline" analysis of 3-D MRI data for clinical trials: application to multiple sclerosis. *IEEE Trans Med Imaging*. 2002;21(10):1280–1291.
48. Aubert-Broche B, Fonov VS, García-Lorenzo D, et al. A new method for structural volume analysis of longitudinal brain MRI data and its application in studying the growth trajectories of anatomical brain structures in childhood. *Neuroimage*. 2013;82:393-402.
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):310-319.
50. Jack CR Jr, Bennett DA, Blennow K, et al. NIA-AA research framework: toward a biological definition of Alzheimer's disease. *Alzheimers Dement*. 2018;14(4):535-562.

51. Shaw LM, Vanderstichele H, Knapik-Czajka M, et al. Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects. *Ann Neurol*. 2009;65(4):403-413.
52. Scriver CR. Human genetics: lessons from Quebec populations. *Annu Rev Genomics Hum Genet*. 2001;2:69-101.
53. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group* under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. 1984;34(7):939-944.
54. Picard C, Nilsson N, Labonté A, et al. Apolipoprotein B is a novel marker for early tau pathology in Alzheimer's disease. *Alzheimers Dement*. 2022;18(5):875-887.
55. van Dyck CH, Swanson CJ, Aisen P, et al. Lecanemab in Early Alzheimer's Disease. *N Engl J Med*. 2023;388(1):9-21.
56. Gluckman P, Klempt N, Guan J, et al. A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun*. 1992;182(2):593-599.
57. Klempt ND, Klempt M, Gunn AJ, Singh K, Gluckman PD. Expression of insulin-like growth factor-binding protein 2 (IGF-BP 2) following transient hypoxia-ischemia in the infant rat brain. *Brain Res Mol Brain Res*. 1992;15(1-2):55-61.
58. Beilharz EJ, Russo VC, Butler G, et al. Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic–ischemic injury. *Brain Res Mol Brain Res*. 1998;59(2):119-134.
59. Sandberg Nordqvist AC, Von Holst H, Holmin S, Sara VR, Bellander BM, Schalling M. Increase of insulin-like growth factor (IGF)-1, IGF binding protein-2 and− 4 mRNAs following cerebral contusion. *Brain Res Mol Brain Res*. 1996;38(2):285-293.
60. Walter HJ, Berry M, Hill DJ, Logan A. Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain. *Endocrinology*. 1997;138(7):3024-3034.
61. Fletcher L, Isgor E, Sprague S, et al. Spatial distribution of insulin-like growth factor binding protein-2 following hypoxic-ischemic injury. *BMC Neurosci*. 2013;14:158.

62. Breese CR, D'Costa A, Rollins YD, et al. Expression of insulin-like growth factor-1 (IGF-1) and IGF-binding protein 2 (IGF-BP2) in the hippocampus following cytotoxic lesion of the dentate gyrus. *J Comp Neurol*. 1996;369(3):388-404.
63. Kar S, Baccichet A, Quirion R, Poirier J. Entorhinal cortex lesion induces differential responses in [125I]insulin-like growth factor I, [125I]insulin-like growth factor II and [125I]insulin receptor binding sites in the rat hippocampal formation. *Neuroscience*. 1993;55(1):69-80.
64. Guan J, Williams CE, Skinner SJ, Mallard EC, Gluckman PD. The effects of insulin-like growth factor (IGF)-1, IGF-2, and des-IGF-1 on neuronal loss after hypoxic-ischemic brain injury in adult rats: evidence for a role for IGF binding proteins. *Endocrinology*. 1996;137(3):893-898.
65. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. *Neurobiol Aging*. 1995;16(3):271-284.
66. Bolós M, Fernandez S, Torres-Aleman I. Oral administration of a GSK3 inhibitor increases brain insulin-like growth factor I levels. *J Biol Chem*. 2010;285(23):17693-17700.
67. Lucas JJ, Hernández F, Gómez-Ramos P, Morán MA, Hen R, Avila J. Decreased nuclear beta-catenin, tau hyperphosphorylation and neurodegeneration in GSK-3beta conditional transgenic mice. *EMBO J*. 2001;20(1-2):27-39.
68. Engel T, Lucas JJ, Gómez-Ramos P, Moran MA, Avila J, Hernández F. Cooexpression of FTDP-17 tau and GSK-3beta in transgenic mice induce tau polymerization and neurodegeneration. *Neurobiol Aging*. 2006;27(9):1258-1268.
69. Hernández F, Borrell J, Guaza C, Avila J, Lucas JJ. Spatial learning deficit in transgenic mice that conditionally over-express GSK-3beta in the brain but do not form tau filaments. *J Neurochem*. 2002;83(6):1529-1533.
70. Hoeflich A, Reyer A, Ohde D, et al. Dissociation of somatic growth, time of sexual maturity, and life expectancy by overexpression of an RGD-deficient IGFBP-2 variant in female transgenic mice. *Aging Cell*. 2016;15(1):111-117.
71. Kanje M, Skottner A, Sjöberg J, Lundborg G. Insulin-like growth factor I (IGF-I) stimulates regeneration of the rat sciatic nerve. *Brain Res*. 1989;486(2):396-398.
72. Near SL, Whalen LR, Miller JA, Ishii DN. Insulin-like growth factor II stimulates motor nerve regeneration. *Proc Natl Acad Sci U S A*. 1992;89(24):11716-11720.

73. Guthrie KM, Nguyen T, Gall CM. Insulin-like growth factor-1 mRNA is increased in deafferented hippocampus: spatiotemporal correspondence of a trophic event with axon sprouting. *J Comp Neurol*. 1995;352(1):147-160.
74. Duan X, Qiao M, Bei F, Kim IJ, He Z, Sanes JR. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. *Neuron*. 2015;85(6):1244-1256.
75. Anderson MA, O'Shea TM, Burda JE, et al. Required growth facilitators propel axon regeneration across complete spinal cord injury. *Nature*. 2018;561(7723):396-400.
76. Liu Y, Wang X, Li W, et al. A Sensitized IGF1 Treatment Restores Corticospinal Axon-Dependent Functions. *Neuron*. 2017;95(4):817-833.e4.
77. Lane EM, Hohman TJ, Jefferson AL, Alzheimer's Disease Neuroimaging Initiative. Insulin-like growth factor binding protein-2 interactions with Alzheimer's disease biomarkers. *Brain Imaging Behav*. 2017;11(6):1779-1786.
78. Lee WH, Michels KM, Bondy CA. Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: correlation with insulin-like growth factors I and II. *Neuroscience*. 1993;53(1):251-265.
79. Khan S, Lu X, Huang Q, et al. IGFBP2 plays an essential role in cognitive development during early life. *Adv Sci (Weinh)*. 2019;6(23):1901152.
80. Burgdorf J, Colechio EM, Ghoreishi-Haack N, et al. IGFBP2 produces rapid-acting and long-lasting effects in rat models of posttraumatic stress disorder via a novel mechanism associated with structural plasticity. *Int J Neuropsychopharmacol*. 2017;20(6):476-484.
81. Brooker GJ, Kalloniatis M, Russo VC, Murphy M, Werther GA, Bartlett PF. Endogenous IGF-1 regulates the neuronal differentiation of adult stem cells. *J Neurosci Res*. 2000;59(3):332-341.
82. Burgdorf JS, Yoon S, Dos Santos M, Lammert CR, Moskal JR, Penzes P. An IGFBP2-derived peptide promotes neuroplasticity and rescues deficits in a mouse model of Phelan-McDermid syndrome. *Mol Psychiatry*. 2023;28(3):1101-1111.
83. Gómez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci*. 1996;16(14):4491-4500.

84. Dickerson BC, Goncharova I, Sullivan MP, et al. MRI-derived entorhinal and hippocampal atrophy in incipient and very mild Alzheimer's disease. *Neurobiol Aging*. 2001;22(5):747-754.
85. Dickerson BC, Bakkour A, Salat DH, et al. The cortical signature of Alzheimer's disease: regionally specific cortical thinning relates to symptom severity in very mild to mild AD dementia and is detectable in asymptomatic amyloid-positive individuals. *Cereb Cortex*. 2009;19(3):497-510.
86. Schwarz CG, Gunter JL, Wiste HJ, et al. A large-scale comparison of cortical thickness and volume methods for measuring Alzheimer's disease severity. *Neuroimage Clin*. 2016;11:802-812.
87. Toledo JB, Da X, Bhatt P, et al. Relationship between plasma analytes and SPARE-AD defined brain atrophy patterns in ADNI. *PLoS One*. 2013;8(2):e55531.
88. McLimans KE, Webb JL, Anantharam V, Kanthasamy A, Willette AA, Alzheimer's Disease Neuroimaging Initiative. Peripheral versus Central Index of Metabolic Dysfunction and Associations with Clinical and Pathological Outcomes in Alzheimer's Disease. *J Alzheimers Dis*. 2017;60(4):1313-1324.
89. Azar WJ, Zivkovic S, Werther GA, Russo VC. IGFBP-2 nuclear translocation is mediated by a functional NLS sequence and is essential for its pro-tumorigenic actions in cancer cells. *Oncogene*. 2014;33(5):578-588.
90. Stein TD, Anders NJ, DeCarli C, Chan SL, Mattson MP, Johnson JA. Neutralization of transthyretin reverses the neuroprotective effects of secreted amyloid precursor protein (APP) in APPSW mice resulting in tau phosphorylation and loss of hippocampal neurons: support for the amyloid hypothesis. *J Neurosci*. 2004;24(35):7707-7717.
91. Schindler N, Mayer J, Saenger S, et al. Phenotype analysis of male transgenic mice overexpressing mutant IGFBP-2 lacking the Cardin-Weintraub sequence motif: Reduced expression of synaptic markers and myelin basic protein in the brain and a lower degree of anxiety-like behaviour. *Growth Horm IGF Res*. 2017;33:1-8.
92. Mahapatra MK, Karuppasamy M, Sahoo BM. Therapeutic Potential of Semaglutide, a Newer GLP-1 Receptor Agonist, in Abating Obesity, Non-Alcoholic Steatohepatitis and Neurodegenerative diseases: A Narrative Review. *Pharm Res*. 2022;39(6):1233-1248.

93. Doré S, Kar S, Quirion R. Rediscovering an old friend, IGF-I: potential use in the treatment of neurodegenerative diseases. *Trends Neurosci*. 1997;20(8):326-331.

2.11 Tables

Table 1 Baseline Participant Demographics

	PREVENT-AD	ADNI-1			QFP	
	CU	CU	MCI	AD	CU	AD
N Sample	109	58	395	111	31	55
Mean Age, Years (SD)	62.60 (5.43)	75.11 (5.77)	74.73 (7.40)	74.73 (8.08)	77.39 (11.37)	80.71 (6.39)
N Female (%)	76 (69.7)	28 (48.3)	140 (35.4)	47 (42.3)	11 (35.5)	23 (41.8)
N *APOE* ε4+ (%)	43 (39.4)	5 (8.6)	210 (53.2)	75 (67.6)	9 (29.0)	32 (58.2)
Mean BMI, kg/m^2 (SD)	27.11 (4.47)	27.02 (4.12)	26.09 (3.97)	25.59 (3.82)	-	-
Mean HbA1C, % (SD)[a]	5.4 (0.4)	-	-	-	-	-
Mean Systolic BP, mmHg (SD)	120.20 (13.85)	131.41 (17.65)	132.79 (18.14)	135.05 (17.11)	-	-
Mean Education, Years (SD)	14.88 (2.94)	15.67 (2.78)	15.64 (3.04)	15.09 (3.21)	-	-
Mean MoCA, Total Score (SD)	27.90 (1.58)	-	-	-	-	-
Mean RBANS, Total Score (SD)[b]	101.10 (10.01)	-	-	-	-	-
Mean CSF $A\beta_{1-42}$, pg/mL (SD)[c**]	1145.73 (277.62)	250.85 (21.08)	163.48 (52.90)	142.56 (39.32)	-	-
Mean CSF p_{181}tau, pg/mL (SD)[c**]	46.83 (18.00)	21.07 (8.43)	36.15 (19.32)	42.05 (19.96)	-	-
Mean CSF t-tau, pg/mL (SD)[c**]	273.09 (129.97)	63.62 (21.76)	102.33 (59.78)	120.47 (56.58)	-	-
Mean CSF IGFBP2, NPX (SD)	6.89 (0.67)	-	-	-	-	-
Mean CSF IGFBP2, ng/mL (SD)	-	100.85 (15.85)	104.93 (18.87)	103.02 (18.76)	-	-
Mean Plasma IGFBP2, log (SD)	-	1.88 (0.20)	1.99 (0.23)	1.91 (0.12)	-	-
Mean Global Aβ, SUVR (SD)[d]	1.30 (0.27)	-	-	-	-	-
Mean Tau metaROI, SUVR (SD)[e]	1.17 (0.07)	-	-	-	-	-
Mean Cortical IGFBP2, log_2 (SD)[f]	-	-	-	-	2.43 (0.56)	2.50 (0.83)
Mean Post-mortem delay, h (SD)	-	-	-	-	30.03 (19.85)	21.07 (10.36)

PREVENT-AD, PResymptomatic EValuation of Experiment or Novel Treatments for Alzheimer's disease; ADNI-1, Alzheimer's Disease Neuroimaging Initiative; QFP, Quebec Founder Population; CU, cognitively unaffected; MCI, mild cognitive impairment; AD, Alzheimer's disease; APOE ε4+, Apolipoprotein ε4 carriers; BMI, body mass index; kg/m^2, kilogram per meter squared; HbA1C, Hemoglobin A1C; BP, blood pressure; mmHg, millimeters of mercury; MoCA, Montreal Cognitive Assessment; RBANS, Repeatable Battery for the Assessment of Neuropsychological Status; CSF, cerebrospinal fluid; $A\beta_{1-42}$, amyloid beta 1-42; pg/mL, picograms per milliliter; p_{181}tau, phosphorylated tau 181; t-tau, total tau; IGFBP2, insulin-like growth factor binding protein 2; NPX, Normalized Protein Expression; SUVR, standardized uptake value ratio; ROI, region of interest; h, hours; SD, standard deviation.

[**]PREVENT-AD and ADNI used different assays to measure CSF AD biomarkers.

[a]107 participants had HbA1c values available.

[b]106 participants had RBANS (Total Score) values available.

[c]101PREVENT-AD participants had CSF $A\beta_{1-42}$, p_{181}tau and t-tau (pg/mL) values available.

[d]46 PREVENT-AD participants had Global Aβ SUVR values available.

[e]49 PREVENT-AD participants had Tau metaROI SUVR values available.

[f]78 QFP participants had cortical IGFBP2 values available.

2.12 Figure legends

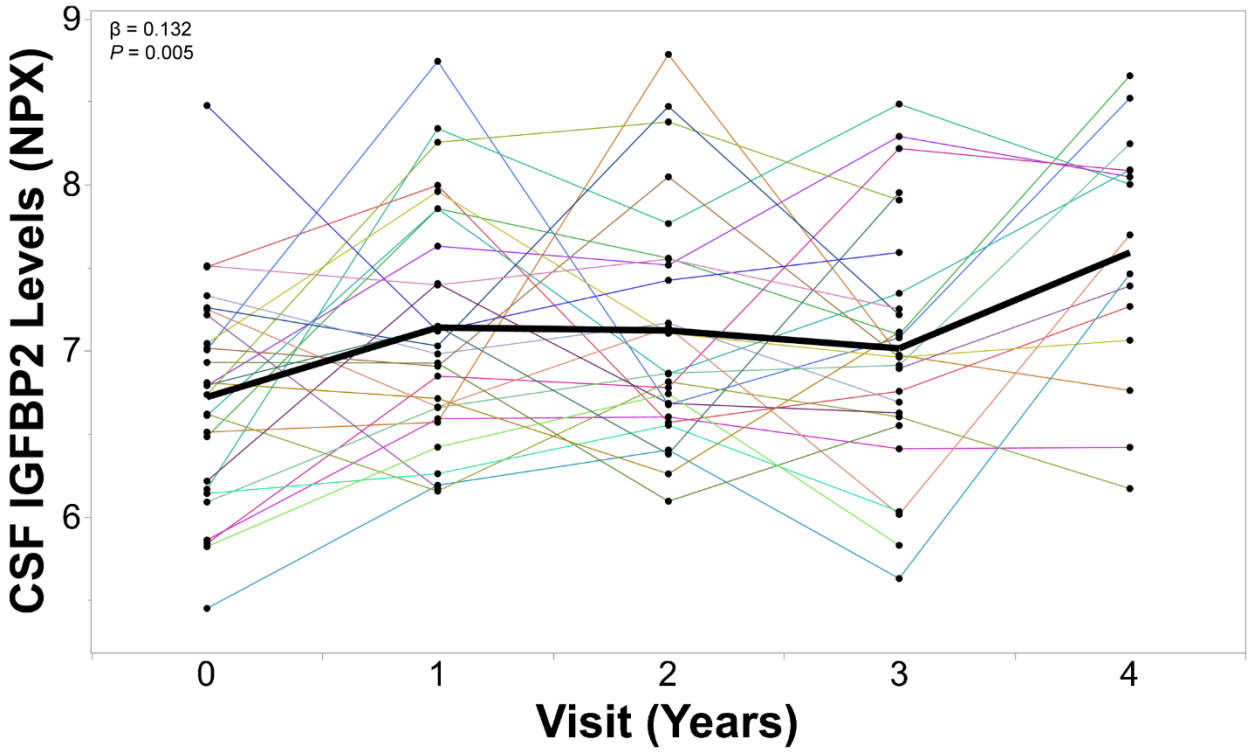


Figure 1 CSF IGFBP2 levels progressively increase over 5 years in asymptomatic PREVENT-AD participants. IGFBP2 was measured in the CSF of a subset of PREVENT-AD participants (n = 27) at baseline and at follow-up visits, using the Olink Proximity Extension Assay. Linear mixed models accounting for participant-specific trajectories demonstrate CSF IGFBP2 levels increase in a subset of at-risk individuals that have been followed for 5 years. β and P values are located in the top left corner.

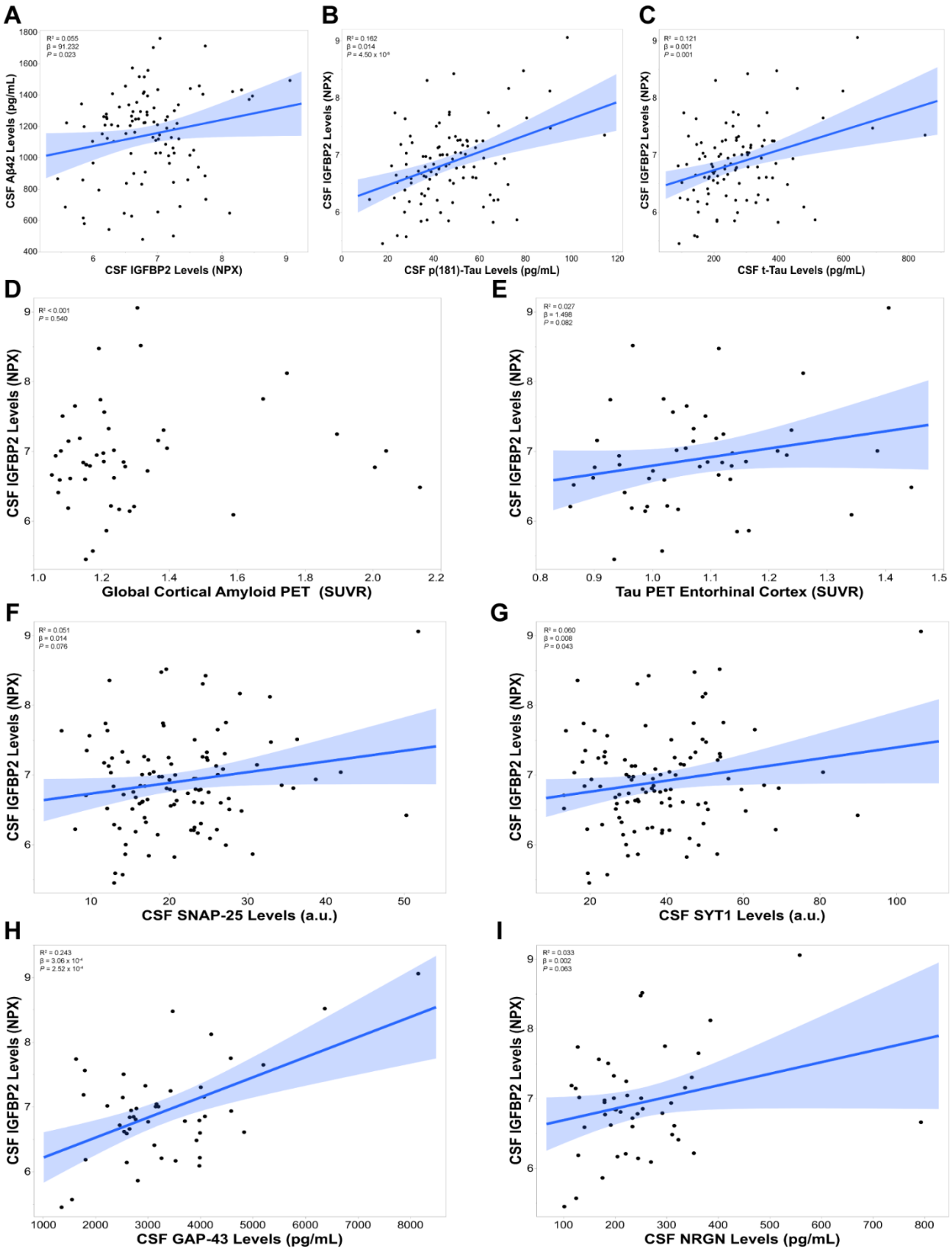

A
R² = 0.055
β = 91.232
P = 0.023
CSF Aβ42 Levels (pg/mL)
CSF IGFBP2 Levels (NPX)
B
R² = 0.162
β = 0.014
P = 4.50 x 10⁻⁶
CSF IGFBP2 Levels (NPX)
CSF p(181)-Tau Levels (pg/mL)
C
R² = 0.121
β = 0.001
P = 0.001
CSF IGFBP2 Levels (NPX)
CSF t-Tau Levels (pg/mL)
D
R² < 0.001
P = 0.540
CSF IGFBP2 Levels (NPX)
Global Cortical Amyloid PET (SUVR)
E
R² = 0.027
β = 1.498
P = 0.082
CSF IGFBP2 Levels (NPX)
Tau PET Entorhinal Cortex (SUVR)
F
R² = 0.051
β = 0.014
P = 0.076
CSF IGFBP2 Levels (NPX)
CSF SNAP-25 Levels (a.u.)
G
R² = 0.060
β = 0.008
P = 0.043
CSF IGFBP2 Levels (NPX)
CSF SYT1 Levels (a.u.)
H
R² = 0.243
β = 3.06 x 10⁻⁴
P = 2.52 x 10⁻⁴
CSF IGFBP2 Levels (NPX)
CSF GAP-43 Levels (pg/mL)
I
R² = 0.033
β = 0.002
P = 0.063
CSF IGFBP2 Levels (NPX)
CSF NRGN Levels (pg/mL)

Figure 2 CSF IGFBP2 is associated with CSF and PET AD biomarkers in the asymptomatic PREVENT-AD cohort. CSF IGFBP2 levels were measured using the Olink Proximity Extension Assay (*n* = 109)**.** CSF AD biomarkers $A\beta_{42}$ (**A**), p_{181}-tau (**B**) and t-tau (**C**) were measured using validated Innotest ELISA kits, following the standardized protocols established by the BIOMARKAPD consortium (*n* = 101). Global cortical Aβ SUVR (**D**) was measured using ^{18}F-NAV4694 (*n* = 46). Tau deposition in the entorhinal cortex **(E)** was measured with flortaucipir (*n* = 49). The synaptic markers SNAP-25 (**F**; *n* = 106), SYT-1 (**G**; *n* = 106), GAP43 (**H**; *n* = 46) and NRGN (**I**; *n* = 46) were quantified using immunoprecipitation followed by mass spectrometry. Significant or trend-level linear regressions are represented with a blue confidence region of the fitted line. R^2 and *P* values are located in the top left corners of each panel. Analyses were adjusted for age, sex and *APOE* ε4 carrier status. SNAP-25, synaptosomal-associated protein 23kDa; SYT1, synaptotagmin-1; GAP43, growth-associated protein 43; NRGN, neurogranin.

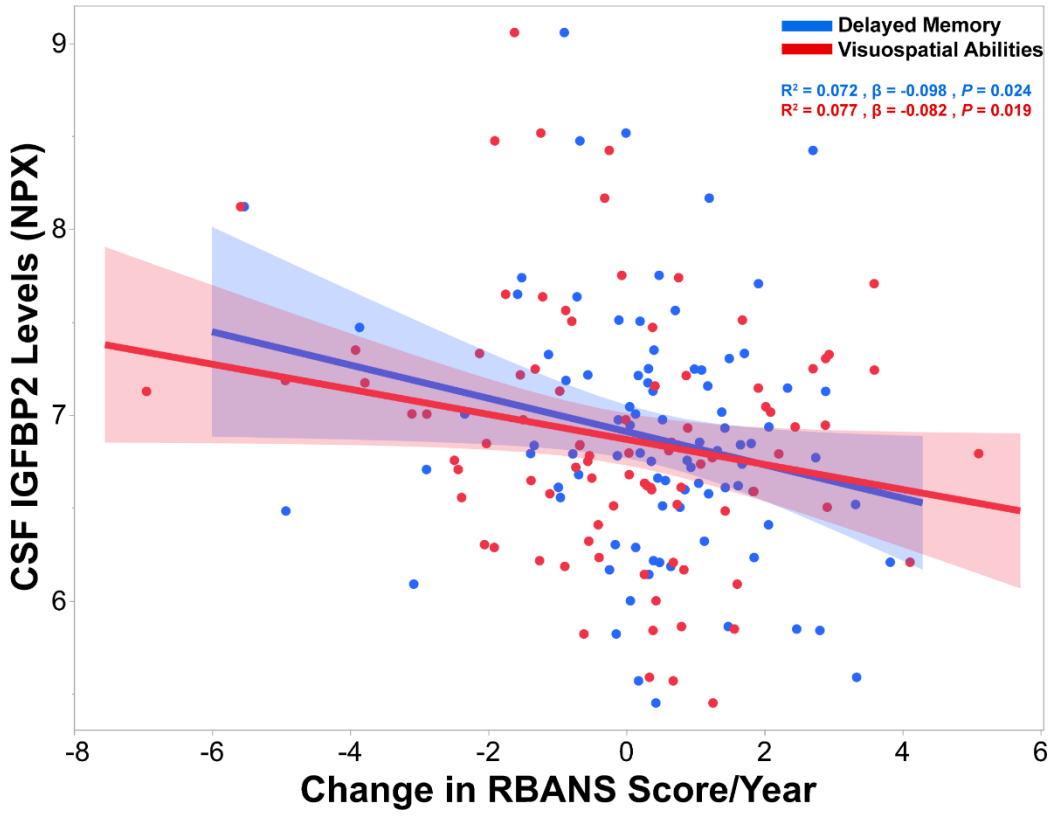


Figure 3 CSF IGFBP2 is associated with longitudinal changes in delayed memory and visuospatial abilities over 5-8 years in PREVENT-AD. CSF IGFBP2 levels were measured using the Olink Proximity Extension Assay (n = 109). Cognitive performance trajectory slopes were computed for each cognitive domain of the RBANS (delayed memory, visuospatial abilities, language, immediate memory and attention) in a subset of PREVENT-AD participants that were followed for 5-8 years (n = 89). Significant linear regressions are represented with a confidence region of the fitted line (blue for delayed memory and red for visuospatial abilities). R^2, β and P values are located in the top right corner. Analyses were adjusted for age, sex, *APOE* ε4 carrier status and years of education.

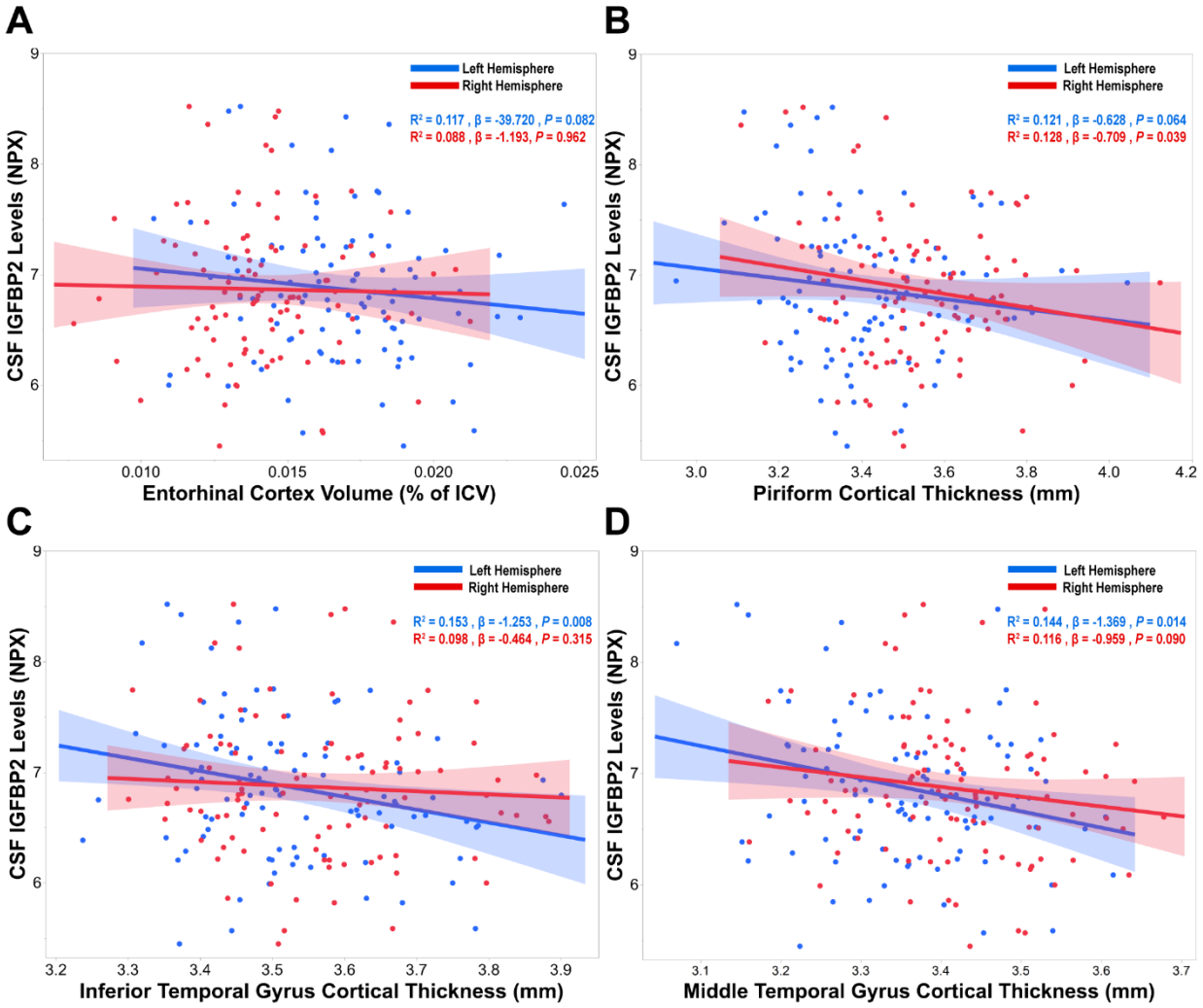


Figure 4 CSF IGFBP2 is associated with atrophy in AD-related brain regions in PREVENT-AD. CSF IGFBP2 levels were measured using the Olink Proximity Extension Assay (n = 109). T1-weighted structural MRI scans were performed on a subset of PREVENT-AD participants (n = 104). The imaging processing pipeline CIVET 1.1.12 was used to analyze neuroimaging data. Entorhinal cortex volumes were normalized by total intracranial volumes (**A**). Cortical thickness measurements were acquired from AD-related brain regions, such as the piriform cortex (**B**), inferior temporal gyrus (**C**) and middle temporal gyrus (**D**). Significant or trend-level linear regressions are represented with a confidence region of the fitted line (blue for left hemisphere and red for right hemisphere). R^2 and P values are located in the top right corner of each panel. Analyses were adjusted for age, sex and *APOE* ε4 carrier status.

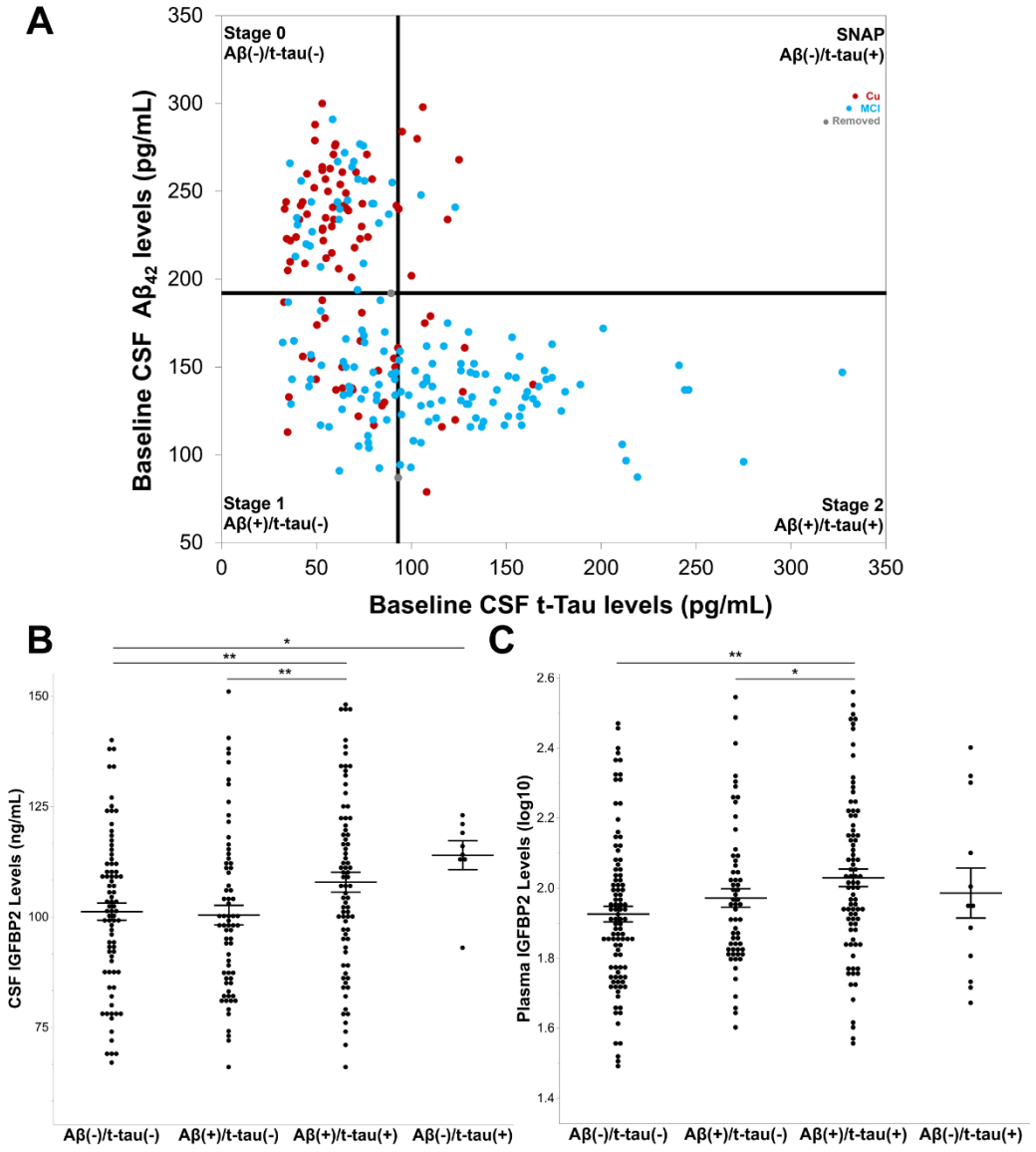


Figure 5 CSF and plasma IGFBP2 is elevated in CSF Aβ(+)/t-tau(+) individuals from the ADNI-1 cohort. Cognitively unaffected participants (red, *n* = 92) and participants with MCI (blue, *n* = 149) from the ADNI-1 cohort were staged as CSF amyloid and/or CSF total tau positive according to the recommended thresholds of 192 pg/mL and 93 pg/mL (**A**). Linear models, adjusted for age, sex and *APOE* ε4 carrier status were used to examine mean differences in IGFBP2 protein levels across stages. CSF IGFBP2 was elevated at Stage 2 (*n* = 77) relative to Stage 0 (*n* = 80) and Stage 1 (*n* = 68) (**B**). Furthermore, CSF IGFBP2 was elevated in SNAP (*n* = 8) compared to Stage 0. Similarly, plasma IGFBP2 was elevated at Stage 2 (*n* = 84) relative to Stage 0 (*n* = 98) and Stage 1 (*n* = 60) (**C**). However, plasma IGFBP2 did not significantly differ between SNAP (*n* = 12) and Stage 0. *P* values are located in the top left corner of each panel. **P* < 0.05; ***P* < 0.01.

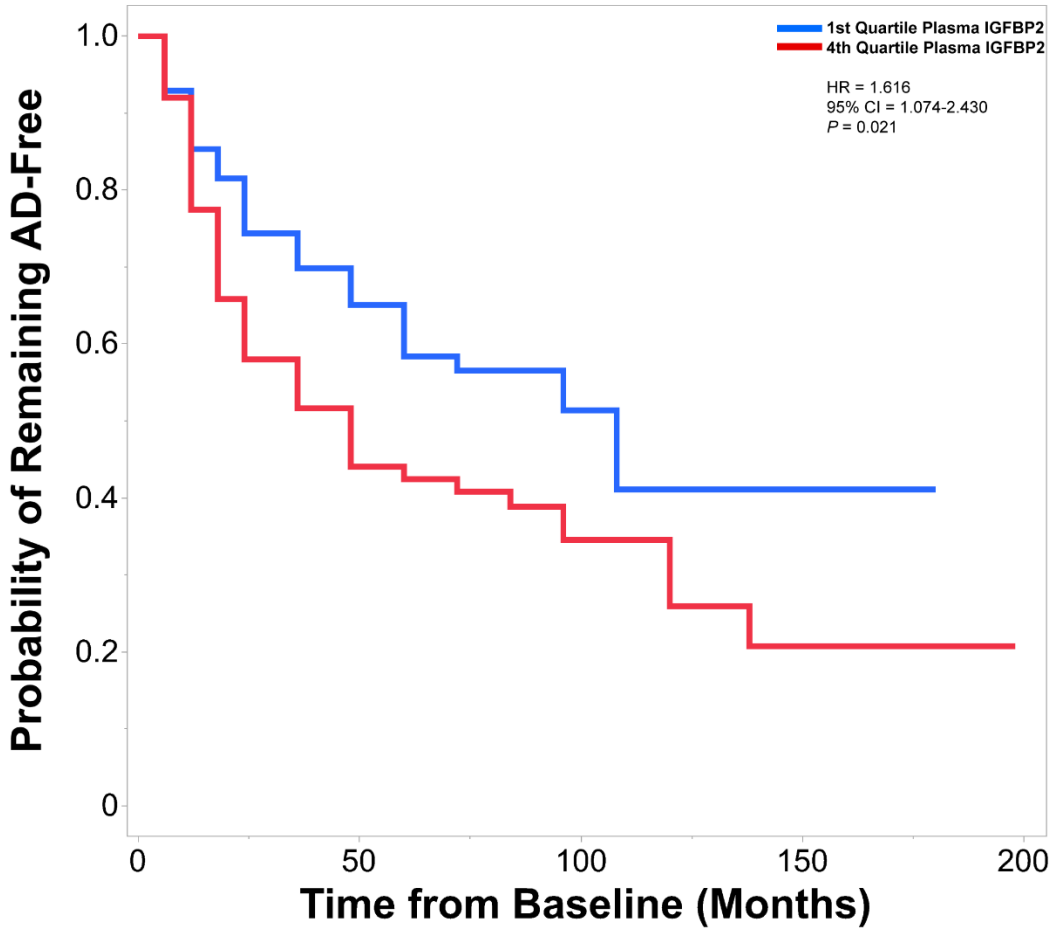


Figure 6 Elevated plasma IGFBP2 is associated with a greater rate of conversion to AD in individuals from the ADNI-1 cohort. Cox proportional hazards models examined the association between baseline plasma IGFBP2 levels and rate of conversion to AD. The first quartile (blue) and fourth (red) values of plasma IGFBP2 were contrasted. Participants were followed from the baseline visit to the time of diagnosis (of AD), or to the time the participant was last confirmed to be free of AD (mean follow-up, 3.8 years; range, 0.5-16.5 years). Of the 226 individuals that were followed longitudinally, 107 individuals progressed to AD. Individuals with plasma IGFBP2 values in the fourth quartile exhibited a greater rate of conversion to AD, compared to the first quartile. HR and *P* values are located in the top right corner. Cox models were adjusted for age, gender and *APOE* ε4 carrier status. HR, Hazard Ratio.

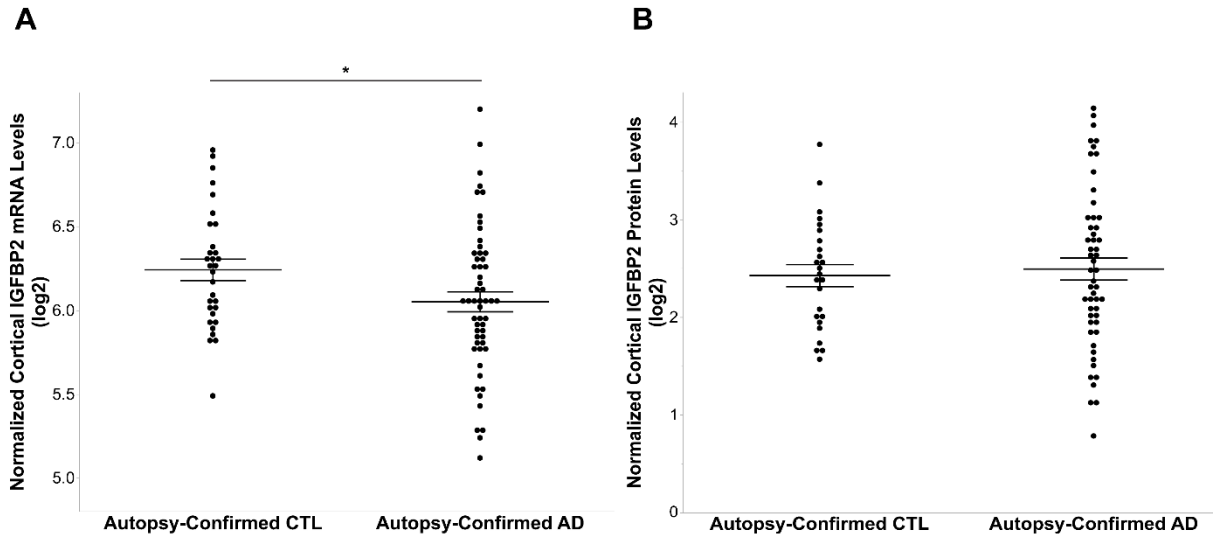


Figure 7 Frontal cortex IGFBP2 gene expression is reduced in autopsy-confirmed AD brains, however protein levels do not differ from elderly controls. Microarray technology was used to measure IGFBP2 mRNA levels in the frontal cortex of autopsy-confirmed AD brains (n = 55) and elderly controls (n = 31) from the QFP cohort (**A**). IGFBP2 protein levels in the frontal cortex were measured in AD brains (n = 53) and control brains (n = 25) using a commercially available ELISA kit (**B**). *P* values are located in the top right corner of each panel. Analyses were adjusted for age, sex, *APOE* ε4 carrier status and post-mortem interval. The data are represented as mean ± SEM. SEM, Standard error of the mean.

2.13 Bridging the manuscripts

The existing literature has demonstrated that the insulin-IGF system shares an intricate relationship with inflammation, which is a pivotal hallmark of AD. Indeed, it has been shown that insulin and IGFs possess anti-inflammatory properties.[157] More specifically, in the next manuscript we were prompted to study the pro-inflammatory protein, osteopontin (OPN), as it has been found to exhibit numerous neuroprotective properties,[158] similar to the insulin-IGF system.[159] Thus, as expected, insulin-IGFs and OPN share similar intracellular signaling pathways, such as the PI3K-AKT and MAPK pathways.[160] Interestingly, when administered in combination with each other, OPN and IGFs promote the regeneration of corticospinal axons,[161] retinal ganglion cell axons,[162] and propriospinal axons,[163] following CNS injury. Furthermore, the insulin-IGF system[164-171] and OPN[172] have been found to be upregulated following several rodent models of stroke and acute brain injury, which suggests that, in response to brain damage, a crosstalk between insulin-IGFs and OPN may exist, and promote brain recovery. Indeed, to support this postulation, both IGFs[166] and OPN[173] have been found to be secreted by the brain's resident immune cells, microglia, which detect and respond to brain injury. Finally, in cell culture experiments, the overexpression of IGFBP2 in preosteoblasts has been associated with a significant increase in OPN expression.[174] Although interesting, this observation must be interpreted with caution and warrants further investigation in the brain. In a similar fashion, OPN has been found to bind to a related IGF-binding protein, notably IGFBP5, and enhance the growth response of smooth muscle cells to IGF-1 and IGFBP5 (together).[175] Overall, the literature surrounding the relationship between the insulin-IGF system and OPN highlights the importance of combination therapies in promoting neuroprotection, potentially during the pre-symptomatic stage of AD.

Submitted to *Molecular Neurodegeneration*

3. Osteopontin exhibits a bidirectional relationship in at-risk individuals and is elevated in autopsy-confirmed Alzheimer brains

Marc James Quesnel,[1,2] Anne Labonté,[2,3] Cynthia Picard,[2,3] Henrik Zetterberg,[4,5,6,7,8,9] Kaj Blennow,[4,5] Ann Brinkmalm,[4,5], Sylvia Villeneuve,[1,2,3] and Judes Poirier[1,2,3] for the Alzheimer's Disease Neuroimaging Initiative[*] and the PREVENT-AD Research Group[†]

Author Affiliations:

1 McGill University, Montréal, Québec, Canada

2 Douglas Mental Health University Institute, Montréal, Québec, Canada

3 Centre for the Studies in the Prevention of Alzheimer's Disease, Douglas Mental Health University Institute, Montréal, Québec, Canada

4 Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

5 Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden

6 Department of Neurodegenerative Disease, UCL Institute of Neurology, London, UK

7 UK Dementia Research Institute at UCL, London, UK

8 Hong Kong Center for Neurodegenerative Diseases, Hong Kong, China

9 Wisconsin Alzheimer's Disease Research Center, University of Wisconsin School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, USA

*Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be

found at:
http://adni.loni.usc.edu/wpcontent/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

†Data used in preparation of this article were obtained from the PRe-symptomatic EValuation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) program at the Centre for Studies on Prevention of Alzheimer's Disease (StoP-AD), Douglas Mental Health University Institute Research Centre (http://douglas.research.mcgill.ca/stop-ad-centre). A complete listing of the PREVENT-AD Research Group can be found at: https://preventad.loris.ca/acknowledgements/acknowledgements.php?date=2023-05-01.

Correspondence to: Dr. 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

Running head: OPN in at-risk individuals and AD brains

Keywords: Osteopontin, Secreted phosphoprotein 1, Alzheimer's disease, cerebrospinal fluid, post-mortem, autopsy, at-risk, pre-symptomatic, mild cognitive impairment

3.1 Abstract

Background: Chronic neuroinflammation is a pivotal hallmark of Alzheimer's disease (AD). Thus, we examined osteopontin (OPN), a key pro-inflammatory protein involved in the recruitment of immune cells, phagocytosis, secretion of pro-inflammatory cytokines, cell survival and tissue repair following injury. In the present study, we analyzed the role of OPN in the cerebrospinal fluid (CSF) of at-risk participants and in autopsy-confirmed AD brains.

Methods: CSF was collected from 109 cognitively unaffected (CU) PREVENT-AD participants that have a parental history of AD. CSF levels of OPN, $A\beta_{42}$, p_{181}-tau, t-tau, SNAP25, SYT1, GAP43 and NRGN were determined using proximity extension assay technology (PEA), validated ELISAs and mass spectrometry. PET cortical amyloid and tau deposition were examined using ^{18}F-NAV4694 and flortaucipir. In PREVENT-AD and in an independent cohort of 241 dementia-free ADNI-1 participants, we allocated individuals into four stages of AD progression based on

the biomarkers CSF $A\beta_{42}$ and t-tau. Finally, OPN mRNA and protein levels were examined in the frontal cortex of 55 autopsy-confirmed AD and 31 control brains from the QFP cohort.

Results: In CU PREVENT-AD participants, CSF OPN was positively correlated with the core AD CSF biomarkers and synaptic markers. Upon staging PREVENT-AD participants according to CSF $A\beta_{42}$ and t-tau levels, CSF OPN exhibited a bidirectional relationship with the stage of AD pathology. PET imaging analyses revealed that CSF OPN was positively correlated with tau deposition in the entorhinal cortex ($P = 0.009$) , fusiform gyrus ($P = 0.030$) and lingual gyrus ($P = 0.002$). Furthermore, CSF OPN was negatively correlated with the volume of the lateral ventricle ($P = 0.001$) and third ventricle ($P = 0.022$). In the ADNI-1 cohort, CSF OPN was elevated in Stage 2 (CSF Aβ(+)/t-tau(+)) ($P = 1.52 \times 10^{-7}$), and was associated with an accelerated rate of conversion to AD (HR 1.925, 95% CI 1.150-3.222, $P = 0.013$) over a mean follow up of 4.3 years. Finally, in the QFP cohort, OPN mRNA and protein levels were significantly upregulated in the frontal cortex of autopsy-confirmed AD brains.

Conclusions: CSF OPN may be a valuable biomarker to identify pre-symptomatic individuals with a significantly greater risk of developing AD and to monitor the effects of novel treatments on neurodegeneration.

3.2 Background

In addition to amyloid-beta (Aβ) plaques, neurofibrillary tangles, neuronal and synaptic loss - chronic neuroinflammation is a cardinal neuropathological hallmark of Alzheimer's disease.[1,2] Indeed, recent genome-wide association studies (GWAS) have identified several genetic risk factors that are associated with immune and inflammatory-related processes,[3,4] thereby highlighting the need to further investigate neuroinflammation as a potential target to delay and/or prevent AD.

Osteopontin (OPN), also known as secreted phosphoprotein 1 (SPP1), is a multifunctional pro-inflammatory protein that is secreted by a diverse collection of cells, ranging from immune cells to non-immune cells.[5] OPN binds to multiple cell-surface receptors, such as integrin molecules (αv (β1, β3, β5, β6), (α4, α5, α8, or α9) β1, α4(β7)) and CD44.[6] Upon binding to its cell-surface receptors, OPN regulates the recruitment of immune cells, cytokine secretion (IL-10, IL-12, IL-

17, IFN-α, IFN-γ, TNF- α), phagocytosis, cell adhesion, cell survival and tissue repair following injury.[6]

In the context of AD, OPN has been found to elevated in CA1 pyramidal neurons of the AD brain[7] as well as in the CSF[8,9,10,11] and plasma[9,12] of AD patients. Several studies suggest that OPN may be a valuable biomarker of disease progression in AD.[13] For instance, OPN is elevated in the CSF of pre-symptomatic amyloid precursor protein (APP) and presenilin-1 (PSEN1) mutation carriers that are 10 or more years away from expected disease onset, compared to non-mutation carriers.[14] Similarly, compared to individuals with stable MCI, MCI patients that progressed to AD over the course of 4-6 years displayed elevated baseline CSF levels of a phosphorylated c-terminal fragment of osteopontin.[15] Furthermore, in a longitudinal study, individuals with MCI that developed AD over a 3 year clinical follow-up exhibited a significant increase in plasma and CSF OPN relative to their baseline visit.[9] Although OPN is elevated in numerous inflammatory and autoimmune diseases afflicting the CNS,[16] there is evidence that suggests that OPN may be a potential marker for differential diagnoses. For instance, CSF OPN levels have been demonstrated to be significantly increased in AD patients, compared to non-AD cognitive impairment cases,[11] such as frontotemporal dementia.[8] Furthermore, plasma OPN levels have been found to be associated with neuroimaging markers of neurodegeneration and cerebrovascular disease, such as medial temporal lobe atrophy and cortical infarcts, as well as with cognitive impairment, in individuals with AD or vascular cognitive impairment.[12]

OPN has been found to be elevated in the brains of several mouse models of AD.[17-20] Single-cell RNA sequencing experiments conducted in mouse models of AD and in human brains, have demonstrated *SPP1* expression is upregulated by disease-associated microglia (DAM), which are believed to respond to CNS damage and promote neuroprotection through both a distinct transcriptional profile and the internalization of Aβ.[21] Indeed, OPN has been found to enhance macrophage-mediated Aβ clearance in cell culture experiments.[18] Furthermore, a recent study found that OPN is secreted by hippocampal perivascular macrophages and facilitates the engulfment of synapses by microglia via a transforming growth factor-beta 1 (TGF-β1) – mediated process.[19]

The aim of the current study is to examine OPN in the CSF of cognitively unaffected (CU) at-risk adults with a parental history of sporadic AD, and in the CSF of an independent cohort of

individuals with MCI. Finally, we were prompted to analyze OPN in the frontal cortex of a large sample of autopsy-confirmed AD and age-matched control brains.

3.3. Methods

3.3.1 PREVENT-AD cohort

3.3.1.1 Study participants

The PRe-symptomatic EValuation of Experimental or Novel Treatments for Alzheimer's Disease (PREVENT-AD) cohort consists of CU individuals that are at-risk of developing AD due to their parental or multi-sibling history of sporadic Alzheimer's disease. The majority of participants were over the age of 60, however individuals aged 55-59 years were included if they were within 15 years of the onset of their youngest-affected relative's symptoms. The PREVENT-AD cohort has been extensively described elsewhere.[22] Each participant and their study partner provided written informed consent. 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.

3.3.1.2 Cerebrospinal fluid measurements

Following an overnight fast, lumbar punctures (LPs) were performed using a Sprotte 24-gauge needle. Within 4 hours, CSF samples were centrifuged (~2000g) for 10 minutes at room temperature, aliquoted and stored at -80°C. OPN protein levels in the CSF were measured in subset of PREVENT-AD participants (n =109) using the Olink proximity extension assay (Uppsala, Sweden). Protein concentrations are expressed in arbitrary Normalized Protein eXpression (NPX) units, which are on a $\log_2$ scale. The validated Innotest enzyme-linked immunosorbent assay (ELISA) kit was used to measure the core AD biomarkers, $A\beta_{42}$, phosphorylated tau (p_{181}-tau) and total tau (t-tau), following the standardized protocols established by the BIOMARKAPD consortium (n = 101). The concentrations of synaptic biomarkers in the CSF, notably SNAP25 and SYT1, were determined using immunoprecipitation followed by mass spectrometry, as previously described (n = 106).[23-25] CSF levels of synaptic markers GAP43 and NRGN were assessed in a subset of PREVENT-AD participants by validated ELISAs (n = 46).[26,27]

3.3.1.3 Pathological staging of participants

Following the recommendations of biological frameworks for defining AD,[28] we used the core AD CSF biomarkers to stage PREVENT-AD participants according to the progression of AD pathology. Individuals were assigned to Stage 0, Aβ(-)/t-tau(-), if they had normal levels of CSF $A\beta_{42}$ and CSF t-tau (n = 53). Participants in Stage 1, Aβ(+)/t-tau(-), exhibited early amyloid pathology, as reflected by reduced levels of CSF $A\beta_{42}$ (n = 14). However, individuals in Stage 1 did not display significant levels of neuronal loss, as reflected by low levels of CSF t-tau. In Stage 2, Aβ(+)/t-tau(+), participants exhibited low levels of CSF $A\beta_{42}$ and elevated levels of CSF t-tau, thus reflecting the final stage of pathology (n = 9). Finally, the Suspected Non-Alzheimer Pathology group (SNAP), Aβ(-)/t-tau(+), exhibited normal levels of CSF $A\beta_{42}$ and elevated levels of CSF t-tau, thus suggesting other causes of neurodegeneration and/or dementia (n = 10).

As previously reported by our group,[29] we defined a CSF $A\beta_{42}$ abnormality threshold at the 25th percentile value (< 989 pg/mL). Furthermore, we specified a CSF t-tau abnormality threshold at the 75th percentile (> 336 pg/mL).[29] These values were generated from the subset of PREVENT-AD participants that had CSF data for the core AD biomarkers and OPN (n = 101). Since our thresholds were slightly arbitrary, and we desired to further differentiate these stages from one another, if possible, we decided a priori to exclude data from 15 individuals whose CSF $A\beta_{42}$ and/or CSF t-tau concentrations were within ± 5% of either threshold. In order to confirm our findings with another core AD biomarker, we further staged PREVENT-AD participants according to CSF levels of p_{181}-tau and CSF $A\beta_{42}$, using a CSF p_{181}-tau abnormality threshold at the 75th percentile value (> 56 pg/mL).

3.3.1.4 Neuroimaging acquisition and processing

Cortical AB and tau pathologies were assessed using ^{18}F-NAV4694 (Navidea Biopharmaceuticals, Dublin, OH, USA) and flortaucipir (^{18}F-AV1451; Eli Lilly & Company, Indianapolis, IN, USA), in a subset of PREVENT-AD participants that also had CSF OPN measurements (n = 46, n = 49 respectively). The PET protocol and image preprocessing pipeline have been previously described.[30] Standardized uptake value ratios (SUVRs) were generated by dividing the signal in the regions of interest (ROI) by the signal in the reference region. Thus, cerebellar grey matter was used as a reference region for ^{18}F-NAV4694, while the inferior cerebellar grey matter was used

for flortaucipir. A global cortical ROI was computed to evaluate Aβ deposition, while tau deposition was assessed by averaging flortaucipir SUVRs in the entorhinal, fusiform and lingual brain regions. Since OPN has been previously associated with central, cortical and medial temporal lobe atrophy, we were prompted to examine the relationship between OPN and a marker of global neurodegeneration, namely cerebral ventricle volume.[12] Thus, lateral, third and fourth ventricle volumes were computed using a volumetric pipeline that has been previously described ($n = 104$).[31]

3.3.1.5 *APOE* genotyping

The protocol for *APOE* genotyping has been previous described.[22] Briefly, the PyroMark Q96 pyrosequencer (Qiagen, Toronto, Ontario, Canada) was used to determine *APOE* genotype in PREVENT-AD. qPCR was used to amplify DNA, with primers rs429358 amplification forward 5'-ACGGCTGTCCAAGGAGCTG-3', rs429358 amplification reverse biotinylated 5'-CACCTCGCCGCGGTACTG-3', rs429358 sequencing 5'CGGACATGGAGGACG-3', rs7412 amplification forward 5'-CTCCGCGATGCCGATGAC-3', rs7412 amplification reverse biotinylated 5'-CCCCGGCCTGGTACACTG-3' and rs7412 sequencing 5'-CGA TGACCTGCAGAAG-3'.

3.3.2 ADNI-1 cohort

3.3.2.1 Study participants

Led by Principal Investigator Michael W. Weiner, MD, the primary objective of the Alzheimer's Disease Neuroimaging Initiative (ADNI) has been to examine the earliest changes associated with AD, and to track the progression of AD pathology. In the current study, we restricted our primary analyses to 241 ADNI-1 participants with CSF data available from 92 cognitively unaffected (CU) individuals and 149 individuals with MCI. Two individuals with ambiguous diagnoses were excluded from subsequent analyses.

3.3.2.2 Cerebrospinal fluid measurements

Following an overnight fast, LPs were performed using a 20- or 24-gauge spinal needle. Within 1 hour after collection, the CSF samples were frozen and shipped on dry ice to the ADNI Biomarker Core laboratory. The samples were thawed for 1h with gentle mixing at room temperature, aliquoted and stored at -80°C. The core AD CSF biomarkers $A\beta_{42}$, p_{181}-tau and t-tau were

measured in ADNI-1 samples using the INNO-BIA AlzBio3 immunoassay kits (Fujirebio, Ghent, Belgium) and the xMap Luminex platform (Austin, Texas, USA). CSF OPN levels had been determined using the Human Discovery Map panel, a multiplex immunoassay developed by Rules Based Medicine. In supplementary analyses, we analyzed mass spectrometry measurements of CSF OPN (AIPVAQDLNAPSDWDSR peptide).

3.3.2.3 Pathological staging of participants

Given our interest in the pre-dementia period, we staged 92 CU individuals and 148 individuals with MCI according to the progression of AD pathology, similar to the analyses performed in the PREVENT-AD cohort. We used the recommended thresholds of 192 pg/mL for CSF $A\beta_{42}$ and 93 pg/mL for CSF t-tau.[32] Two individuals that had core AD biomarker values on the thresholds were excluded from further analyses. To replicate our findings, we further staged ADNI-1 participants based on the combination of CSF p_{181}-tau (recommended threshold >23 pg/mL) and CSF $A\beta_{42}$.[32]

3.3.2.4 *APOE* genotyping

The ABI 7900 real-time thermo-cycler (Applied Biosystems, Foster City, CA) was used to determine the *APOE* genotype of ADNI participants from DNA prepared from EDTA whole blood. TaqMan qPCR assays were used to determine *APOE* nucleotides.[32]

3.3.3 QFP Cohort

3.3.3.1 Study participants

The Quebec Founder Population (QFP) is a unique population isolate from Eastern Canada, which has been previously described in detail.[33] We examined the brains of 55 autopsy-confirmed AD cases and 31 autopsy-confirmed age-matched controls, which were obtained from the Douglas-Bell Canada Brain Bank. According to medical record reviews, neuropsychological examinations and caregiver interviews, there was no evidence of memory problems, neurological or neuropsychiatric diseases in the elderly control group. Controls exhibited neuropathology that is associated with healthy aging (plaque and tangle densities $<10/mm^3$ and $< 20/mm^3$ in at least one hippocampal and neocortical section). AD cases had to fulfill the histopathological NINCDS-ADRDA criteria for definite AD.[34] This study was conformed to the Code of Ethics of the World Medical Association and was approved by the Ethics Board of the Douglas Mental Health

University Institute. This study complied with the ethical principles of the Declaration of Helsinki. Each participant provided written informed consent.

3.3.3.2 OPN mRNA levels in the frontal cortex

Transcriptome-wide gene expression was measured using the human Clariom D Assay, by Génome Québec. Briefly, the Nanodrop Spectrophotometer ND-1000 was used to measure total RNA (NanoDrop Technologies, Inc.). RNA integrity was evaluated with the Agilent 2100 Bioanalyzer. 10 ng of total RNA was used to synthesize sense-strand cDNA. The GeneChip WT Terminal Labeling Kit was used to fragment and label single-stranded cDNA, following the manufacture's instructions. 5 µg cDNA was hybridized on the GeneChip cartridge array and incubated at 45°C for 17 hours in the GeneChip Hybridization Oven 640, at 60 rpm. The microarrays were washed in the GeneChip Fluidics Station 450, using the GeneChip Hybridization, Wash, and Stain Kit, according to the manufacture's instructions. Finally, microarrays were scanned in a GeneChip Scanner 3000. OPN mRNA levels are presented on a $\log_2$ scale.

3.3.3.3 OPN protein levels in the frontal cortex

Of the 86 brains that had OPN mRNA levels measured, OPN protein levels were measured in 78 brains (n = 25 Controls, n = 53 AD). Frontal cortex brain samples were placed in pre-filled tubes containing 2.8 mm ceramic beads (Omni International, GA, USA). One tablet of protease inhibitor was dissolved in 50 mL of cold phosphate-buffered saline. 1 mL of protease inhibitor solution was added to each tube. The Bead Ruptor 24 (Omni International, Kennesaw, GA, USA) was used to mechanically homogenize brain samples. The Bead Ruptor 24 was set twice at 5.65m/s for 30 seconds, with a 15 second pause between runs. Following homogenization, the samples were stored overnight at -20°C. Two freeze-thaw cycles were performed. Finally, the homogenates were centrifuged for 5 minutes at 5000 rpm and 4°C. The supernatant was collected and stored for future use at -80°C.

Frontal cortex OPN protein levels were determined using a commercially available ELISA kit (Cat.# ELH-OPN) developed by RayBiotech (Peachtree Corners, GA, USA). Protocols were performed according to the manufacturers' instructions and results were obtained using the BioTek Synergy H1 microplate reader (Winooski, Vermont, USA). Sample replicates had a coefficient of

variability (CV) of less than 20%. However, three samples with a CV greater than 20% were excluded from analyses. Finally, in order to normalize cortical OPN protein levels, total protein concentration was measured using a commercially available bicinchoninic acid assay developed by Pierce (Cat.# 23225). Finally, in order to meet model assumptions, normalized OPN protein ratios were $\log_2$ transformed.

3.3.3.4 *APOE* genotyping

DNA was extracted from brain tissue with the DNeasy Tissue Kit (Qiagen). As previously described in PREVENT-AD,[22] *APOE* genotype was determined using the PyroMark Q96 pyrosequencer.

3.3.4 Statistical Analyses

In the PREVENT-AD cohort, linear regression models adjusted for age, gender and *APOE* ε4 carrier status were used to examine the associations between baseline CSF OPN and baseline measurements of CSF AD biomarkers ($A\beta_{42}$, p_{181}-tau, t-tau), CSF synaptic proteins (SNAP25, SYT1, GAP43, NRGN) as well as PET and volumetric neuroimaging data. Across all analyses, OPN was assigned as a dependent variable, except for the association between OPN, CSF $A\beta_{42}$ and ventricular volume. *P* values ≤ 0.05 were considered statistically significant.

In both the PREVENT-AD and ADNI-I cohorts, ANCOVA was used to examine mean differences in CSF OPN levels across pathological stages, as determined by CSF $A\beta_{42}$, CSF t-tau, and p_{181}-tau positivity. These analyses were adjusted for age, gender and *APOE* ε4 carrier status. To correct for multiple planned comparisons between pathological stages, *P* values ≤ 0.01 were considered statistically significant.

In the ADNI-I cohort, Cox proportional hazards models examined the association between baseline CSF OPN levels and rate of conversion to AD. CU participants and individuals with MCI were followed from the baseline visit to the time of diagnosis (of AD), or to the time the participant was last confirmed to be free of AD. Cox models were adjusted for age, gender and *APOE* ε4 carrier status. *P* values ≤ 0.05 were considered statistically significant

In the QFP cohort, the relationship between frontal cortex OPN mRNA, protein levels, and diagnosis was evaluated with ANCOVA, adjusted for age at death, sex, *APOE* ε4 carrier status

and post-mortem interval. Statistical significance was considered at $P \leq 0.05$. R^2 values are presented as adjusted R^2. All Analyses were two-tailed, and performed in SPSS 23 (IBM) and JMP Pro 16 (SAS).

3.3.5 Data availability

Data pertaining to the PREVENT-AD cohort can be downloaded from data release 6.0 at https://openpreventad.loris.ca/. CSF, plasma, genetic and clinical data from the ADNI-1 cohort were downloaded from the ADNI website (http://adni.loni.usc.edu/). Data collected from the QFP cohort are not publicly available, however the data are available from the corresponding author upon reasonable request.

3.4 Results

3.4.1 Demographics

Table 1 summarizes the demographic characteristics of the three cohorts that were used to analyze the role of OPN in the CSF of asymptomatic (PREVENT-AD) and symptomatic individuals (ADNI-1), as well as in the frontal cortex of autopsy-confirmed AD and age-matched control brains (QFP).

3.4.2 PREVENT-AD cohort

3.4.2.1 CSF OPN is associated with CSF AD and synaptic biomarkers in asymptomatic AD

In CU PREVENT-AD participants ($n = 101$), CSF OPN levels were positively correlated with CSF $A\beta_{42}$ ($R^2 = 0.200$, $\beta = 266.462$, $P = 0.004$, Fig. 1A), CSF p_{181}-tau ($R^2 = 0.430$, $\beta = 0.010$, $P = 1.69 \times 10^{-13}$, Fig. 1B) and CSF t-tau ($R^2 = 0.338$, $\beta = 0.001$, $P = 2.45 \times 10^{-10}$, Fig. 1C). Furthermore, CSF OPN was associated with synaptic markers in the CSF, notably SNAP25 ($R^2 = 0.104$, $\beta = 0.011$, $P = 0.001$, Fig. 1D), SYT1 ($R^2 = 0.181$, $\beta = 0.008$, $P = 8.00 \times 10^{-6}$, Fig. 1E), GAP43 ($R^2 = 0.427$, $\beta = 1.31 \times 10^{-4}$, $P = 4.00 \times 10^{-6}$, Fig. 1F), and NRGN ($R^2 = 0.215$, $\beta = 0.001$, $P = 0.004$, Fig. 1G).

3.4.2.2 CSF OPN exhibits a bidirectional relationship with stage of AD pathology in asymptomatic AD

86 CU PREVENT-AD participants were staged according to CSF $A\beta_{42}$ and CSF t-tau thresholds of 989 pg/mL and 336 pg/mL (Fig. 2A). CSF OPN was significantly reduced at Stage 1 Aβ(+)/t-tau(-), relative to Stage 0 Aβ(-)/t-tau(-) ($P = 0.001$, Fig. 2B). Furthermore, CSF OPN was markedly increased at Stage 2 Aβ(+)/t-tau(+) relative to Stage 1 ($P = 1.23 \times 10^{-4}$), but not to Stage 0 ($P = 0.071$). Finally, OPN was significantly increased in SNAP Aβ(-)/t-tau(+) relative to Stage 0 ($P = 0.008$).

To reproduce our findings, we further staged PREVENT-AD participants ($n = 85$) according to CSF $A\beta_{42}$ and CSF p_{181}-tau thresholds of 989 pg/mL and 56 pg/mL, respectively (data not shown). Once more, we observed a significant reduction in CSF OPN in levels in Stage 1 relative to Stage 0 ($P = 0.001$), as well as a marked increase in Stage 2 relative to Stage 1 ($P = 1.40 \times 10^{-5}$), and to Stage 0 (trend, $P = 0.013$). Finally, the elevated p_{181}-tau only Stage, Aβ(-)/p_{181}-tau(+), displayed a significant increase in CSF OPN relative to Stage 0 ($P = 0.001$).

Next, we verified the bidirectional nature of additional proteins in response to the progression of AD pathology in a subset of cardiovascular and inflammatory proteins that were also included in the Olink Cardiovascular III panel. In identical analyses involving CSF $A\beta_{42,}$ t-tau and p_{181}-tau staging, 5 proteins (TNFRSF14, ALCAM, UPAR, SHPS1, and KLK6) exhibited a significant reduction in Stage 1 relative to Stage 0, and a significant increase in Stage 2 relative to Stage 1. Finally, the SNAP group displayed elevated levels of these supplementary proteins (excluding KLK6), relative to Stage 0 (Figure 2B).

Finally, we removed the additional ± 5% threshold ranges and verified our results. Indeed, we observed a significant reduction at Stage 1 relative to Stage 0 and a marked increase at Stage 2 relative to Stage 1 in the following proteins from the Olink Cardiovascular III panel: TNFRSF14, ALCAM, CSTB, BLM Hydrolase, LTBR, CNTN1, IL6RA, AXL, UPAR, OPN, SHPS1, CHI3L1, EGFR, IL18BP, KLK6 and CXCL16. However, as with our previous analyses, a large number of Stage 0 vs Stage 1 reductions were at a trend-level ($0.01 < P < 0.05$) after adjusting for multiple comparisons at $P = 0.01$. This had been previously reported by our group, and is most likely due to a limited number of individuals in Stages 1 and 2.

3.4.2.3 CSF OPN is associated with tau PET burden in Braak stages 2-3 in asymptomatic AD

A subset of CU PREVENT-AD participants that had CSF OPN measurements also underwent NAV4696 (n = 46) and flortaucipir (n = 49) PET imaging to assess early Aβ and tau deposition. CSF OPN was not associated with global cortical Aβ deposition (P = 0.374, Fig. 3A). However, CSF OPN was positively correlated with tau deposition in brain regions representing Braak Stages 2-3,[35] most notably, the entorhinal cortex (R^2 = 0.159, β = 0.814 , P = 0.009, Fig. 3B), fusiform (R^2 = 0.118, β = 1.205, P = 0.030, Fig. 3C) and lingual gyri (R^2 = 0.204, β = 1.320, P = 0.002, Fig. 3D).

3.4.2.4 CSF OPN is associated with reduced cerebral ventricular volume in asymptomatic AD

We analyzed baseline structural neuroimaging data collected from a subset of PREVENT-AD individuals (n = 104) in a cross-sectional fashion. Four individuals were omitted from analyses due to failed quality control regarding subject-specific stereotaxic registration and/or brain masking. After adjusting for total intracranial volume, baseline CSF OPN was negatively correlated with the volume of the lateral ventricle (n = 100, R^2 = 0.356, β = -0.956 P = 0.001, Fig. 4A) and with the volume of the third ventricle (R^2 = 0.313, β = -0.033 , P = 0.022, Fig. 4B*)*. However, CSF OPN levels were not correlated with the volume of the fourth ventricle (P = 0.10, Fig. 4C).

3.4.3 ADNI-1 cohort

3.4.3.1 CSF OPN is elevated in CSF Aβ(+)/t-tau(+) individuals

We staged 91 CU individuals and 147 individuals with MCI as amyloid and/or tau positive according to recommended CSF $A\beta_{42}$ and CSF t-tau thresholds of 192 pg/mL and 93 pg/mL respectively (Fig. 5A).[32] The results from the multiplex immunoassay (Fig. 5B) revealed that CSF OPN levels did not significantly differ between Stages 0 (n = 81) Aβ(-)/t-tau(-) and Stage 1 (n = 69) Aβ(+)/t-tau(-), P = 0.642. However, CSF OPN was significantly elevated at Stage 2 (n = 79) Aβ(+)/t-tau(+) relative to Stage 0 ($P = 1.52 \times 10^{-7}$) and Stage 1 ($P = 9.44 \times 10^{-10}$). Finally, CSF OPN did not significantly differ between Stage 0 and SNAP (n = 9) Aβ(-)/t-tau(+) (P = 0.108).

In order to verify our findings with phosphorylated tau pathology, we further staged 89 CU individuals and 147 individuals with MCI based on the recommended CSF $A\beta_{42}$ and CSF p_{181}-tau thresholds of 192 pg/mL and 23 pg/mL.[32] While using this staging criteria for p_{181}-tau, we obtained similar results; CSF OPN was significantly elevated at Stage 2 $A\beta(+)/p_{181}$-tau(+) compared to Stage 0 $A\beta(-)/p_{181}$-tau(-) (trend, $P = 0.015$) and Stage 1 $A\beta(+)/p_{181}$-tau(-) ($P = 0.004$). However, CSF OPN did not differ between Stage 0 and Stage 1 ($P = 0.394$), or Stage 0 and the p_{181}-tau only group $A\beta(-)/p_{181}$-tau(+) ($P = 0.261$).

In the end, we performed similar analyses using the CSF mass spectrometry data for OPN (AIPVAQDLNAPSDWDSR peptide), which yielded similar results to the CSF immunoassay data. However, in contrast with the immunoassay data, we observed a significant increase in the CSF OPN mass spectrometry peptide in the SNAP and p_{181}-tau only group $A\beta(-)/p_{181}$-tau(+) compared to Stage 0 ($P = 1.00 \times 10^{-5}$ and $P = 0.001$, respectively). Furthermore, we observed a significant reduction in the CSF OPN mass spectrometry peptide at Stage 1 compared to Stage 0 (for p_{181}-tau staging, $P = 0.005$, but not for t-tau staging, $P = 0.098$). This latter finding replicates the reduction in CSF OPN that we observed in Stage 1 in PREVENT-AD.

3.4.3.2 Elevated CSF OPN is associated with a faster rate of conversion to AD

In our primary analysis for conversion to AD in ADNI-1, we established CSF OPN threshold values at the 25th ($\leq$ 27 ng/mL, first quartile) and 75th percentiles ($\geq$ 39.75 ng/mL, fourth quartile). A total of 126 individuals that were either CU or had MCI at the baseline visit, were included in these analyses. Of these dementia-free participants, 62 individuals eventually met the clinical criteria for a diagnosis of AD (mean follow-up, 4.3 years; range, 0.5-16 years). Cox proportional hazards models that were adjusted for age and gender revealed that individuals with CSF OPN values in the fourth quartile (>75th percentile) exhibited a faster rate of conversion to AD, than individuals with CSF OPN values in the first quartile (<25th percentile) (HR 1.925, 95% CI 1.150-3.222, $P = 0.013$, Fig. 6). However, it is important to recognize that this association was attenuated when adjusting for *APOE* ε4 carrier status ($P = 0.109$). Finally, using the MRM mass spectrometry data, we observed a similar trend when adjusting for age and gender (HR = 1.707, $P = 0.087$).

3.4.4 QFP cohort

3.4.4.1 OPN mRNA and protein levels are increased in the frontal cortex of autopsy-confirmed AD brains

OPN gene expression was assessed by DNA microarray in the QFP cohort, and demonstrated to be significantly increased in the frontal cortex of autopsied AD brains ($n = 55$), compared to age-matched controls ($n = 31$) ($P = 1.00 \times 10^{-6}$, Fig. 7A). Furthermore, as demonstrated through ELISA, after controlling for total protein levels, OPN protein levels were also elevated in the frontal cortex of AD cases, relative to controls ($P = 0.006$, Fig. 7B).

3.5 Discussion

Chronic neuroinflammation is a cardinal neuropathological hallmark of AD. Furthermore, recent GWAS studies have identified several genetic risk factors that are associated with immune and inflammatory-related processes,[3,4] thereby highlighting the need to further investigate neuroinflammation as a potential target to delay and/or prevent AD. To this end, we investigated the role of OPN in the pre- and symptomatic stages of AD.

First, we observed a positive relationship between CSF OPN and CSF $A\beta_{42}$, in CU individuals with a parental history of AD. This finding is certainly consistent with the finding that OPN enhances the phagocytosis of Aβ by macrophages in cell culture experiments.[18] Furthermore, it has been previously suggested that OPN may regulate the expression and/or activity of matrix metalloproteinase-9 (MMP-9),[18,36,37] which has been found to both degrade fibrillar Aβ[38] and cleave OPN.[39] It has been further demonstrated that the overexpression of MMP-9 rescues impairments in insulin signaling that contribute to neuronal loss in mouse models of AD.[40] Hence, we postulate that OPN may exert neuroprotection through an intricate relationship with MMP-9. However, in both the PREVENT-AD and ADNI-1 cohorts CSF MMP-9 measurements were below the limit of detection. Finally, it is important to recognize that OPN was not associated with global cortical Aβ deposition. This observation emphasizes the importance of OPN in the earliest stages of AD, as changes in CSF Aβ are believed to precede changes in PET Aβ.[41]

Our results suggest that OPN may be upregulated as a result of early CSF p_{181}-tau production and deposition in brain regions associated with early Braak Stages 2-3, in CU individuals.[35] This

finding is consistent with a longitudinal study that demonstrated that elevated CSF OPN was associated with an accelerated rate of tau PET accumulation (Braak III-IV and Braak V-VI) and concomitant cognitive decline, in Aβ(+)/Tau(+) individuals.[42] Indeed, it is has been established that OPN promotes PI3K/AKT signalling,[43] which regulates the activity of Glycogen synthase kinase-3, which is a major producer of phosphorylated tau.[44] Hence, it is conceivable that OPN may play a pivotal role in the detection of elevated phospho-tau production, and ultimately, dampen NFT formation. However, given the role of OPN in enhancing phagocytosis, it is equally possible that OPN may contribute to the microglia-mediated internalization of secreted exosomes containing phosphorylated tau.[45] Hence, we postulate OPN may regulate microglia-mediated degradation of pathological tau species.[46] However, further studies are required to support these hypotheses.

Finally, we found OPN is positively associated with CSF t-tau, a marker of neuronal damage and death. This finding is in agreement with the upregulation of OPN and its cell-surface receptors following ischemic brain injury in rodents.[47] Furthermore, in cell culture experiments and in mouse models of stroke, the administration of OPN has been demonstrated to directly protect neurons from cell death induced by oxygen and glucose deprivation, and reduce infarct size.[48] Altogether, our results suggest that OPN may offer neuroprotection by promoting neuronal survival, during the pre-symptomatic stages of AD.

Following the emergence of biological frameworks for defining AD,[28] we staged CU PREVENT-AD participants as CSF amyloid and/or total tau positive according to CSF $A\beta_{42}$ and CSF t-tau. We found that CSF OPN exhibits a bidirectional relationship with stage of AD pathology, which is in accordance with our analyses that kept CSF $A\beta_{42}$ and t-tau as numerical, continuous variables. In secondary analyses, five other inflammatory/immune related proteins from the Olink Cardiovascular Pannel (TNFRSF14, ALCAM, UPAR, SHPS1, and KLK6) also displayed this bidirectional relationship. Finally, we replicated our analyses when we used p_{181}tau to stage participants, and when we removed the additional 5% thresholds. These supplementary analyses did not interfere with the statistical significance of the original results, and ultimately permitted us to discover additional proteins that exhibit a bidirectional relationship with the stage of AD pathology. Overall, our data confirm the bidirectional nature of certain proteins that we observed

in a previous study,[29] and suggest that the relationship between neuroinflammation and pre-symptomatic AD is more complex than previously anticipated.

Considering the prominent loss of synapses in AD, which correlates most strongly with cognitive decline,[49] we examined the synaptic markers SNAP25, SYT1, GAP43 and NRGN in the CSF of CU individuals with a parental history of AD.[23-27] Our results suggest that CSF OPN may be upregulated in response to early synaptic dysfunction and/or loss in asymptomatic adults. Thus, it is possible that OPN may promote the regeneration of axons and synapses in these CU individuals that are at a greater risk of developing AD. This view is certainly consistent with the fact that OPN, in combination therapies, promotes the regeneration of axons following rodent models of spinal cord injury,[50] stroke[51] and nerve transections.[52] The compensatory role of OPN in synaptic processes underlying cognition is further highlighted by a report that CSF OPN was positively correlated with mini-mental state examination scores in elderly controls and in patients with AD.[8,9] Finally, given the intricate relationship between OPN and MMP proteins, it is important to recognize that several matrix metalloproteinases such as MMP-2,[36] MMP-3[53] and MMP-9,[36,54] which cleave OPN into distinct fragments, have been demonstrated to be upregulated following acute brain injury. Overall, these observations further emphasize the importance of cleaved OPN fragments during the recovery phases of brain injury.

In order to verify our CSF findings, we examined the relationship between CSF OPN and anatomical changes in the brain in the asymptomatic PREVENT-AD cohort. Interestingly, CSF OPN was negatively correlated with the volume of the lateral and third ventricles. This observation is critical, as the enlargement of the cerebral ventricles is used as a marker of brain atrophy and AD progression.[55] Hence, our results contrast with those of a previous report, that found elevated plasma levels of OPN accompanied cortical and medial temporal lobe atrophy, in patients with AD or vascular cognitive impairment.[12] However, our findings emphasize the protective effects of OPN, as elevated CSF levels of OPN were associated with reductions in cerebral ventricle volume. Overall, our neuroimaging results confirm our CSF observations, and further suggest that OPN plays a pivotal role in the brain, by preserving neuronal and synaptic integrity, during the pre-symptomatic stage of the disease.

To independently validate our observations, we further analyzed data from a well-characterized cohort of CU individuals and individuals with MCI from ADNI-1. Our results suggest that CSF

OPN may be a valuable biomarker for identifying CSF Aβ(+)/t-tau(+) and/or CSF Aβ(+)/p_{181}-tau(+) individuals and thus, facilitate screening for suitable high-risk patients for clinical trials. Similarly, CSF OPN may be used as an outcome measure for therapies designed to reduce neurodegeneration. Unfortunately, due to a limited number of individuals that underwent PET imaging and had CSF OPN measurements, we were unable to stage participants as amyloid and/or phospho-tau positive based on PET scans, however, it is important to recognize that changes in CSF Aβ and t-tau/p_{181}tau are believed to occur prior to changes in Aβ and tau PET.[41] Thus, our findings are critical, as they capture the very beginning of the disease process. Furthermore, although the mass spectrometry and immunoassay results were similar, we replicated the bidirectional relationship of OPN with AD pathology (observed in PREVENT-AD), with the mass spectrometry data from ADNI-1 cohort. Finally, upon conducting survival analyses, we found elevated CSF OPN was associated with a greater rate of conversion to AD over the course of 0.5-16 years, which is consistent with the existing literature.[13-15] However, it is important to recognize this association was statistically significant when adjusting for age and gender, but not when *APOE* ε4 carrier status was added. Hence, this finding needs to be interpreted with caution, and warrants further investigation in an independent cohort.

Although several pilot studies have reported that OPN is elevated in the frontal cortex of AD brains,[18,20,56,57] we verified these observations in a large sample of autopsy-confirmed AD brains from the QFP cohort. Consistent with previous studies, OPN mRNA and protein levels were markedly increased in the frontal cortex of AD brains, compared to age-matched elderly control brains. However, it is important to recognize that we were unable to differentiate between extracellular OPN and intracellular OPN, which is believed to be generated by an alternative translational initiation site.[58] In contrast to the extracellular OPN, studies suggest that intracellular OPN enhances IFN-α expression, inhibit IL-27 release, and promote T helper 17 cell responses.[6,58]

Through the interpretation of the present study's results, we believe OPN exerts a protective role in the brain during the pre-symptomatic stage of AD. However, it cannot be dismissed that chronic neuroinflammation mediated by OPN may be harmful to the brain, during the later stages of the disease. Indeed, there are several lines of evidence that suggest this may be true. For instance, the genetic ablation of OPN and the administration of anti-OPN antibodies have been shown to reduce plaque formation, reduce the number of dystrophic neurites and ultimately, improve cognition in

5XFAD mice.[20] In a similar fashion, siRNA and antibodies targeted against CD44V10, a splice variant of the OPN receptor, protect primary neuronal cultures from Aβ-induced cell death.[59] Finally, in mouse models of additional neurodegenerative diseases, such as multiple sclerosis, OPN deficiency has been associated with a significant reduction in the severity of the disease, as manifested by a greater frequency of remissions.[60]

3.6 Conclusions

In summary, we have demonstrated that CSF OPN may be a valuable biomarker to identify individuals with a significantly greater risk of developing AD. As suggested by conflicting findings in the literature, it is possible an upregulation in CSF OPN may be beneficial or detrimental, depending on the stage of the disease.[2] We postulate acute increases in OPN-mediated neuroinflammation may promote Aβ clearance,[18] inhibit phospho-tau production and/or propagation,[45,46] promote axonal/synaptic reinnervation[50-52] and enhance cell survival during the pre-symptomatic stages of AD.[47,48] Several lines of evidence suggest this may be the case, as CSF OPN levels have been found to be elevated in recently diagnosed AD patients (disease duration ≤ 2 years), and negatively correlated with disease duration.[8,9] However, further studies are required to examine the clinical implications of spliced isoforms, cleaved isoforms and phosphorylation states of OPN, which are all believed to influence OPN function. In a similar fashion, further investigating the cell-type specific effects of OPN as well as the plethora of cell-surface receptors that recognize OPN, is necessary to uncover the signaling pathways that may confer neuroprotection to OPN. Conversely, during the later stages of AD, we speculate chronic increases in OPN-mediated neuroinflammation may be harmful to the brain, by potentially enhancing the release of toxic pro-inflammatory cytokines, promoting the aberrant elimination of synapses, and inhibiting the apoptosis of reactive glial cells.[2,6,19] Hence, acquiring a thorough understanding of the distinct stages of AD through the use of biomarkers, is critical to develop therapies to upregulate or downregulate OPN expression, proteolytic cleavage, and/or phosphorylation status.

3.7 Acknowledgements

The authors would like to thank Dr. Naguib Mechawar at the Douglas Institute/Bell Canada Brain Bank for providing human brain tissues from the Quebec Founder Population. We also wish to

thank Mrs. Jennifer Tremblay-Mercier, Marie-Elyse Lafaille-Magnan and Melissa Savard as well as Drs. Pedro Rosa-Neto, Daniel Auld and David Lafontaine for their technical expertise.

3.8 Funding

JP is supported by the Fonds de recherche du Québec-Santé (FRQS), the Canadian Institutes of Health Research (CIHR #PJT 153287) and the J.L. Levesque Foundation. SV is supported by a Canada Research Chair and a Canada Fund for Innovation grant, the FRQS, the CIHR, Brain Canada, McGill University and the Alzheimer's Association. HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2022-01018 and #2019-02397), the European Union's Horizon Europe research and innovation programme under grant agreement No 101053962, Swedish State Support for Clinical Research (#ALFGBG-71320), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), the AD Strategic Fund and the Alzheimer's Association (#ADSF-21-831376-C, #ADSF-21-831381-C, and #ADSF-21-831377-C), the Bluefield Project, the Olav Thon Foundation, the Erling-Persson Family Foundation, Stiftelsen för Gamla Tjänarinnor, Hjärnfonden, Sweden (#FO2022-0270), the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No 860197 (MIRIADE), the European Union Joint Programme – Neurodegenerative Disease Research (JPND2021-00694), the National Institute for Health and Care Research University College London Hospitals Biomedical Research Centre, and the UK Dementia Research Institute at UCL (UKDRI-1003). KB is supported by the Swedish Research Council (#2017-00915 and #2022-00732), the Swedish Alzheimer Foundation (#AF-930351, #AF-939721 and #AF-968270), Hjärnfonden, Sweden (#FO2017-0243 and #ALZ2022-0006), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986 and #ALFGBG-965240), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), the Alzheimer's Association 2021 Zenith Award (ZEN-21-848495), and the Alzheimer's Association 2022-2025 Grant (SG-23-1038904 QC). MJQ is supported by the FRQS.

PREVENT-AD was launched in 2011 as a $13.5 million, 7-year public-private partnership using funds provided by McGill University, FRQS, an unrestricted research grant from Pfizer Canada, the Levesque Foundation, the Douglas Hospital Research Centre and Foundation, the Government

of Canada, and the Canada Fund for Innovation. Private sector contributions are facilitated by the Development Office of the McGill University Faculty of Medicine and by the Douglas Hospital Research Centre Foundation (http://www.douglas.qc.ca/).

Data collection and sharing for this project was funded by the ADNI (National Institutes of Health Grant U01 AG024904) and DOD ADNI (Department of Defense award number W81XWH-12-2-0012). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: AbbVie, Alzheimer's Association; Alzheimer's Drug Discovery Foundation; Araclon Biotech; BioClinica, Inc.; Biogen; Bristol-Myers Squibb Company; CereSpir, Inc.; Cogstate; Eisai Inc.; Elan Pharmaceuticals, Inc.; Eli Lilly and Company; EuroImmun; F. Hoffmann-La Roche Ltd and its affiliated company Genentech, Inc.; Fujirebio; GE Healthcare; IXICO Ltd.;Janssen Alzheimer Immunotherapy Research & Development, LLC.; Johnson & Johnson Pharmaceutical Research & Development LLC.; Lumosity; Lundbeck; Merck & Co., Inc.;Meso Scale Diagnostics, LLC.; NeuroRx Research; Neurotrack Technologies; Novartis Pharmaceuticals Corporation; Pfizer Inc.; Piramal Imaging; Servier; Takeda Pharmaceutical Company; and Transition Therapeutics. The CIHR is providing funds to support ADNI clinical sites in Canada. Private sector contributions are facilitated by the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Therapeutic Research Institute at the University of Southern California. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of Southern California.

3.9 Competing interests

JP serves as a scientific advisor to the Alzheimer Society of France. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Optoceutics, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures

Incubator Program (outside submitted work). KB has served as a consultant and at advisory boards for Acumen, ALZPath, BioArctic, Biogen, Eisai, Lilly, Moleac Pte. Ltd, Novartis, Ono Pharma, Prothena, Roche Diagnostics, and Siemens Healthineers; has served at data monitoring committees for Julius Clinical and Novartis; has given lectures, produced educational materials and participated in educational programs for AC Immune, Biogen, Celdara Medical, Eisai and Roche Diagnostics; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

3.10 References

1. Serrano-Pozo A, Frosch MP, Masliah E, Hyman BT. Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor Perspect Med*. 2011;1(1):a006189.
2. Akiyama H, Barger S, Barnum S, et al. Inflammation and Alzheimer's disease. *Neurobiol Aging*. 2000;21(3):383-421.
3. Bellenguez C, Küçükali F, Jansen IE, et al. New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat Genet*. 2022;54(4):412-436.
4. Guerreiro R, Wojtas A, Bras J, et al. TREM2 variants in Alzheimer's disease. *N Engl J Med*. 2013;368(2):117-127.
5. Mazzali M, Kipari T, Ophascharoensuk V, Wesson JA, Johnson R, Hughes J. Osteopontin--a molecule for all seasons. *QJM*. 2002;95(1):3-13.
6. Lund SA, Giachelli CM, Scatena M. The role of osteopontin in inflammatory processes. *J Cell Commun Signal*. 2009;3(3-4):311-322.
7. Wung JK, Perry G, Kowalski A, et al. Increased expression of the remodeling- and tumorigenic-associated factor osteopontin in pyramidal neurons of the Alzheimer's disease brain. *Curr Alzheimer Res*. 2007;4(1):67-72.
8. Comi C, Carecchio M, Chiocchetti A, et al. Osteopontin is increased in the cerebrospinal fluid of patients with Alzheimer's disease and its levels correlate with cognitive decline. *J Alzheimers Dis*. 2010;19(4):1143-1148.
9. Sun Y, Yin XS, Guo H, Han RK, He RD, Chi LJ. Elevated osteopontin levels in mild cognitive impairment and Alzheimer's disease. *Mediators Inflamm*. 2013;2013:615745.

10. Paterson RW, Heywood WE, Heslegrave AJ, et al. A targeted proteomic multiplex CSF assay identifies increased malate dehydrogenase and other neurodegenerative biomarkers in individuals with Alzheimer's disease pathology. *Transl Psychiatry*. 2016;6(11):e952.
11. Zhou M, Haque RU, Dammer EB, et al. Targeted mass spectrometry to quantify brain-derived cerebrospinal fluid biomarkers in Alzheimer's disease. *Clin Proteomics*. 2020;17:19.
12. Chai YL, Chong JR, Raquib AR, et al. Plasma osteopontin as a biomarker of Alzheimer's disease and vascular cognitive impairment. *Sci Rep*. 2021;11(1):4010.
13. Spellman DS, Wildsmith KR, Honigberg LA, et al. Development and evaluation of a multiplexed mass spectrometry based assay for measuring candidate peptide biomarkers in Alzheimer's Disease Neuroimaging Initiative (ADNI) CSF. *Proteomics Clin Appl*. 2015;9(7-8):715-731.
14. Ringman JM, Schulman H, Becker C, et al. Proteomic changes in cerebrospinal fluid of presymptomatic and affected persons carrying familial Alzheimer disease mutations. *Arch Neurol*. 2012;69(1):96-104.
15. Simonsen AH, McGuire J, Hansson O, et al. Novel panel of cerebrospinal fluid biomarkers for the prediction of progression to Alzheimer dementia in patients with mild cognitive impairment. *Arch Neurol*. 2007;64(3):366-370.
16. Cappellano G, Vecchio D, Magistrelli L, et al. The *Yin-Yang* of osteopontin in nervous system diseases: damage *versus* repair. *Neural Regen Res*. 2021;16(6):1131-1137.
17. Wirths O, Breyhan H, Marcello A, Cotel MC, Brück W, Bayer TA. Inflammatory changes are tightly associated with neurodegeneration in the brain and spinal cord of the APP/PS1KI mouse model of Alzheimer's disease. *Neurobiol Aging*. 2010;31(5):747-757.
18. Rentsendorj A, Sheyn J, Fuchs DT, et al. A novel role for osteopontin in macrophage-mediated amyloid-β clearance in Alzheimer's models. *Brain Behav Immun*. 2018;67:163-180.
19. De Schepper S, Ge JZ, Crowley G, et al. Perivascular cells induce microglial phagocytic states and synaptic engulfment via SPP1 in mouse models of Alzheimer's disease. *Nat Neurosci*. 2023;26(3):406-415.

20. Qiu Y, Shen X, Ravid O, et al. Definition of the contribution of an Osteopontin-producing CD11c^{+} microglial subset to Alzheimer's disease. *Proc Natl Acad Sci U S A*. 2023;120(6):e2218915120.
21. Keren-Shaul H, Spinrad A, Weiner A, et al. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell*. 2017;169(7):1276-1290.e17.
22. Tremblay-Mercier J, Madjar C, Das S, et al. Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. *Neuroimage Clin*. 2021;31:102733.
23. Brinkmalm A, Brinkmalm G, Honer WG, et al. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Mol Neurodegener*. 2014;9:53.
24. Öhrfelt A, Brinkmalm A, Dumurgier J, et al. The pre-synaptic vesicle protein synaptotagmin is a novel biomarker for Alzheimer's disease. *Alzheimers Res Ther*. 2016;8(1):41.
25. Tible M, Sandelius Å, Höglund K, et al. Dissection of synaptic pathways through the CSF biomarkers for predicting Alzheimer disease. *Neurology*. 2020;95(8):e953-e961.
26. Sandelius Å, Portelius E, Källén Å, 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.
27. Portelius E, Olsson B, Höglund K, et al. Cerebrospinal fluid neurogranin concentration in neurodegeneration: relation to clinical phenotypes and neuropathology. *Acta Neuropathol*. 2018;136(3):363-376.
28. Jack CR Jr, Bennett DA, Blennow K, et al. NIA-AA research framework: toward a biological definition of Alzheimer's disease. *Alzheimers Dement*. 2018;14(4):535-562.
29. Meyer PF, Savard M, Poirier J, et al. Bi-directional Association of Cerebrospinal Fluid Immune Markers with Stage of Alzheimer's Disease Pathogenesis. *J Alzheimers Dis*. 2018;63(2):577-590.
30. McSweeney M, Binette AP, Meyer PF, et al. Intermediate flortaucipir uptake is associated with Aβ-PET and CSF tau in asymptomatic adults. *Neurology*. 2020;94(11):e1190-1200

31. Aubert-Broche B, Fonov VS, García-Lorenzo D, et al. A new method for structural volume analysis of longitudinal brain MRI data and its application in studying the growth trajectories of anatomical brain structures in childhood. *Neuroimage*. 2013;82:393-402.
32. Shaw LM, Vanderstichele H, Knapik-Czajka M, et al. Cerebrospinal fluid biomarker signature in Alzheimer's disease neuroimaging initiative subjects. *Ann Neurol*. 2009;65(4):403-413.
33. Scriver CR. Human genetics: lessons from Quebec populations. *Annu Rev Genomics Hum Genet*. 2001;2:69-101.
34. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group* under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. 1984;34(7):939-944.
35. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. *Neurobiol Aging*. 1995;16(3):271-284.
36. Chan JL, Reeves TM, Phillips LL. Osteopontin expression in acute immune response mediates hippocampal synaptogenesis and adaptive outcome following cortical brain injury. *Exp Neurol*. 2014;261:757-771.
37. Morisaki Y, Niikura M, Watanabe M, et al. Selective Expression of Osteopontin in ALS-resistant Motor Neurons is a Critical Determinant of Late Phase Neurodegeneration Mediated by Matrix Metalloproteinase-9. *Sci Rep*. 2016;6:27354.
38. Yan P, Hu X, Song H, et al. Matrix metalloproteinase-9 degrades amyloid-beta fibrils in vitro and compact plaques in situ. *J Biol Chem*. 2006;281(34):24566-24574.
39. Lindsey ML, Zouein FA, Tian Y, Padmanabhan Iyer R, de Castro Brás LE. Osteopontin is proteolytically processed by matrix metalloproteinase 9. *Can J Physiol Pharmacol*. 2015;93(10):879-886.
40. Kaminari A, Giannakas N, Tzinia A, Tsilibary EC. Overexpression of matrix metalloproteinase-9 (MMP-9) rescues insulin-mediated impairment in the 5XFAD model of Alzheimer's disease. *Sci Rep*. 2017;7(1):683.
41. Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119-128.

42. Pereira JB, Janelidze S, Strandberg O, et al. Microglial activation protects against accumulation of tau aggregates in nondemented individuals with underlying Alzheimer's disease pathology. *Nat Aging*. 2022;2(12):1138-1144.
43. Dai J, Peng L, Fan K, et al. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. *Oncogene*. 2009;28(38):3412-3422.
44. Hooper C, Killick R, Lovestone S. The GSK3 hypothesis of Alzheimer's disease. *J Neurochem*. 2008;104(6):1433-1439.
45. Wang Y, Balaji V, Kaniyappan S, et al. The release and trans-synaptic transmission of Tau via exosomes. *Mol Neurodegener*. 2017;12(1):5.
46. Luo W, Liu W, Hu X, Hanna M, Caravaca A, Paul SM. Microglial internalization and degradation of pathological tau is enhanced by an anti-tau monoclonal antibody. *Sci Rep*. 2015;5:11161.
47. Ellison JA, Velier JJ, Spera P, et al. Osteopontin and its integrin receptor alpha(v)beta3 are upregulated during formation of the glial scar after focal stroke. *Stroke*. 1998;29(8):1698-1707.
48. Meller R, Stevens SL, Minami M, et al. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab*. 2005;25(2):217-225.
49. Terry RD, Masliah E, Salmon DP, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*. 1991;30(4):572-580.
50. Anderson MA, O'Shea TM, Burda JE, et al. Required growth facilitators propel axon regeneration across complete spinal cord injury. *Nature*. 2018;561(7723):396-400.
51. Liu Y, Wang X, Li W, et al. A Sensitized IGF1 Treatment Restores Corticospinal Axon-Dependent Functions. *Neuron*. 2017;95(4):817-833.e4
52. Duan X, Qiao M, Bei F, Kim IJ, He Z, Sanes JR. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. *Neuron*. 2015;85(6):1244-1256.
53. Kim HJ, Fillmore HL, Reeves TM, Phillips LL. Elevation of hippocampal MMP-3 expression and activity during trauma-induced synaptogenesis. *Exp Neurol*. 2005;192(1):60-72.

54. Szklarczyk A, Lapinska J, Rylski M, McKay RD, Kaczmarek L. Matrix metalloproteinase-9 undergoes expression and activation during dendritic remodeling in adult hippocampus. *J Neurosci*. 2002;22(3):920-930.
55. Nestor SM, Rupsingh R, Borrie M, et al. Ventricular enlargement as a possible measure of Alzheimer's disease progression validated using the Alzheimer's disease neuroimaging initiative database. *Brain*. 2008;131(Pt 9):2443-2454.
56. Sathe G, Mangalaparthi KK, Jain A, et al. Multiplexed Phosphoproteomic Study of Brain in Patients with Alzheimer's Disease and Age-Matched Cognitively Healthy Controls. *OMICS*. 2020;24(4):216-227.
57. Sathe G, Albert M, Darrow J, et al. Quantitative proteomic analysis of the frontal cortex in Alzheimer's disease. *J Neurochem*. 2021;156(6):988-1002.
58. Inoue M, Shinohara ML. Intracellular osteopontin (iOPN) and immunity. *Immunol Res*. 2011;49(1-3):160-172.
59. Pinner E, Gruper Y, Ben Zimra M, et al. CD44 Splice Variants as Potential Players in Alzheimer's Disease Pathology. *J Alzheimers Dis*. 2017;58(4):1137-1149.
60. Chabas D, Baranzini SE, Mitchell D, et al. The influence of the proinflammatory cytokine, osteopontin, on autoimmune demyelinating disease. *Science*. 2001;294(5547):1731-1735.
61. Anborgh PH, Mutrie JC, Tuck AB, Chambers AF. Pre- and post-translational regulation of osteopontin in cancer. *J Cell Commun Signal*. 2011;5(2):111-122.

3.11 Tables

Table 1 Baseline Participant Demographics

	PREVENT-AD	ADNI-1			QFP	
	CU	CU	MCI	AD	CU	AD
N Sample	109	92	148	68	31	55
Mean Age, Years (SD)	62.60 (5.43)	75.70 (5.45)	74.79 (7.20)	74.89 (7.65)	77.39 (11.37)	80.71 (6.39)
N Female (%)	76 (69.7)	46 (50.0)	47 (31.8)	30 (44.1)	11 (35.5)	23 (41.8)
N *APOE* ε4+ (%)	43 (39.4)	22 (23.9)	79 (53.4)	48 (70.6)	9 (29.0)	32 (58.2)
Mean CSF $A\beta_{42}$, pg/mL (SD)[a][a]	1145.73 (277.62)	208 (53.38)	160 (49.34)	141.20 (35.17)	-	-
Mean CSF p_{181}tau, pg/mL (SD)[a][a]	46.83 (18.00)	24.79 (13.43)	36.20 (15.55)	41.28 (20.73)	-	-
Mean CSF t-tau, pg/mL (SD)[a][a]	273.09 (129.97)	68.85 (26.37)	105.10 (52.71)	122.52 (59.40)	-	-
Mean CSF OPN, NPX (SD)	10.22 (0.28)	-	-	-	-	-
Mean CSF OPN , ng/mL (SD)	-	31.02 (10.09)	34.13 (9.87)	34.79(8.99)	-	-
Mean Global Aβ, SUVR (SD)[b]	1.30 (0.27)	-	-	-	-	-
Mean Tau metaROI, SUVR (SD)[c]	1.17 (0.07)	-	-	-	-	-
Mean Cortical OPN Protein, log_2 (SD)[d]	-	-	-	-	1.42 (1.01)	2.17 (0.97)
Mean Post-mortem delay, h (SD)	-	-	-	-	30.03 (19.85)	21.07 (10.36)

PREVENT-AD, PResymptomatic EValuation of Experiment or Novel Treatments for Alzheimer's disease; ADNI-1, Alzheimer's Disease Neuroimaging Initiative; QFP, Quebec Founder Population; CU, cognitively unaffected; MCI, mild cognitive impairment; AD, Alzheimer's disease; *APOE* ε4+, Apolipoprotein ε4 carriers; CSF, cerebrospinal fluid; $A\beta_{42}$, amyloid-beta 42; pg/mL, picograms per milliliter; p_{181}tau, phosphorylated tau 181; t-tau, total tau; OPN, osteopontin; NPX, Normalized Protein eXpression; SUVR, standardized uptake value ratio; ROI, region of interest; h, hours; SD, standard deviation.

[a][a] PREVENT-AD and ADNI used different assays to measure CSF AD biomarkers.

[a] 101PREVENT-AD participants had CSF $A\beta_{42}$, p_{181}tau and t-tau (pg/mL) values available

[b] 46 PREVENT-AD participants had Global Aβ SUVR values available.

[c] 49 PREVENT-AD participants had Tau metaROI SUVR values available

[d] 75 QFP participants had cortical OPN values available

3.12 Figure Legends

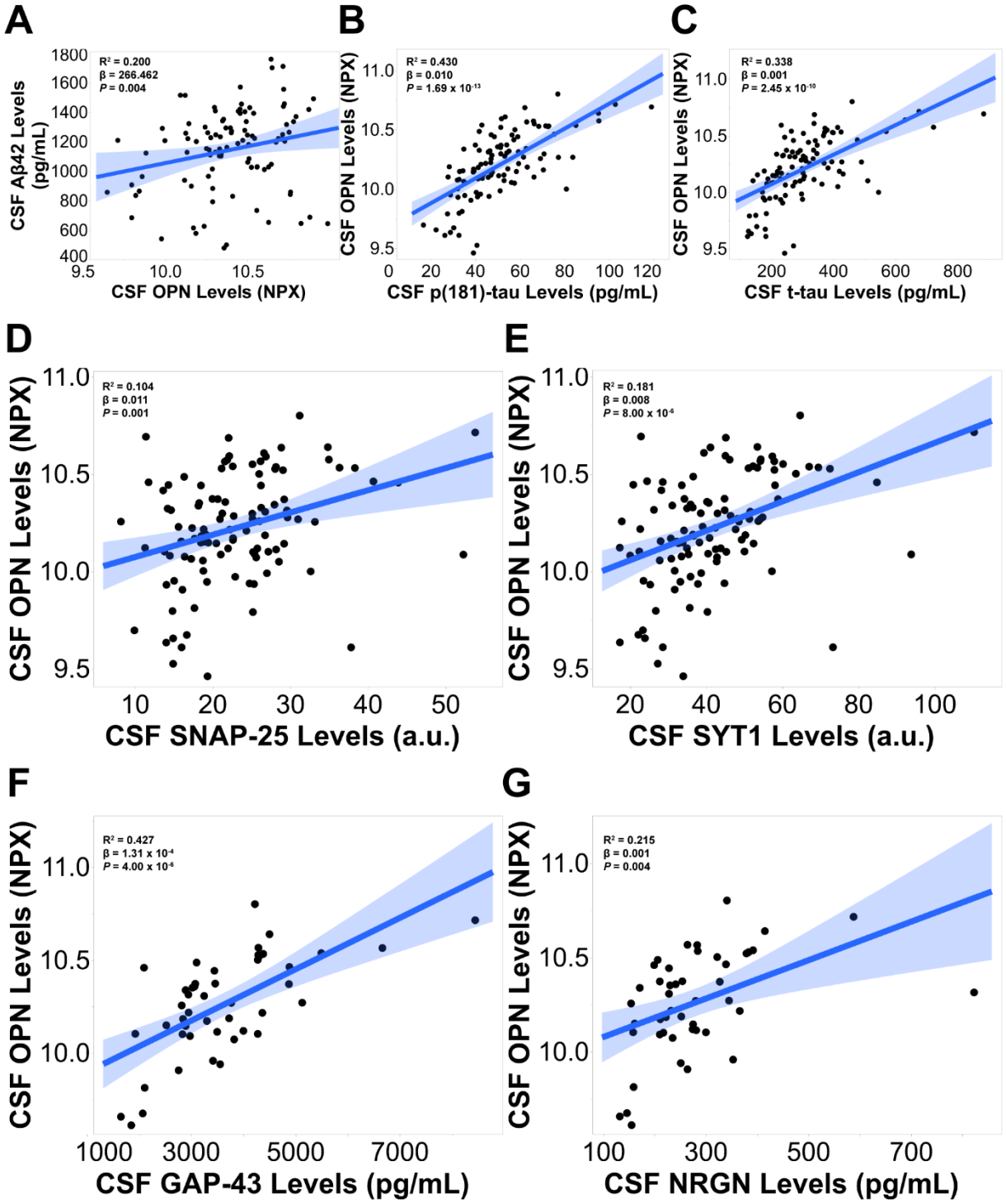


Figure 1 CSF OPN is associated with the core CSF AD biomarkers and with synaptic markers in the asymptomatic PREVENT-AD cohort. CSF OPN levels were measured using the Olink Proximity Extension Assay (n = 109)**.** CSF AD biomarkers $A\beta_{42}$ (**A**), p_{181}-tau (**B**) and t-tau (**C**) were measured using validated Innotest ELISA kits, following the standardized protocols established by the BIOMARKAPD consortium (n = 101). The synaptic markers SNAP-25 (**D**; n = 106), SYT-1 (**E**; n = 106), GAP43 (**F**; n = 46) and NRGN (**G**; n = 46) were quantified using immunoprecipitation followed by mass spectrometry. Significant or trend-level linear regressions are represented with a blue confidence region of the fitted line. R^2 and P values are located in the top left corners of each panel. Analyses were adjusted for age, sex and *APOE* ε4 carrier status. SNAP-25, synaptosomal-associated protein 23kDa; SYT1, synaptotagmin-1; GAP43, growth-associated protein 43; NRGN, neurogranin.

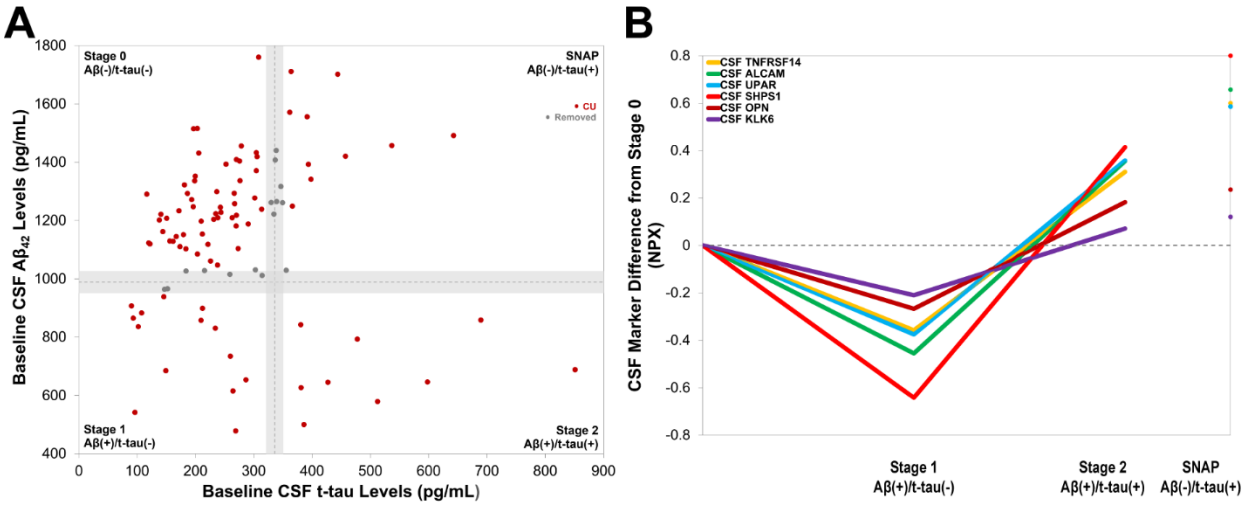


Figure 2 CSF OPN exhibits a bidirectional relationship with AD pathology in CU PREVENT-AD participants. CU participants (n = 85) were staged as CSF amyloid and/or CSF total tau positive according to the thresholds of 989 pg/mL and 336 pg/mL (**A**). Linear models, adjusted for age, sex and *APOE* ε4 carrier status were used to examine mean differences in OPN protein levels across stages. CSF OPN was reduced at Stage 1 (n = 14) relative to Stage 0 (n = 53). Furthermore, CSF OPN was elevated at Stage 2 (n = 9) compared to Stage 0 (trend) and Stage 1. Finally, CSF OPN was increased in SNAP (n = 10) relative to Stage 0. This bidirectional relationship was also seen in five other inflammatory / immune-related proteins (TNFRSF14, ALCAM, UPAR, SHPS1, KLK6) from the Olink Cardiovascular Panel.

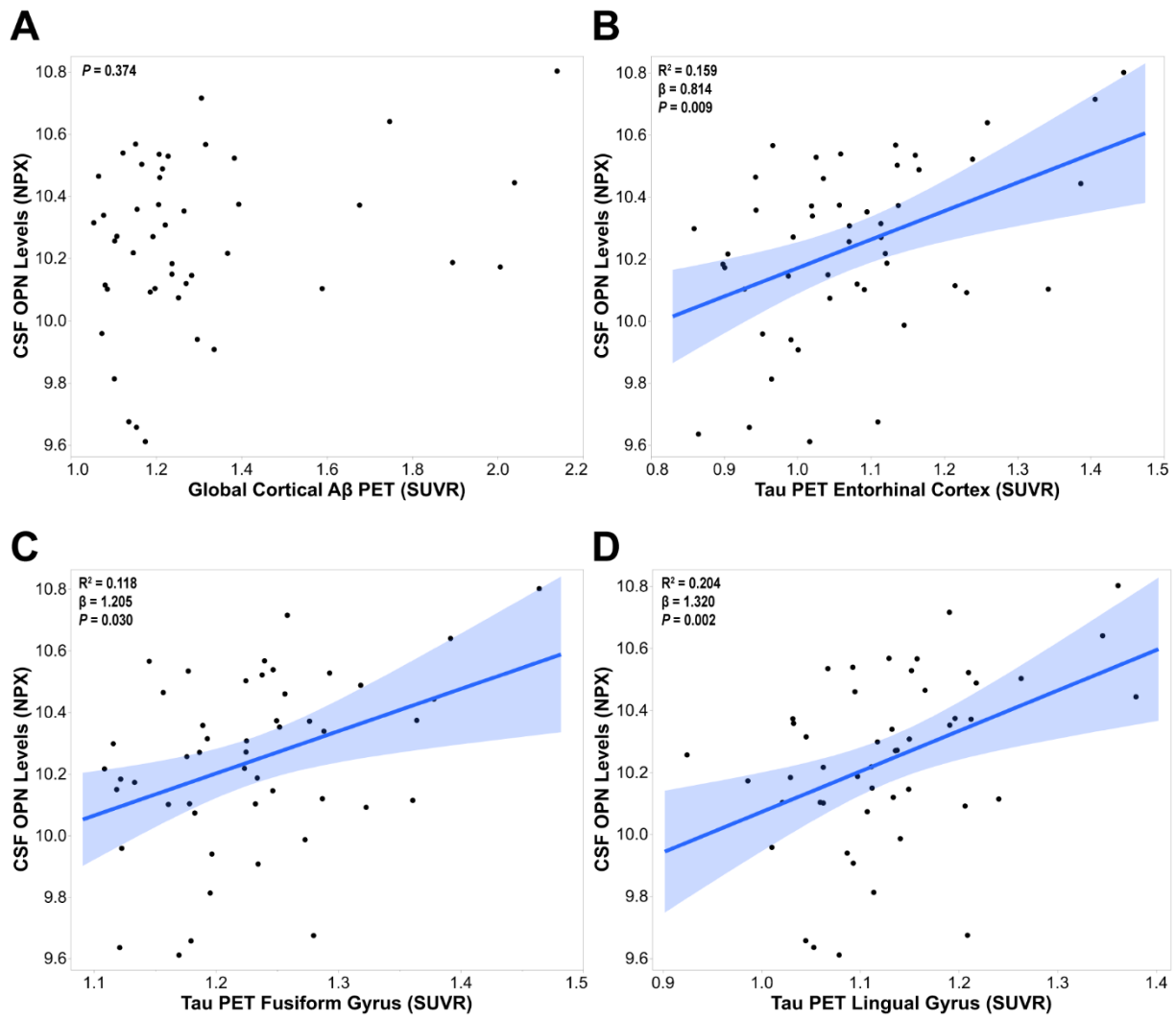


Figure 3 CSF OPN is associated with PET tau burden in Braak stages 2-3 in the asymptomatic PREVENT-AD cohort. CSF OPN levels were measured using the Olink Proximity Extension Assay (n = 109)**.** Global cortical Aβ SUVR was measured using ^{18}F-NAV4694 (n = 46) (**A**). Tau deposition in the entorhinal cortex **(B)**, fusiform gyrus **(C)** and lingual gyrus **(D)** was measured with flortaucipir (n = 49). Significant linear regressions are represented with a blue confidence region of the fitted line. R^2 and P values are located in the top left corners of each panel. Analyses were adjusted for age, sex and *APOE* ε4 carrier status.

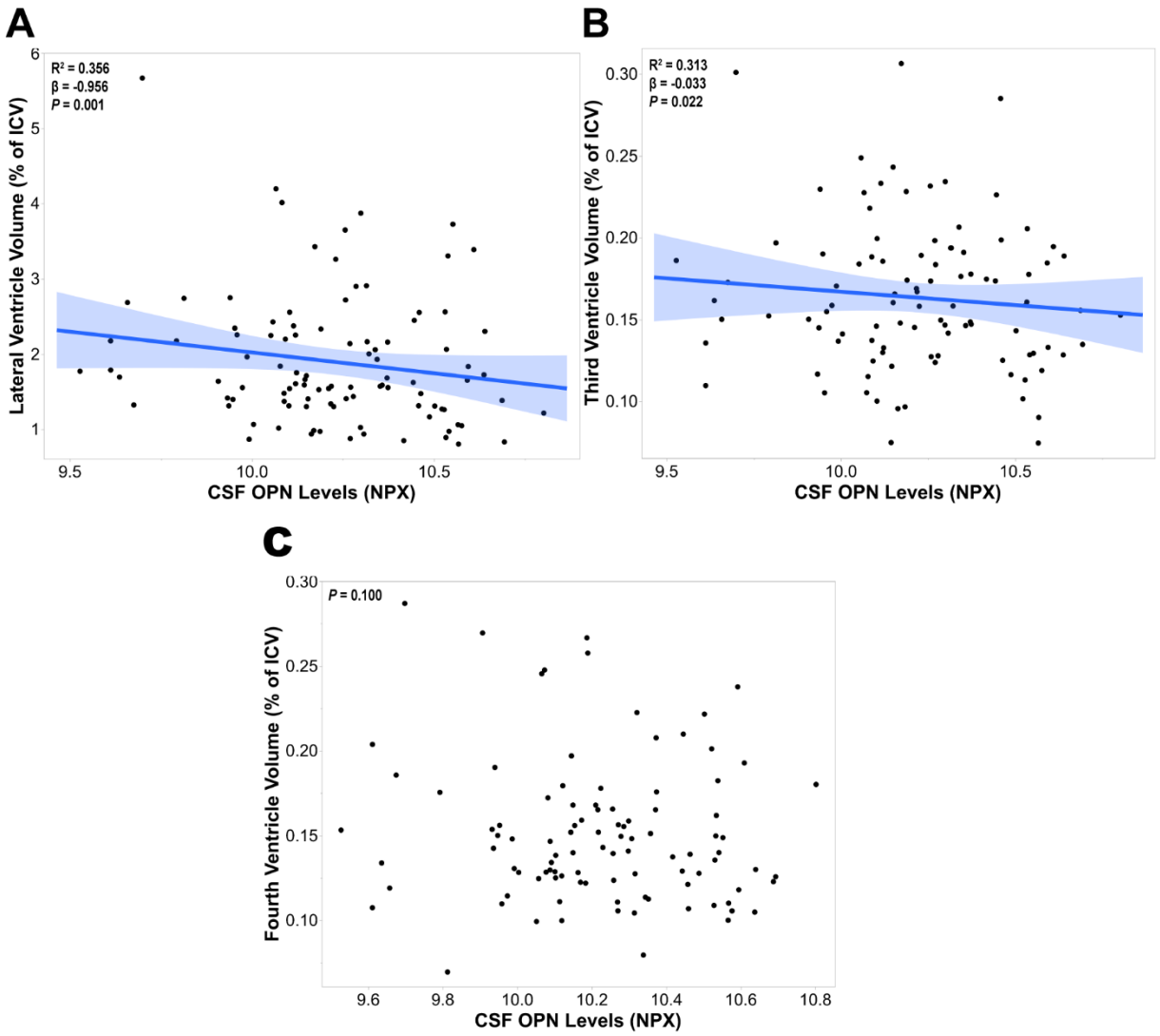


Figure 4 CSF OPN is associated with reduced cerebral ventricle volume in asymptomatic PREVENT-AD Participants. CSF OPN levels were measured using the Olink Proximity Extension Assay (*n* = 109)**.** Lateral ventricle **(A)**, third ventricle **(B)** and fourth ventricle **(C)** Brain volumes were computed using a volumetric pipeline that has been previously described (*n* = 104).[31]. Significant linear regressions are represented with a blue confidence region of the fitted line. R^2 and *P* values are located in the top right corners of each panel. Analyses were adjusted for total intracranial volume (ICV), age, sex and *APOE* ε4 carrier status.

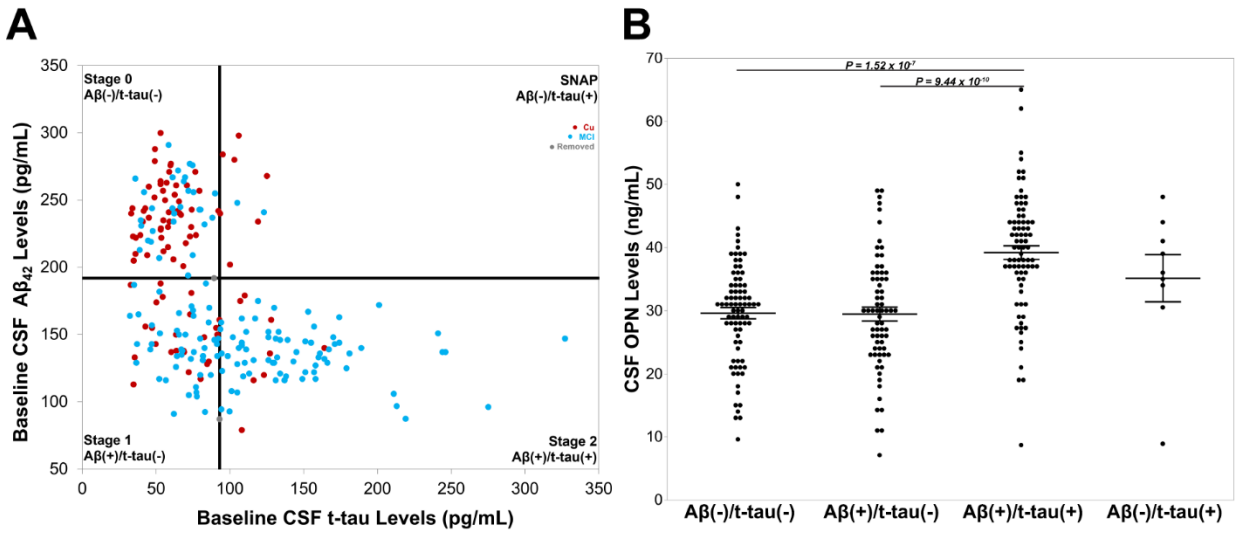


Figure 5 CSF OPN is elevated in CSF Aβ(+)/t-tau(+) individuals from the ADNI-1 cohort. CU participants (red, *n* = 91) and participants with MCI (blue, *n* = 147) from the ADNI-1 cohort were staged as CSF amyloid and/or CSF total tau positive according to the recommended thresholds of 192 pg/mL and 93 pg/mL (**A**). Linear models, adjusted for age, sex and *APOE* ε4 carrier status were used to examine mean differences in OPN protein levels across stages. CSF OPN was elevated at Stage 2 (*n* = 79) relative to Stage 0 (*n* = 81) and Stage 1 (*n* = 69). Furthermore, CSF IGFBP2 was elevated in SNAP (*n* = 9) compared to Stage 0 (**B**).

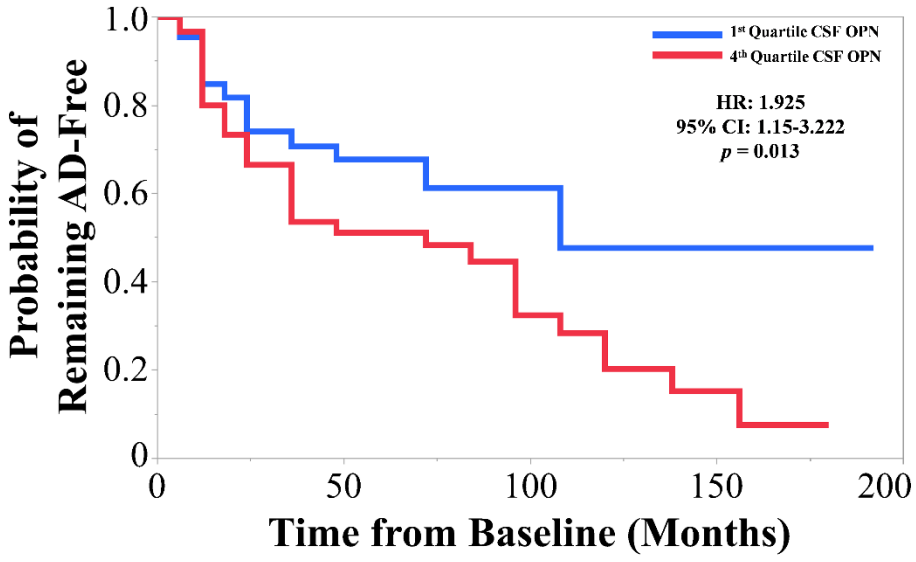


Figure 6 Elevated CSF OPN is associated with a greater rate of conversion to AD in individuals from the ADNI-1 cohort. Cox proportional hazards models examined the association between baseline CSF OPN levels and rate of conversion to AD. The first quartile (blue) and fourth (red) values of CSF OPN were contrasted. CU participants and individuals with MCI were followed from the baseline visit to the time of diagnosis (of AD), or to the time the participant was last confirmed to be free of AD (mean follow-up, 4.3 years; range, 0.5-16 years). Of the 126 individuals that were followed longitudinally, 62 individuals progressed to AD. HR and *P* values are located in the top right corner. Cox models were adjusted for age, gender and *APOE* ε4 carrier status. HR, Hazard Ratio.

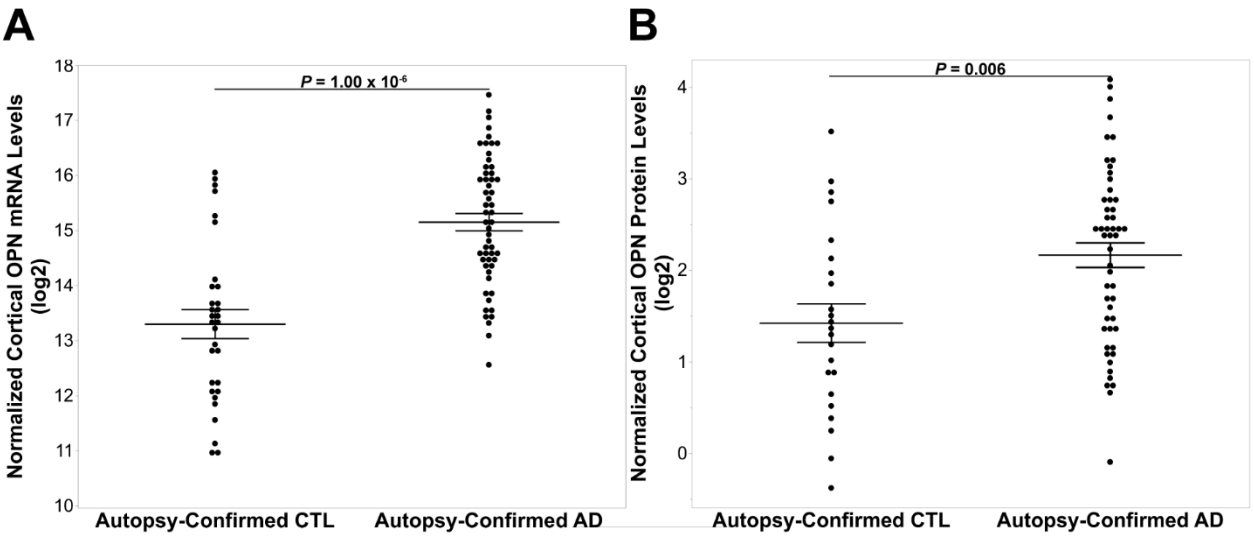


Figure 7 OPN gene expression and protein levels are significantly elevated in the frontal cortex of autopsy-confirmed AD brains. Microarray technology was used to measure OPN mRNA levels in the frontal cortex of autopsy-confirmed AD brains (n = 55) and age-matched elderly controls (n = 31) from the QFP cohort (**A**). OPN protein levels in the frontal cortex were measured in AD brains (n = 52) and control brains (n = 23) using a commercially available ELISA kit (**B**). *P* values are located in the top left corner of each panel. Analyses were adjusted for age, sex, *APOE* ε4 carrier status and post-mortem delay. The data are represented as mean ± SEM. SEM, Standard error of the mean.

4. Discussion

4.1 Implications

4.1.1 Discovery of novel therapeutic targets for pre-symptomatic AD

The recent emergence of Aβ clearing therapies represents a promising beginning to addressing the underlying biology of AD.[4-6] However, these monoclonal antibody therapies that target Aβ (aducanumab, lecanemab, donanemab) only slow down the progression of the disease by 22-35% in patients with MCI or mild AD. [4-6] Unfortunately, it has been argued that such a difference may be difficult to observe in the clinic. Furthermore, similar monoclonal antibody therapies, such as solanezumab, have failed in patients with mild-moderate sporadic AD[151] and notably, in participants with preclinical AD.[152] Importantly, solanezumab and gantenerumab (another antibody treatment) did not offer cognitive or clinical benefits to asymptomatic and symptomatic familial AD mutation carriers.[176] Finally, it is important to recognize these treatments are associated with concerning rates of amyloid-related imaging abnormalities such as cerebral edema and cerebral microhemorrhages (especially amongst *APOE* ε4 carriers and homozygotes*)*.[4-6] Thus, these limited benefits highlight the need to identify additional therapeutic targets (perhaps related to phospho-tau production, axonal/synaptic regeneration, neurogenesis, etc) that may be used in combination therapies. Furthermore, considering that neuropathological changes associated with AD begin decades before the onset of symptoms,[7] it is possible these antibody treatments have been administered to patients too late in the disease process.[81] As an alternative, administering a treatment earlier in the disease that could delay the onset of symptoms by 5 years would markedly reduce the prevalence of AD by nearly 50%.[177]

4.1.2 Discovery of novel biomarkers for screening for pre-symptomatic individuals that are at-risk of developing AD

It is clear that studying the pre-symptomatic stage of AD remains the best strategy to delay, slow down and/or prevent AD.[7,81] However, identifying pre-symptomatic individuals that will convert to AD with complete accuracy remains the great challenge. To this end, the Dominantly Inherited Alzheimer Network (DIAN)[178] and the Alzheimer's Prevention Initiative (API)[179] have been established to study pre-symptomatic (*APP, PSEN1, PSEN2)* mutation carriers that will develop

familial AD with 100% certainty. However, familial AD cases remain rare, as they account for 1% of total AD cases.[3] Furthermore, it has been shown that neuropathological alterations differ between familial AD and sporadic AD.[180] For instance, a recent cryo-electron microscopy study demonstrated that type I $A\beta_{42}$ filaments were present in post-mortem brains of patients with sporadic AD, while type II $A\beta_{42}$ filaments were present in the brains of patients with familial AD (and in the APP^{NL-F} mouse model of AD).[180] Besides these differences in pathology, genetic analyses of the familial genes (*APP*, *PSEN1*, *PSEN2*) in large cohorts of sporadic cases (GWAS studies) failed to identify any significant polymorphism or mutation linked to disease risk in the sporadic form of the illness. Thus, taken altogether, it is possible that the pre-symptomatic stage of familial AD may not be representative of the pre-symptomatic stage of sporadic AD. Alternatively, using parental and/or multi-sibling history as an inclusion criterion (as in the PREVENT-AD cohort) is a major advancement to investigate the pre-symptomatic stage of sporadic AD.[181] In fact, many of the PREVENT-AD participants are likely to be in this pre-symptomatic stage, as 39% of individuals with a first-degree relative affected by AD are expected to develop AD (by the age of 96), which is two times greater than the general population.[98] Furthermore, using a family history of AD alongside genetic risk factors such as *APOE* ε4, are cost-effective alternatives to Aβ PET scans (as well as structural MRI scans), which are often required for screening for clinical trials.

To this end, in pre-symptomatic PREVENT-AD participants, we have found that CSF IGFBP2 is a valuable marker of longitudinal decline in delayed memory and visuospatial abilities, which are cognitive domains of the RBANS scale that are affected early on in AD.[182] Furthermore, we have shown that IGFBP2 is a valuable marker of brain atrophy in AD-vulnerable brain regions such as the entorhinal cortex (trend-level), inferior and middle temporal gyrus, piriform cortex and precuneus. The latter two brain regions are of clinical importance, as the piriform cortex is responsible for olfaction, which is dysfunctional early on in AD.[183] Furthermore, transcranial magnetic stimulation of the precuneus over 24 weeks has been shown to reduce cognitive and functional decline in mild and moderate AD patients, compared to sham stimulation.[184] In an independent and well-characterized cohort, we demonstrated that CSF and plasma IGFBP2 are elevated in CSF Aβ(+)/t-tau(+) individuals from the ADNI-1 cohort. This staging criterion is vital for predicting disease progression and screening for clinical trials.[90] Nevertheless, due to a limited number of ADNI-1 participants that underwent both lumbar punctures and PET scans, we were

unable to stage participants as amyloid and/or phospho-tau positive based on PET scans. However, it is important to recognize that changes in CSF Aβ, p_{181}-tau and t-tau are believed to occur prior to changes in Aβ PET, tau PET and MRI brain atrophy.[81] Thus, our findings are critical, as they capture the very beginning of the disease process. Furthermore, as we were interested in the stages leading up to dementia, we included ADNI-1 participants with MCI in these staging analyses. Indeed, nearly 15% of individuals living with MCI develop AD after 2 years.[3] Within 5 years, approximately 33% of people living with MCI (due to AD) progress to AD.[3] Finally, from a longitudinal perspective, we found that baseline plasma IGFBP2 is associated with a faster rate of near-term conversion to AD.

Besides studying the insulin-IGF feature of AD, we also examined the neuroinflammation aspect of AD. To this end, we analyzed a pro-inflammatory protein, namely, OPN. First, we demonstrated that CSF OPN exhibits a peculiar bidirectional relationship with stage of AD pathology, and is ultimately elevated in pre-symptomatic CSF Aβ(+)/t-tau(+) PREVENT-AD participants. Although unexpected, the reduction of CSF OPN in CSF Aβ(+)/t-tau(-) individuals suggests that Aβ plaques may dampen the normal immune response in the brain. We have previously observed this unusual relationship in an independent analysis of immune/inflammatory proteins in PREVENT-AD.[47] In addition to these biofluid findings, neuroimaging analyses revealed that CSF OPN may be upregulated as a result of significant tau deposition in the entorhinal, fusiform and lingual regions in PREVENT-AD participants. Furthermore, CSF OPN was negatively correlated with the volume of the lateral and third ventricles (exception of the fourth ventricle). This latter observation is critical, as it emphasizes the protective role of OPN in pre-symptomatic PREVENT-AD individuals, as it appears OPN inhibits the enlargement of the ventricles in the brain. Indeed, these results suggest that OPN may preserve the integrity of the brain, by promoting the maintenance of healthy neurons and synapses. In the ADNI-1 cohort, CSF OPN was also elevated in CSF Aβ(+)/t-tau(+) individuals, which further validates our observation in the PREVENT-AD cohort. Finally, from a longitudinal perspective, we also found that baseline CSF OPN is associated with an accelerated rate of conversion to AD.

Overall, our data suggest that incorporating IGFBP2 and OPN in established biomarker panels for AD may help identify vulnerable and pre-symptomatic individuals with more accuracy. In a

similar fashion, we believe IGFBP2 and OPN may open up new avenues of research into protective therapies against AD and related dementias.

4.2 Limitations

4.2.1 Correlations do not imply causation

Although we have presented exciting novel insights into the pre-symptomatic stage of AD, we recognize that correlations/associations do not imply causation. Based on the existing literature, we hypothesize that IGFBP2 and OPN may be upregulated in an attempt to stave off early AD pathology. However, we have not proven any definite causality. For instance, it cannot be dismissed that upregulations in IGFBP2 and/or OPN may be harmful to the brain and contribute to the progression of AD. First, it is possible to argue that an upregulation in IGFBP2 may be detrimental to the brain, as IGFBP2 may sequester excessive amounts of IGFs and potentially inhibit neuroprotective IGF signaling in the brain. However, one might question why IGFBP2 would be upregulated following acute brain injury (rodent models of stroke, etc.),[165-170] if the role of IGFBP2 was to merely sequester IGFs and hinder IGF signaling during the recovery phase. This scenario appears unlikely, considering that IGFs have been found to be upregulated following acute brain injury[164,166-168,170] and causally promote neuronal survival and repair.[185] Nevertheless, several studies highlight the protective properties of IGFBP2. For instance, the overexpression of IGFBP2 in mice has been found to inhibit insulin resistance.[186] Similarly, *IGFBP2* and *IGF2* expression were found to be upregulated following the administration of estrogen into the hippocampus of female rats.[187] This finding suggests that IGFBP2 and IGF2 may account for some of the protective effects of estrogen against AD.[187] Finally, studies have shown that intracellular IGFBP2 regulates the expression of vascular endothelial growth factor,[188] which has been argued to be protective against AD.[189] Nevertheless, it is important to consider that during mid/late stage AD, it is possible that an upregulation in IGFBP2 may sequester the dwindling IGFs (which are known to be reduced in post-mortem AD brains), and ultimately inhibit neuroprotective IGF signaling.

In a similar fashion to the insulin-IGF system, OPN has been found to be upregulated following acute brain injury[172] and causally promote neuronal survival[158] and repair.[161-163] Thus, these findings suggest that OPN may play a protective role in acute conditions, such as during the pre-

symptomatic stage of AD. Indeed, in a partial model of AD pathology, OPN gene expression and secretion has been found to be significantly upregulated in the hippocampus of rats that received unilateral entorhinal cortex lesions (UECLs).[190] Furthermore, OPN knockout mice with UECLs displayed cognitive impairments during the recovery phase, compared to wild-type mice with UECLs.[190] Taken altogether, these results suggests that an increase in OPN secretion (by microglia) is necessary for the clearance of degenerating axon terminals, such that compensatory sprouting can occur, and new synapses can be formed.[190]

On the other hand, it is possible the pro-inflammatory nature of OPN may eventually be harmful to the brain as the disease progresses. Indeed, there are several lines of evidence that suggest this may be the case. For instance, it has been shown that inflammation exacerbates the production of Aβ peptides and phosphorylated tau, and promotes insulin resistance/type 2 diabetes.[191,192] It is possible OPN may exert these detrimental effects by promoting the chronic release of various pro-inflammatory cytokines such as IL-12 and IL-17.[48] More specifically, studies have found that the genetic ablation and neutralization of the IL-12/IL-23 subunits p35, p40 or p19, leads to reductions in Aβ burden and improvements in cognition in APP/PS1 mice.[193] Furthermore, it has been demonstrated that the administration of anti-IL-17 antibodies reverses short-term memory and synaptic plasticity deficits in 3xTg-AD mice.[194] Moreover, it is important to recognize that OPN also inhibits the secretion of IL-10, which is a prominent anti-inflammatory cytokine.[48] However, several studies have found conflicting results regarding IL-10 in AD.[195,196] For instance, the sustained expression of IL-10 in APP+PS1 mice has been demonstrated to increase plasma Aβ levels, reduce astro/microgliosis, enhance neurogenesis and improve spatial learning.[195] Conversely, IL-10 deficiency has been shown to reduce amyloid deposition, preserve synaptic integrity and improve memory in APP/PS1 mice.[196] Finally, OPN enhances the release of interferons (IFN) such as IFN-α and IFN-γ, which may exert detrimental effects on the brain.[197,198] For instance, the genetic ablation and antibody blockade of the interferon alpha/beta receptor 1 protects primary neurons from Aβ-induced cell death,[197] and reduces synaptic loss in mouse models of AD.[198]

4.2.2 Potential discrepancies between CSF and brain tissue protein levels

Unfortunately, we were unable to correlate CSF IGFBP2 and OPN protein levels with post-mortem brain tissue IGFBP2 and OPN protein levels, within the same individuals. However, it would be a

lengthy and challenging feat to acquire biofluids from living participants and brain tissue from the same individuals, once they were deceased. Thus, based off of independent cohorts, we postulated that CSF IGFBP2 and OPN protein levels were representative of IGFBP2 and OPN protein levels in brain tissue. However, it cannot be dismissed that CSF and/or plasma IGFBP2 and OPN protein levels may, in fact, be inversely correlated with brain tissue IGFBP2 and OPN protein levels. For instance, this phenomenon has been observed with the synaptic proteins SYT1, SNAP25, GAP43 and NRGN, which are reduced in AD brains[67-69] yet are elevated in the CSF of AD patients.[70] Such a scenario is not inconceivable for IGFBP2. For instance, an accumulation of IGFBP2 in the CSF and a concomitant lack of IGFBP2 in brain tissue may be detrimental by promoting IGF degradation and/or IGF clearance through the BBB. In fact, we did observe a modest reduction in IGFBP2 mRNA levels in the frontal cortex of autopsy-confirmed AD brains from the QFP cohort, compared to age-matched controls. However, we were unable to detect any changes in IGFBP2 at the protein level. Thus, it is possible the discrepancy between IGFBP2 mRNA and IGFBP2 protein levels may be due to post-transcriptional modifications such as RNA methylation, which influences the splicing, export, translation and degradation of mRNA.[199] In a similar fashion, post-translational modifications may also affect the stability of proteins, and thus, help explain the observed inconsistencies between IGFBP2 mRNA and IGFBP2 protein levels.[200] Alternatively, it is possible that we did not observe any differences in IGFBP2 protein levels as we merely examined "end-stage" AD through post-mortem brains. Thus, it is plausible that changes in IGFBP2 might have occurred earlier on in the disease process, most likely during the pre-symptomatic stage (as observed in PREVENT-AD participants). Finally, given the well-established literature surrounding OPN in AD and other neurodegenerative diseases,[201] it is highly likely that CSF OPN protein levels reflect OPN protein levels in brain tissues.

4.2.3 AD-specific markers or general-neurodegeneration markers

Although the present thesis has focused solely on AD, it is important to address the issue of differential diagnoses. Indeed, several forms of dementia, such as VD, FTD and LBD share overlapping symptoms with AD.[14-16] Furthermore, as previously mentioned, a clinical diagnosis of AD can have a specificity as low as 44%.[17] Thus, it is imperative to identify biomarkers that may help differentiate AD from other neurodegenerative diseases. To this end, in exploratory analyses using CSF samples from the CCNA cohort, we contrasted CSF levels of IGFBP2 and

OPN across AD, FTD, LBD and PD (data not shown). Interestingly, we observed a large increase in CSF OPN levels in AD cases compared to FTD, LBD and PD cases, however this difference was not statistically significant. It is possible this difference in CSF OPN did not reach statistical significance due to limitations in sample size for the non-AD cases. Nevertheless, our observations are certainly consistent with a previous finding that CSF OPN was elevated in patients with AD compared to patients with FTD.[202] Thus, we argue that additional studies are needed to assess CSF OPN levels across multiple neurodegenerative diseases.

4.2.4 Potential contributions of comorbidities

Similar to the issue of differential diagnoses, the issue of comorbidities must be addressed. Indeed, post-mortem brain tissue studies have demonstrated that only 44% of AD patients in their 60s exhibit pure AD pathology, as Lewy bodies and vascular anomalies are abundant in this age group.[203] Furthermore, pure AD pathology is only present in 20% of AD patients in their 70s and beyond, as TDP-43 pathology, non-AD tau pathology and vascular-associated infarcts accumulate with age.[203] Thus, it is important to consider that clinically undetectable comorbidities present in the PREVENT-AD and ADNI-1 cohorts might have influenced the variability in IGFBP2, OPN, biomarker and neuroimaging measurements. Thus, it is important to recognize that a definite diagnosis of AD can only be performed upon conducting autopsies on post-mortem brains.[13] Hence, acquiring a definite diagnosis for relatively young cohorts such as PREVENT-AD would be a lengthy challenge. Nevertheless, it is critical to address the issue of neurological (and systemic) comorbidities, as they can likely explain why current amyloid-lowering treatments for AD only reduce the progression of the disease by 22-35%.[4-6] Taken altogether, the issue of comorbidities emphasizes the importance of holistic therapeutic approaches, as targeting multiple molecules should be the ultimate goal.

4.2.5 Generalizability of results

Although we replicated our findings across three distinct cohorts, namely, PREVENT-AD, ADNI-1 and QFP, the generalizability of our results may be restricted to well-educated Caucasian individuals. Thus, it is of the utmost importance to reproduce our findings in diverse and underrepresented populations, which have been shown to exhibit the highest prevalence of dementia.[96] For instance, the prevalence of dementia in Blacks/African Americans above the age

of 65 is 19.3%, 16.7% in Latinx/ Hispanics and 7.4% in Caucasians.[96] However, the PREVENT-AD cohort is composed of 98.9% Caucasians,[181] while the ADNI cohort is primarily comprised of Caucasians at 79.3%, Blacks/African Americans at 11.5% and Latinx/Hispanics at 5.6%.[204] Furthermore, it must be taken into consideration that as a population isolate from Eastern Canada, the QFP is a highly homogeneous cohort in terms of genetics and environmental factors. Thus, we recommend that IGFBP2 and OPN should be further analyzed in autopsied brains from diverse populations, before reaching any definitive conclusions. Finally, educational attainment is also believed to influence the risk of developing AD.[101,102] However, several studies of AD (such as PREVENT-AD and ADNI) have recruited well-educated individuals that possess a mean education of 15.4 ± 3.4 years, which may not be representative of the general population.[181] Thus, in order to develop effective screening tools and treatments for AD, it is necessary to establish cohorts that reflect the ethnocultural and socioeconomic diversity of the general population.

4.2.6 The grand picture of AD

Overall, in the grand picture of AD, it is likely that microglia and astrocytes are the key players that detect the initial brain changes occurring during the pre-symptomatic stage of AD. Thus, it is possible that an increase in OPN synthesis and secretion by disease-associated microglia might be the triggering factor that is needed to promote brain repair following the earliest signs of brain injury in AD.[173] Subsequently, we postulate that OPN may promote an increase in IGFBP2 secretion by astrocytes,[205,206] which therefore, may inhibit IGF degradation and facilitate IGF transport, ultimately enhancing IGF signaling (particularly in the hippocampus). Hence, in the end, we hypothesize that IGFBP2 may partially mediate and/or potentiate some of the neuroprotective effects of OPN (Aβ clearance, synaptic/axonal regeneration, neuronal survival, etc).[158,161-163,207] In fact, in the PREVENT-AD cohort, we observed a positive relationship between CSF IGFBP2 and CSF OPN (data not shown, $R^2 = 0.24$, $\beta = 1.13$, $P = 2.60 \times 10^{-7}$). Furthermore, when pooling AD and control brains from the QFP cohort, we also discovered a positive correlation between IGFBP2 and OPN protein levels in the frontal cortex (data not shown, $R^2 = 0.03$, $\beta = 0.209$, $P = 0.024$). However, further experiments are needed to ascertain the cause-and-effect relationship between IGFBP2, OPN and AD pathology.

4.3 Future Directions

4.3.1 Post-mortem studies on autopsied AD brains

Although we have quantified IGFBP2 and OPN in the frontal cortex of autopsy-confirmed AD brains, the next step would be to examine these proteins in temporal and parietal brain regions that are particularly vulnerable to early AD pathology, such as the entorhinal cortex, hippocampus, fusiform gyrus, piriform cortex, precuneus and inferior parietal lobule. However, as previously mentioned, unfortunately, these tissues remain scarce and were unavailable for the QFP cohort. Thus, we would have to acquire these tissues from an independent cohort to run these analyses.

4.3.2 Mouse models of AD and acute brain injury

Although *IGFBP2* gene expression is elevated in the cortex of AD (TASTPM) and tau transgenic mice (P301L) during late life,[208] it would be interesting to cross *IGFBP2*$^{KO/KO}$ mice to 3xTg mice, which possess mutations in *APP*, *PSEN1* and *MAPT*.[209] It would be in our interest to examine whether the absence of IGFBP2 accelerates the disease process in 3xTg * IGFBP2$^{KO/KO}$ mice, compared to 3xTg mice. In an independent set of experiments, we would perform intracerebroventricular (ICV) injections of neutralizing anti-IGFBP2 antibodies in 3xTg mice alone. We hypothesize that IGFBP2 deficiency and neutralization may result in insufficient IGF-mediated compensation. In order to verify our observations, in a subsequent series of experiments, we would perform ICV injections of IGFBP2 in 3xTg * IGFBP2$^{KO/KO}$ mice to examine whether IGF signaling could be restored. In the end, although AD mouse models are a practical way to study amyloid and tau pathologies, it is important to bear in mind that these mouse models reflect the familial form of AD and may not be representative of sporadic AD.[209,210] Furthermore, individuals affected by familial AD only carry a single genetic mutation, and do not carry 3 to 5 mutations, as in several mouse models.[209,210]

Next, considering the protective role of IGFs in rescuing neurons and regenerating axons/synapses following hypoxic-ischemic brain injury and CNS/PNS injury,[211-213] we would like to test whether combination therapies of IGF-1 and IGFBP2 may be effective in reducing neuronal and synaptic loss. In order to accomplish this goal, we would expose rodents to hypoxic-ischemic conditions (and in separate experiments, to unilateral lesions of the entorhinal cortex) and perform ICV

injections of IGF-1 and IGFBP2 prior (prevention) or following (rescue) injury to one hemisphere. Control groups of rodents would receive saline or IGF-1 injections. We hypothesize that IGF-1 and IGFBP2 together would rescue neuronal and synaptic loss to the greatest extent, as IGFBP2 would prevent IGF degradation and enhance the transport of IGFs to their receptors.

Since OPN is known to be cleaved by several proteases such as MMPs (2,3,7,9), thrombin, enterokinase, cathepsin D and plasmin, it would be interesting to identify which OPN peptide fragment(s) and/or splice variant(s) confer neuroprotection to OPN.[214] This would be an intriguing question to answer, since different OPN fragments bind to different receptors and possess distinct biological functions.[214] To this end, we would use mass spectrometry experiments to determine which OPN peptide fragment(s) and/or splice variant(s) are upregulated following hypoxic-ischemic brain injury and verify these observations using entorhinal cortex lesion experiments. Similarly, we would use mass spectrometry to identify which function-modifying post-translational modifications (such as phosphorylation, glycosylation, sulfation and transglutamination) are upregulated on OPN following brain injury.[214] Finally, it would be in our interest to identify which OPN receptor(s) (integrins or CD44 variants) are upregulated following acute brain damage.[172] Taken altogether, we would verify whether treatment with the identified OPN peptide fragment(s) and associated post-translational modifications grants neuroprotection to OPN in rodent models of brain injury.

Finally, considering the remarkable potential of combination therapies of IGF-1 and OPN to regenerate axons and synapses following stroke, spinal cord injuries, and optic nerve transections in rodents,[161-163] it would be fascinating to examine whether incorporating IGFBP2 (to combination therapies of IGF-1 and OPN) would amplify the observed neuroregenerative effects of such therapies. Encouraging support for this hypothesis stems from previous experiments in which the combination of OPN, IGF-1 and IGFBP5 significantly enhanced the growth of smooth muscle cells, compared to treatment with only OPN and IGF-1.[175] Overall, if this effect is replicated in the CNS using OPN, IGF-1 and IGFBP2, such a finding would be instrumental not only for AD, but for other neurological conditions as well.

4.4 Summary

Brain changes associated with AD begin decades before the onset of symptoms. Thus, this pre-symptomatic period provides an excellent window of opportunity to identify vulnerable individuals, unravel mechanism implicated in AD, and develop treatments to delay, slow down and/or prevent AD. To this end, in at-risk individuals, we discovered that IGFBP2 and OPN are valuable markers of future decline in delayed memory and visuospatial abilities, AD-related brain atrophy and CSF Aβ(+)/t-tau(+) profiles. From a longitudinal perspective, we found that IGFBP2 and OPN are effective in predicting a future conversion to AD. Taken altogether, we hope our findings spark interest in therapies targeting insulin-IGF signaling (IGFBP2) and/or neuroinflammation (OPN) during pre-symptomatic AD.

5. References

1. GBD 2019 Dementia Forecasting Collaborators. Estimation of the global prevalence of dementia in 2019 and forecasted prevalence in 2050: an analysis for the Global Burden of Disease Study 2019. *Lancet Public Health*. 2022;7(2):e105-e125.
2. Nandi A, Counts N, Chen S, et al. Global and regional projections of the economic burden of Alzheimer's disease and related dementias from 2019 to 2050: A value of statistical life approach. *EClinicalMedicine*. 2022;51:101580.
3. Alzheimer's Association. 2023 Alzheimer's disease facts and figures. *Alzheimers Dement*. 2023;19(4):1598-1695.
4. Budd Haeberlein S, Aisen PS, Barkhof F, et al. Two Randomized Phase 3 Studies of Aducanumab in Early Alzheimer's Disease. *J Prev Alzheimers Dis*. 2022;9(2):197-210.
5. van Dyck CH, Swanson CJ, Aisen P, et al. Lecanemab in Early Alzheimer's Disease. *N Engl J Med*. 2023;388(1):9-21.
6. Sims JR, Zimmer JA, Evans CD, et al. Donanemab in Early Symptomatic Alzheimer Disease: The TRAILBLAZER-ALZ 2 Randomized Clinical Trial. *JAMA*. 2023;330(6):512-527.
7. Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7(3):280-292.

8. Maurer K, Volk S, Gerbaldo H. Auguste D and Alzheimer's disease. *Lancet*. 1997;349(9064):1546-1549.
9. Hippius H, Neundörfer G. The discovery of Alzheimer's disease. *Dialogues Clin Neurosci*. 2003;5(1):101-108.
10. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition. 2013.
11. 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):189-198.
12. Nasreddine ZS, Phillips NA, Bédirian V, et al. The Montreal Cognitive Assessment, MoCA: a brief screening tool for mild cognitive impairment. *J Am Geriatr Soc*. 2005;53(4):695-699.
13. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology*. 1984;34(7):939-944.
14. Kalaria R. Similarities between Alzheimer's disease and vascular dementia. *J Neurol Sci*. 2002;203-204:29-34.
15. Harciarek M, Jodzio K. Neuropsychological differences between frontotemporal dementia and Alzheimer's disease: a review. *Neuropsychol Rev*. 2005;15(3):131-145.
16. Gnanalingham KK, Byrne EJ, Thornton A, Sambrook MA, Bannister P. Motor and cognitive function in Lewy body dementia: comparison with Alzheimer's and Parkinson's diseases. *J Neurol Neurosurg Psychiatry*. 1997;62(3):243-252.
17. Beach TG, Monsell SE, Phillips LE, Kukull W. Accuracy of the clinical diagnosis of Alzheimer disease at National Institute on Aging Alzheimer Disease Centers, 2005-2010. *J Neuropathol Exp Neurol*. 2012;71(4):266-273.
18. Haass C, Kaether C, Thinakaran G, Sisodia S. Trafficking and proteolytic processing of APP. *Cold Spring Harb Perspect Med*. 2012;2(5):a006270.
19. Milward EA, Papadopoulos R, Fuller SJ, et al. The amyloid protein precursor of Alzheimer's disease is a mediator of the effects of nerve growth factor on neurite outgrowth. *Neuron*. 1992;9(1):129-137.

20. Vassar R, Bennett BD, Babu-Khan S, et al. Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE. *Science*. 1999;286(5440):735-741.
21. Barrow CJ, Yasuda A, Kenny PT, Zagorski MG. Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *J Mol Biol*. 1992;225(4):1075-1093.
22. Kang J, Lemaire HG, Unterbeck A, et al. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*. 1987;325(6106):733-736.
23. Cohen SI, Linse S, Luheshi LM, et al. Proliferation of amyloid-β42 aggregates occurs through a secondary nucleation mechanism. *Proc Natl Acad Sci U S A*. 2013;110(24):9758-9763.
24. Sakono M, Zako T. Amyloid oligomers: formation and toxicity of Abeta oligomers. *FEBS J*. 2010;277(6):1348-1358.
25. Lammich S, Kojro E, Postina R, et al. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. *Proc Natl Acad Sci U S A*. 1999;96(7):3922-3927.
26. Guedes-Dias P, Holzbaur ELF. Axonal transport: Driving synaptic function. *Science*. 2019;366(6462):eaaw9997.
27. Ballatore C, Lee VM, Trojanowski JQ. Tau-mediated neurodegeneration in Alzheimer's disease and related disorders. *Nat Rev Neurosci*. 2007;8(9):663-672.
28. Alonso AD, Grundke-Iqbal I, Barra HS, Iqbal K. Abnormal phosphorylation of tau and the mechanism of Alzheimer neurofibrillary degeneration: sequestration of microtubule-associated proteins 1 and 2 and the disassembly of microtubules by the abnormal tau. *Proc Natl Acad Sci U S A*. 1997;94(1):298-303.
29. Shin RW, Iwaki T, Kitamoto T, Sato Y, Tateishi J. Massive accumulation of modified tau and severe depletion of normal tau characterize the cerebral cortex and white matter of Alzheimer's disease. Demonstration using the hydrated autoclaving method. *Am J Pathol*. 1992;140(4):937-945.
30. Moloney CM, Lowe VJ, Murray ME. Visualization of neurofibrillary tangle maturity in Alzheimer's disease: A clinicopathologic perspective for biomarker research. *Alzheimers Dement*. 2021;17(9):1554-1574.

31. Braak H, Braak E. Staging of Alzheimer's disease-related neurofibrillary changes. *Neurobiol Aging*. 1995;16(3):271-284.
32. Clavaguera F, Bolmont T, Crowther RA, et al. Transmission and spreading of tauopathy in transgenic mouse brain. *Nat Cell Biol*. 2009;11(7):909-913.
33. Liu L, Drouet V, Wu JW, et al. Trans-synaptic spread of tau pathology in vivo. *PLoS One*. 2012;7(2):e31302.
34. Gómez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT. Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci*. 1996;16(14):4491-4500.
35. Van Hoesen GW, Hyman BT, Damasio AR. Entorhinal cortex pathology in Alzheimer's disease. *Hippocampus*. 1991;1(1):1-8.
36. Masliah E, Mallory M, Hansen L, DeTeresa R, Alford M, Terry R. Synaptic and neuritic alterations during the progression of Alzheimer's disease. *Neurosci Lett*. 1994;174(1):67-72.
37. Sze CI, Troncoso JC, Kawas C, Mouton P, Price DL, Martin LJ. Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *J Neuropathol Exp Neurol*. 1997;56(8):933-944.
38. Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: a review of progress. *J Neurol Neurosurg Psychiatry*.
39. Whitehouse PJ, Price DL, Struble RG, Clark AW, Coyle JT, Delon MR. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. *Science*. 1982;215(4537):1237-1239.
40. Akiyama H, Barger S, Barnum S, et al. Inflammation and Alzheimer's disease. *Neurobiol Aging*. 2000;21(3):383-421.
41. Guerreiro R, Wojtas A, Bras J, et al. TREM2 variants in Alzheimer's disease. *N Engl J Med*. 2013;368(2):117-127.
42. Walker KA, Hoogeveen RC, Folsom AR, et al. Midlife systemic inflammatory markers are associated with late-life brain volume: The ARIC study. *Neurology*. 2017;89(22):2262-2270.
43. in t' Veld BA, Ruitenberg A, Hofman A, et al. Nonsteroidal antiinflammatory drugs and the risk of Alzheimer's disease. *N Engl J Med*. 2001;345(21):1515-1521.

44. Aisen PS, Davis KL, Berg JD, et al. A randomized controlled trial of prednisone in Alzheimer's disease. Alzheimer's Disease Cooperative Study. *Neurology*. 2000;54(3):588-593.
45. Thal LJ, Ferris SH, Kirby L, et al. A randomized, double-blind, study of rofecoxib in patients with mild cognitive impairment. *Neuropsychopharmacology*. 2005;30(6):1204-1215.
46. 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.
47. Meyer PF, Savard M, Poirier J, et al. Bi-directional Association of Cerebrospinal Fluid Immune Markers with Stage of Alzheimer's Disease Pathogenesis. *J Alzheimers Dis*. 2018;63(2):577-590.
48. Lund SA, Giachelli CM, Scatena M. The role of osteopontin in inflammatory processes. *J Cell Commun Signal*. 2009;3(3-4):311-322.
49. Blennow K. Cerebrospinal fluid protein biomarkers for Alzheimer's disease. *NeuroRx*. 2004;1(2):213-225.
50. Palmqvist S, Zetterberg H, Mattsson N, et al. Detailed comparison of amyloid PET and CSF biomarkers for identifying early Alzheimer disease. *Neurology*. 2015;85(14):1240-1249.
51. Camporesi E, Nilsson J, Brinkmalm A, et al. Fluid Biomarkers for Synaptic Dysfunction and Loss. *Biomark Insights*. 2020;15:1177271920950319.
52. 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):652-656.
53. Tapiola T, Alafuzoff I, Herukka SK, et al. Cerebrospinal fluid {beta}-amyloid 42 and tau proteins as biomarkers of Alzheimer-type pathologic changes in the brain. *Arch Neurol*. 2009;66(3):382-389.
54. Noble W, Hanger DP, Miller CC, Lovestone S. The importance of tau phosphorylation for neurodegenerative diseases. *Front Neurol*. 2013;4:83.
55. Vanmechelen E, Vanderstichele H, Davidsson P, et al. Quantification of tau phosphorylated at threonine 181 in human cerebrospinal fluid: a sandwich ELISA with a synthetic phosphopeptide for standardization. *Neurosci Lett*. 2000;285(1):49-52.

56. Kohnken R, Buerger K, Zinkowski R, et al. Detection of tau phosphorylated at threonine 231 in cerebrospinal fluid of Alzheimer's disease patients. *Neurosci Lett*. 2000;287(3):187-190.
57. Milà-Alomà M, Ashton NJ, Shekari M, et al. Plasma p-tau231 and p-tau217 as state markers of amyloid-β pathology in preclinical Alzheimer's disease. *Nat Med*. 2022;28(9):1797-1801.
58. Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol. 2006;5(3):228-234.
59. Scheff SW, Price DA, Schmitt FA, DeKosky ST, Mufson EJ. Synaptic alterations in CA1 in mild Alzheimer disease and mild cognitive impairment. *Neurology*. 2007;68(18):1501-1508.
60. Terry RD, Masliah E, Salmon DP, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol*. 1991;30(4):572-580.
61. Öhrfelt A, Brinkmalm A, Dumurgier J, et al. The pre-synaptic vesicle protein synaptotagmin is a novel biomarker for Alzheimer's disease. *Alzheimers Res Ther*. 2016;8(1):41.
62. Brinkmalm A, Brinkmalm G, Honer WG, et al. SNAP-25 is a promising novel cerebrospinal fluid biomarker for synapse degeneration in Alzheimer's disease. *Mol Neurodegener*. 2014;9:53.
63. Zhang H, Therriault J, Kang MS, et al. Cerebrospinal fluid synaptosomal-associated protein 25 is a key player in synaptic degeneration in mild cognitive impairment and Alzheimer's disease. *Alzheimers Res Ther*. 2018;10(1):80.
64. Sandelius Å, Portelius E, Källén Å, 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.
65. Kester MI, Teunissen CE, Crimmins DL, et al. Neurogranin as a Cerebrospinal Fluid Biomarker for Synaptic Loss in Symptomatic Alzheimer Disease. *JAMA Neurol*. 2015;72(11):1275-1280.

66. Portelius E, Zetterberg H, Skillbäck T, et al. Cerebrospinal fluid neurogranin: relation to cognition and neurodegeneration in Alzheimer's disease. *Brain*. 2015;138(Pt 11):3373-3385.
67. Masliah E, Mallory M, Alford M, et al. Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease. *Neurology*. 2001;56(1):127-129.
68. Greber S, Lubec G, Cairns N, Fountoulakis M. Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease. *Electrophoresis*. 1999;20(4-5):928-934.
69. Davidsson P, Blennow K. Neurochemical dissection of synaptic pathology in Alzheimer's disease. *Int Psychogeriatr*. 1998;10(1):11-23.
70. Tible M, Sandelius Å, Höglund K, et al. Dissection of synaptic pathways through the CSF biomarkers for predicting Alzheimer disease. *Neurology*. 2020;95(8):e953-e961.
71. Milà-Alomà M, Brinkmalm A, Ashton NJ, et al. CSF Synaptic Biomarkers in the Preclinical Stage of Alzheimer Disease and Their Association With MRI and PET: A Cross-sectional Study. *Neurology*. 2021;97(21):e2065-e2078.
72. Klunk WE, Engler H, Nordberg A, et al. Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. *Ann Neurol*. 2004;55(3):306-319.
73. Rabinovici GD, Furst AJ, O'Neil JP, et al. 11C-PIB PET imaging in Alzheimer disease and frontotemporal lobar degeneration. *Neurology*. 2007;68(15):1205-1212.
74. Okello A, Koivunen J, Edison P, et al. Conversion of amyloid positive and negative MCI to AD over 3 years: an 11C-PIB PET study. *Neurology*. 2009;73(10):754-760.
75. Landau SM, Breault C, Joshi AD, et al. Amyloid-β imaging with Pittsburgh compound B and florbetapir: comparing radiotracers and quantification methods. *J Nucl Med*. 2013;54(1):70-77.
76. Clark CM, Pontecorvo MJ, Beach TG, et al. Cerebral PET with florbetapir compared with neuropathology at autopsy for detection of neuritic amyloid-β plaques: a prospective cohort study. *Lancet Neurol*. 2012;11(8):669-678.
77. Chételat G, La Joie R, Villain N, et al. Amyloid imaging in cognitively normal individuals, at-risk populations and preclinical Alzheimer's disease. *Neuroimage Clin*. 2013;2:356-365.
78. Schöll M, Lockhart SN, Schonhaut DR, et al. PET Imaging of Tau Deposition in the Aging Human Brain. *Neuron*. 2016;89(5):971-982.

79. Fleisher AS, Pontecorvo MJ, Devous MD Sr, et al. Positron Emission Tomography Imaging With [18F]flortaucipir and Postmortem Assessment of Alzheimer Disease Neuropathologic Changes. *JAMA Neurol*. 2020;77(7):829-839.
80. Phelps ME, Huang SC, Hoffman EJ, Selin C, Sokoloff L, Kuhl DE. Tomographic measurement of local cerebral glucose metabolic rate in humans with (F-18)2-fluoro-2-deoxy-D-glucose: validation of method. *Ann Neurol*. 1979;6(5):371-388.
81. Jack CR Jr, Knopman DS, Jagust WJ, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol*. 2010;9(1):119-128.
82. Rocher AB, Chapon F, Blaizot X, Baron JC, Chavoix C. Resting-state brain glucose utilization as measured by PET is directly related to regional synaptophysin levels: a study in baboons. *Neuroimage*. 2003;20(3):1894-1898.
83. Hoffman JM, Welsh-Bohmer KA, Hanson M, et al. FDG PET imaging in patients with pathologically verified dementia. *J Nucl Med*. 2000;41(11):1920-1928.
84. Pagani M, Nobili F, Morbelli S, et al. Early identification of MCI converting to AD: a FDG PET study. *Eur J Nucl Med Mol Imaging*. 2017;44(12):2042-2052.
85. Jack CR Jr, Petersen RC, Xu YC, et al. Prediction of AD with MRI-based hippocampal volume in mild cognitive impairment. *Neurology*. 1999;52(7):1397-1403.
86. Nelson MD, Saykin AJ, Flashman LA, Riordan HJ. Hippocampal volume reduction in schizophrenia as assessed by magnetic resonance imaging: a meta-analytic study. *Arch Gen Psychiatry*. 1998;55(5):433-440.
87. Dickerson BC, Bakkour A, Salat DH, et al. The cortical signature of Alzheimer's disease: regionally specific cortical thinning relates to symptom severity in very mild to mild AD dementia and is detectable in asymptomatic amyloid-positive individuals. *Cereb Cortex*. 2009;19(3):497-510.
88. Schwarz CG, Gunter JL, Wiste HJ, et al. A large-scale comparison of cortical thickness and volume methods for measuring Alzheimer's disease severity. *Neuroimage Clin*. 2016;11:802-812.
89. Bateman RJ, Xiong C, Benzinger TL, et al. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N Engl J Med*. 2012;367(9):795-804.
90. Jack CR Jr, Bennett DA, Blennow K, et al. A/T/N: An unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology*. 2016;87(5):539-547.

91. Pike CJ, Carroll JC, Rosario ER, Barron AM. Protective actions of sex steroid hormones in Alzheimer's disease. *Front Neuroendocrinol*. 2009;30(2):239-258.
92. Vegeto E, Benedusi V, Maggi A. Estrogen anti-inflammatory activity in brain: a therapeutic opportunity for menopause and neurodegenerative diseases. *Front Neuroendocrinol*. 2008;29(4):507-519.
93. Tang MX, Jacobs D, Stern Y, et al. Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet*. 1996;348(9025):429-432.
94. Pourhadi N, Mørch LS, Holm EA, Torp-Pedersen C, Meaidi A. Menopausal hormone therapy and dementia: nationwide, nested case-control study. *BMJ*. 2023;381:e072770.
95. Whitmer RA, Quesenberry CP, Zhou J, Yaffe K. Timing of hormone therapy and dementia: the critical window theory revisited. *Ann Neurol*. 2011;69(1):163-169.
96. Chen C, Zissimopoulos JM. Racial and ethnic differences in trends in dementia prevalence and risk factors in the United States. *Alzheimers Dement (N Y)*. 2018;4:510-520.
97. Bekris LM, Yu CE, Bird TD, Tsuang DW. Genetics of Alzheimer disease. *J Geriatr Psychiatry Neurol*. 2010;23(4):213-227.
98. Lautenschlager NT, Cupples LA, Rao VS, et al. Risk of dementia among relatives of Alzheimer's disease patients in the MIRAGE study: What is in store for the oldest old?. *Neurology*. 1996;46(3):641-650.
99. Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet*. 1993;342(8873):697-699.
100. Bellenguez C, Küçükali F, Jansen IE, et al. New insights into the genetic etiology of Alzheimer's disease and related dementias. *Nat Genet*. 2022;54(4):412-436.
101. Livingston G, Huntley J, Sommerlad A, et al. Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. *Lancet*. 2020;396(10248):413-446
102. Stern Y, Gurland B, Tatemichi TK, Tang MX, Wilder D, Mayeux R. Influence of education and occupation on the incidence of Alzheimer's disease. *JAMA*. 1994;271(13):1004-1010..
103. Stern Y. Cognitive reserve in ageing and Alzheimer's disease. *Lancet Neurol*. 2012;11(11):1006-1012.
104. Steiner P. Brain Fuel Utilization in the Developing Brain. *Ann Nutr Metab*. 2019;75 Suppl 1:8-18.

105. Honig LS, Tang MX, Albert S, et al. Stroke and the risk of Alzheimer disease. *Arch Neurol*. 2003;60(12):1707-1712.
106. Mahley RW. Apolipoprotein E: from cardiovascular disease to neurodegenerative disorders. *J Mol Med (Berl)*. 2016;94(7):739-746.
107. Whitmer RA, Sidney S, Selby J, Johnston SC, Yaffe K. Midlife cardiovascular risk factors and risk of dementia in late life. *Neurology*. 2005;64(2):277-281.
108. Wolozin B, Kellman W, Ruosseau P, Celesia GG, Siegel G. Decreased prevalence of Alzheimer disease associated with 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors. *Arch Neurol*. 2000;57(10):1439-1443.
109. Feldman HH, Doody RS, Kivipelto M, et al. Randomized controlled trial of atorvastatin in mild to moderate Alzheimer disease: LEADe. *Neurology*. 2010;74(12):956-964.
110. Sano M, Bell KL, Galasko D, et al. A randomized, double-blind, placebo-controlled trial of simvastatin to treat Alzheimer disease. *Neurology*. 2011;77(6):556-563.
111. Skoog I, Lernfelt B, Landahl S, et al. 15-year longitudinal study of blood pressure and dementia. *Lancet*. 1996;347(9009):1141-1145.
112. Qiu C, Winblad B, Fratiglioni L. The age-dependent relation of blood pressure to cognitive function and dementia. *Lancet Neurol*. 2005;4(8):487-499.
113. Walker KA, Sharrett AR, Wu A, et al. Association of Midlife to Late-Life Blood Pressure Patterns With Incident Dementia. *JAMA*. 2019;322(6):535-545.
114. Forette F, Seux ML, Staessen JA, et al. The prevention of dementia with antihypertensive treatment: new evidence from the Systolic Hypertension in Europe (Syst-Eur) study. *Arch Intern Med*. 2002;162(18):2046-2052.
115. Khachaturian AS, Zandi PP, Lyketsos CG, et al. Antihypertensive medication use and incident Alzheimer disease: the Cache County Study. *Arch Neurol*. 2006;63(5):686-692.
116. Albanese E, Launer LJ, Egger M, et al. Body mass index in midlife and dementia: Systematic review and meta-regression analysis of 589,649 men and women followed in longitudinal studies. *Alzheimers Dement (Amst)*. 2017;8:165-178.
117. Ott A, Stolk RP, van Harskamp F, Pols HA, Hofman A, Breteler MM. Diabetes mellitus and the risk of dementia: The Rotterdam Study. *Neurology*. 1999;53(9):1937-1942.
118. Moran C, Phan TG, Chen J, et al. Brain atrophy in type 2 diabetes: regional distribution and influence on cognition. *Diabetes Care*. 2013;36(12):4036-4042

119. Steen E, Terry BM, J Rivera E, et al. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease–is this type 3 diabetes?. *J Alzheimers Dis*. 2005;7(1):63-80.
120. Moloney AM, Griffin RJ, Timmons S, O'Connor R, Ravid R, O'Neill C. Defects in IGF-1 receptor, insulin receptor and IRS-1/2 in Alzheimer's disease indicate possible resistance to IGF-1 and insulin signalling. *Neurobiol Aging*. 2010;31(2):224-243.
121. Talbot K, Wang HY, Kazi H, et al. Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resistance, IRS-1 dysregulation, and cognitive decline. *J Clin Invest*. 2012;122(4):1316-1338.
122. Kar S, Poirier J, Guevara J, et al. Cellular distribution of insulin-like growth factor-II/mannose-6-phosphate receptor in normal human brain and its alteration in Alzheimer's disease pathology. *Neurobiol Aging*. 2006;27(2):199-210.
123. Reger MA, Watson GS, Green PS, et al. Intranasal insulin improves cognition and modulates beta-amyloid in early AD. *Neurology*. 2008;70(6):440-448.
124. Craft S, Baker LD, Montine TJ, et al. Intranasal insulin therapy for Alzheimer disease and amnestic mild cognitive impairment: a pilot clinical trial. *Arch Neurol*. 2012;69(1):29-38.
125. Reger MA, Watson GS, Green PS, et al. Intranasal insulin administration dose-dependently modulates verbal memory and plasma amyloid-beta in memory-impaired older adults. *J Alzheimers Dis*. 2008;13(3):323-331.
126. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL. Sniffing neuropeptides: a transnasal approach to the human brain. *Nat Neurosci*. 2002;5(6):514-516.
127. Loughrey DG, Lavecchia S, Brennan S, Lawlor BA, Kelly ME. The Impact of the Mediterranean Diet on the Cognitive Functioning of Healthy Older Adults: A Systematic Review and Meta-Analysis. *Adv Nutr*. 2017;8(4):571-586.
128. Ahlskog JE, Geda YE, Graff-Radford NR, Petersen RC. Physical exercise as a preventive or disease-modifying treatment of dementia and brain aging. *Mayo Clin Proc*. 2011;86(9):876-884.
129. Zotcheva E, Bergh S, Selbæk G, et al. Midlife Physical Activity, Psychological Distress, and Dementia Risk: The HUNT Study. *J Alzheimers Dis*. 2018;66(2):825-833.

130. Kivimäki M, Singh-Manoux A, Pentti J, et al. Physical inactivity, cardiometabolic disease, and risk of dementia: an individual-participant meta-analysis. *BMJ*. 2019;365:l1495.
131. Bubu OM, Brannick M, Mortimer J, et al. Sleep, Cognitive impairment, and Alzheimer's disease: A Systematic Review and Meta-Analysis. *Sleep*. 2017;40(1):10.1093/sleep/zsw032.
132. Ohara T, Honda T, Hata J, et al. Association Between Daily Sleep Duration and Risk of Dementia and Mortality in a Japanese Community. *J Am Geriatr Soc*. 2018;66(10):1911-1918.
133. Graves AB, van Duijn CM, Chandra V, et al. Alcohol and tobacco consumption as risk factors for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group. *Int J Epidemiol*. 1991;20 Suppl 2:S48-S57.
134. Ott A, Slooter AJ, Hofman A, et al. Smoking and risk of dementia and Alzheimer's disease in a population-based cohort study: the Rotterdam Study. *Lancet*. 1998;351(9119):1840-1843.
135. Peters R, Ee N, Peters J, Booth A, Mudway I, Anstey KJ. Air Pollution and Dementia: A Systematic Review. *J Alzheimers Dis*. 2019;70(s1):S145-S163.
136. Schwarzinger M, Pollock BG, Hasan OSM, Dufouil C, Rehm J; QalyDays Study Group. Contribution of alcohol use disorders to the burden of dementia in France 2008-13: a nationwide retrospective cohort study. *Lancet Public Health*. 2018;3(3):e124-e132.
137. Ilomaki J, Jokanovic N, Tan EC, Lonnroos E. Alcohol Consumption, Dementia and Cognitive Decline: An Overview of Systematic Reviews. *Curr Clin Pharmacol*. 2015;10(3):204-212.
138. Sommerlad A, Ruegger J, Singh-Manoux A, Lewis G, Livingston G. Marriage and risk of dementia: systematic review and meta-analysis of observational studies. *J Neurol Neurosurg Psychiatry*. 2018;89(3):231-238.
139. Elser H, Horváth-Puhó E, Gradus JL, et al. Association of Early-, Middle-, and Late-Life Depression With Incident Dementia in a Danish Cohort. *JAMA Neurol*. 2023;e232309.
140. Bartels C, Wagner M, Wolfsgruber S, Ehrenreich H, Schneider A; Alzheimer's Disease Neuroimaging Initiative. Impact of SSRI Therapy on Risk of Conversion From Mild

Cognitive Impairment to Alzheimer's Dementia in Individuals With Previous Depression. *Am J Psychiatry*. 2018;175(3):232-241.

141. Fann JR, Ribe AR, Pedersen HS, et al. Long-term risk of dementia among people with traumatic brain injury in Denmark: a population-based observational cohort study. *Lancet Psychiatry*. 2018;5(5):424-431.
142. Mortimer JA, van Duijn CM, Chandra V, et al. Head trauma as a risk factor for Alzheimer's disease: a collaborative re-analysis of case-control studies. EURODEM Risk Factors Research Group. *Int J Epidemiol*. 1991;20 Suppl 2:S28-S35.
143. Yiannopoulou KG, Papageorgiou SG. Current and Future Treatments in Alzheimer Disease: An Update. *J Cent Nerv Syst Dis*. 2020;12:1179573520907397.
144. Rogers SL, Doody RS, Pratt RD, Ieni JR. Long-term efficacy and safety of donepezil in the treatment of Alzheimer's disease: final analysis of a US multicentre open-label study. *Eur Neuropsychopharmacol*. 2000;10(3):195-203.
145. Poirier J, Delisle MC, Quirion R, 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):12260-12264.
146. Rogawski MA, Wenk GL. The neuropharmacological basis for the use of memantine in the treatment of Alzheimer's disease. *CNS Drug Rev*. 2003;9(3):275-308.
147. Winblad B, Poritis N. Memantine in severe dementia: results of the 9M-Best Study (Benefit and efficacy in severely demented patients during treatment with memantine). *Int J Geriatr Psychiatry*. 1999;14(2):135-146.
148. McShane R, Westby MJ, Roberts E, et al. Memantine for dementia. *Cochrane Database Syst Rev*. 2019;3(3):CD003154.
149. Egan MF, Kost J, Tariot PN, et al. Randomized Trial of Verubecestat for Mild-to-Moderate Alzheimer's Disease. *N Engl J Med*. 2018;378(18):1691-1703.
150. Doody RS, Raman R, Farlow M, et al. A phase 3 trial of semagacestat for treatment of Alzheimer's disease. *N Engl J Med*. 2013;369(4):341-350.
151. Doody RS, Thomas RG, Farlow M, et al. Phase 3 trials of solanezumab for mild-to-moderate Alzheimer's disease. *N Engl J Med*. 2014;370(4):311-321.
152. Sperling RA, Donohue MC, Raman R, et al. Trial of Solanezumab in Preclinical Alzheimer's Disease. *N Engl J Med*. 2023;10.1056/NEJMoa2305032.

153. Beach TG, Wilson JR, Sue LI, et al. Circle of Willis atherosclerosis: association with Alzheimer's disease, neuritic plaques and neurofibrillary tangles. *Acta Neuropathol*. 2007;113(1):13-21.
154. Greenberg SM, Bacskai BJ, Hernandez-Guillamon M, Pruzin J, Sperling R, van Veluw SJ. Cerebral amyloid angiopathy and Alzheimer disease - one peptide, two pathways. *Nat Rev Neurol*. 2020;16(1):30-42.
155. Roher AE, Debbins JP, Malek-Ahmadi M, et al. Cerebral blood flow in Alzheimer's disease. *Vasc Health Risk Manag*. 2012;8:599-611.
156. Zenaro E, Piacentino G, Constantin G. The blood-brain barrier in Alzheimer's disease. *Neurobiol Dis*. 2017;107:41-56.
157. Park SE, Dantzer R, Kelley KW, McCusker RH. Central administration of insulin-like growth factor-I decreases depressive-like behavior and brain cytokine expression in mice. *J Neuroinflammation*. 2011;8:12.
158. Meller R, Stevens SL, Minami M, et al. Neuroprotection by osteopontin in stroke. *J Cereb Blood Flow Metab*. 2005;25(2):217-225.
159. Guan J, Williams CE, Skinner SJ, Mallard EC, Gluckman PD. The effects of insulin-like growth factor (IGF)-1, IGF-2, and des-IGF-1 on neuronal loss after hypoxic-ischemic brain injury in adult rats: evidence for a role for IGF binding proteins. *Endocrinology*. 1996;137(3):893-898.
160. Dai J, Peng L, Fan K, et al. Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. *Oncogene*. 2009;28(38):3412-3422.
161. Liu Y, Wang X, Li W, et al. A Sensitized IGF1 Treatment Restores Corticospinal Axon-Dependent Functions. *Neuron*. 2017;95(4):817-833.e4.
162. Duan X, Qiao M, Bei F, Kim IJ, He Z, Sanes JR. Subtype-specific regeneration of retinal ganglion cells following axotomy: effects of osteopontin and mTOR signaling. *Neuron*. 2015;85(6):1244-1256.
163. Anderson MA, O'Shea TM, Burda JE, et al. Required growth facilitators propel axon regeneration across complete spinal cord injury. *Nature*. 2018;561(7723):396-400.
164. Gluckman P, Klempt N, Guan J, et al. A role for IGF-1 in the rescue of CNS neurons following hypoxic-ischemic injury. *Biochem Biophys Res Commun*. 1992;182(2):593-599.

165. Klempt ND, Klempt M, Gunn AJ, Singh K, Gluckman PD. Expression of insulin-like growth factor-binding protein 2 (IGF-BP 2) following transient hypoxia-ischemia in the infant rat brain. *Brain Res Mol Brain Res*. 1992;15(1-2):55-61.
166. Beilharz EJ, Russo VC, Butler G, et al. Co-ordinated and cellular specific induction of the components of the IGF/IGFBP axis in the rat brain following hypoxic–ischemic injury. *Brain Res Mol Brain Res*. 1998;59(2):119-134.
167. Sandberg Nordqvist AC, Von Holst H, Holmin S, Sara VR, Bellander BM, Schalling M. Increase of insulin-like growth factor (IGF)-1, IGF binding protein-2 and− 4 mRNAs following cerebral contusion. *Brain Res Mol Brain Res*. 1996;38(2):285-293.
168. Walter HJ, Berry M, Hill DJ, Logan A. Spatial and temporal changes in the insulin-like growth factor (IGF) axis indicate autocrine/paracrine actions of IGF-I within wounds of the rat brain. *Endocrinology*. 1997;138(7):3024-3034.
169. Fletcher L, Isgor E, Sprague S, et al. Spatial distribution of insulin-like growth factor binding protein-2 following hypoxic-ischemic injury. *BMC Neurosci*. 2013;14:158.
170. Breese CR, D'Costa A, Rollins YD, et al. Expression of insulin-like growth factor-1 (IGF-1) and IGF-binding protein 2 (IGF-BP2) in the hippocampus following cytotoxic lesion of the dentate gyrus. *J Comp Neurol*. 1996;369(3):388-404.
171. Kar S, Baccichet A, Quirion R, Poirier J. Entorhinal cortex lesion induces differential responses in [125I]insulin-like growth factor I, [125I]insulin-like growth factor II and [125I]insulin receptor binding sites in the rat hippocampal formation. *Neuroscience*. 1993;55(1):69-80.
172. Ellison JA, Velier JJ, Spera P, et al. Osteopontin and its integrin receptor alpha(v)beta3 are upregulated during formation of the glial scar after focal stroke. *Stroke*. 1998;29(8):1698-1707.
173. Keren-Shaul H, Spinrad A, Weiner A, et al. A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease. *Cell*. 2017;169(7):1276-1290.e17.
174. Xi G, Wai C, DeMambro V, Rosen CJ, Clemmons DR. IGFBP-2 directly stimulates osteoblast differentiation. *J Bone Miner Res*. 2014;29(11):2427-2438.
175. Nam TJ, Busby WH Jr, Rees C, Clemmons DR. Thrombospondin and osteopontin bind to insulin-like growth factor (IGF)-binding protein-5 leading to an alteration in IGF-I-stimulated cell growth. *Endocrinology*. 2000;141(3):1100-1106.

176. Salloway S, Farlow M, McDade E, et al. A trial of gantenerumab or solanezumab in dominantly inherited Alzheimer's disease. *Nat Med*. 2021;27(7):1187-1196.
177. Breitner JC. Clinical genetics and genetic counseling in Alzheimer disease. *Ann Intern Med*. 1991;115(8):601-606.
178. Mills SM, Mallmann J, Santacruz AM, et al. Preclinical trials in autosomal dominant AD: implementation of the DIAN-TU trial. *Rev Neurol (Paris)*. 2013;169(10):737-743.
179. Reiman EM, Langbaum JB, Fleisher AS, et al. Alzheimer's Prevention Initiative: a plan to accelerate the evaluation of presymptomatic treatments. *J Alzheimers Dis*. 2011;26 Suppl 3(Suppl 3):321-329.
180. Yang Y, Arseni D, Zhang W, et al. Cryo-EM structures of amyloid-β 42 filaments from human brains. *Science*. 2022;375(6577):167-172.
181. Tremblay-Mercier J, Madjar C, Das S, et al. Open science datasets from PREVENT-AD, a longitudinal cohort of pre-symptomatic Alzheimer's disease. *Neuroimage Clin*. 2021;31:102733.
182. Duff K, Humphreys Clark JD, O'Bryant SE, Mold JW, Schiffer RB, Sutker PB. Utility of the RBANS in detecting cognitive impairment associated with Alzheimer's disease: sensitivity, specificity, and positive and negative predictive powers. *Arch Clin Neuropsychol*. 2008;23(5):603-612.
183. Lafaille-Magnan ME, Poirier J, Etienne P, et al. Odor identification as a biomarker of preclinical AD in older adults at risk. *Neurology*. 2017;89(4):327-335.
184. Koch G, Casula EP, Bonnì S, et al. Precuneus magnetic stimulation for Alzheimer's disease: a randomized, sham-controlled trial. *Brain*. 2022;145(11):3776-3786.
185. Doré S, Kar S, Quirion R. Insulin-like growth factor I protects and rescues hippocampal neurons against beta-amyloid- and human amylin-induced toxicity. *Proc Natl Acad Sci U S A*. 1997;94(9):4772-4777.
186. Wheatcroft SB, Kearney MT, Shah AM, et al. IGF-binding protein-2 protects against the development of obesity and insulin resistance. *Diabetes*. 2007;56(2):285-294.
187. Takeo C, Ikeda K, Horie-Inoue K, Inoue S. Identification of Igf2, Igfbp2 and Enpp2 as estrogen-responsive genes in rat hippocampus. *Endocr J*. 2009;56(1):113-120.

188. Azar WJ, Zivkovic S, Werther GA, Russo VC. IGFBP-2 nuclear translocation is mediated by a functional NLS sequence and is essential for its pro-tumorigenic actions in cancer cells. *Oncogene*. 2014;33(5):578-588.
189. Hohman TJ, Bell SP, Jefferson AL; Alzheimer's Disease Neuroimaging Initiative. The role of vascular endothelial growth factor in neurodegeneration and cognitive decline: exploring interactions with biomarkers of Alzheimer disease. *JAMA Neurol*. 2015;72(5):520-529.
190. Chan JL, Reeves TM, Phillips LL. Osteopontin expression in acute immune response mediates hippocampal synaptogenesis and adaptive outcome following cortical brain injury. *Exp Neurol*. 2014;261:757-771.
191. Kinney JW, Bemiller SM, Murtishaw AS, Leisgang AM, Salazar AM, Lamb BT. Inflammation as a central mechanism in Alzheimer's disease. *Alzheimers Dement (N Y)*. 2018;4:575-590.
192. Kahles F, Findeisen HM, Bruemmer D. Osteopontin: A novel regulator at the cross roads of inflammation, obesity and diabetes. *Mol Metab*. 2014;3(4):384-393.
193. Vom Berg J, Prokop S, Miller KR, et al. Inhibition of IL-12/IL-23 signaling reduces Alzheimer's disease-like pathology and cognitive decline. *Nat Med*. 2012;18(12):1812-1819.
194. Brigas HC, Ribeiro M, Coelho JE, et al. IL-17 triggers the onset of cognitive and synaptic deficits in early stages of Alzheimer's disease. *Cell Rep*. 2021;36(9):109574.
195. Kiyota T, Ingraham KL, Swan RJ, Jacobsen MT, Andrews SJ, Ikezu T. AAV serotype 2/1-mediated gene delivery of anti-inflammatory interleukin-10 enhances neurogenesis and cognitive function in APP+PS1 mice. *Gene Ther*. 2012;19(7):724-733.
196. Guillot-Sestier MV, Doty KR, Gate D, et al. Il10 deficiency rebalances innate immunity to mitigate Alzheimer-like pathology. *Neuron*. 2015;85(3):534-548.
197. Taylor JM, Minter MR, Newman AG, Zhang M, Adlard PA, Crack PJ. Type-1 interferon signaling mediates neuro-inflammatory events in models of Alzheimer's disease. *Neurobiol Aging*. 2014;35(5):1012-1023.
198. Roy ER, Wang B, Wan YW, et al. Type I interferon response drives neuroinflammation and synapse loss in Alzheimer disease. *J Clin Invest*. 2020;130(4):1912-1930.

199. Jiang X, Liu B, Nie Z, et al. The role of m6A modification in the biological functions and diseases. *Signal Transduct Target Ther*. 2021;6(1):74.
200. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet*. 2012;13(4):227-232.
201. Cappellano G, Vecchio D, Magistrelli L, et al. The *Yin-Yang* of osteopontin in nervous system diseases: damage *versus* repair. *Neural Regen Res*. 2021;16(6):1131-1137.
202. Comi C, Carecchio M, Chiocchetti A, et al. Osteopontin is increased in the cerebrospinal fluid of patients with Alzheimer's disease and its levels correlate with cognitive decline. *J Alzheimers Dis*. 2010;19(4):1143-1148.
203. Beach TG, Malek-Ahmadi M. Alzheimer's Disease Neuropathological Comorbidities are Common in the Younger-Old. *J Alzheimers Dis*. 2021;79(1):389-400.
204. Weiner MW, Veitch DP, Miller MJ, et al. Increasing participant diversity in AD research: Plans for digital screening, blood testing, and a community-engaged approach in the Alzheimer's Disease Neuroimaging Initiative 4. *Alzheimers Dement*. 2023;19(1):307-317.
205. Lee WH, Michels KM, Bondy CA. Localization of insulin-like growth factor binding protein-2 messenger RNA during postnatal brain development: correlation with insulin-like growth factors I and II. *Neuroscience*. 1993;53(1):251-265.
206. Khan S, Lu X, Huang Q, et al. IGFBP2 plays an essential role in cognitive development during early life. *Adv Sci (Weinh)*. 2019;6(23):1901152.
207. Rentsendorj A, Sheyn J, Fuchs DT, et al. A novel role for osteopontin in macrophage-mediated amyloid-β clearance in Alzheimer's models. *Brain Behav Immun*. 2018;67:163-180.
208. Bonham LW, Geier EG, Steele NZ, et al. Insulin-like growth factor binding protein 2 is associated with biomarkers of Alzheimer's disease pathology and shows differential expression in transgenic mice. *Front Neurosci*. 2018;12:476.
209. Sterniczuk R, Antle MC, Laferla FM, Dyck RH. Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 2. Behavioral and cognitive changes. *Brain Res*. 2010;1348:149-155.

210. Forner S, Kawauchi S, Balderrama-Gutierrez G, et al. Systematic phenotyping and characterization of the 5xFAD mouse model of Alzheimer's disease. *Sci Data*. 2021;8(1):270.
211. Kanje M, Skottner A, Sjöberg J, Lundborg G. Insulin-like growth factor I (IGF-I) stimulates regeneration of the rat sciatic nerve. *Brain Res*. 1989;486(2):396-398.
212. Near SL, Whalen LR, Miller JA, Ishii DN. Insulin-like growth factor II stimulates motor nerve regeneration. *Proc Natl Acad Sci U S A*. 1992;89(24):11716-11720.
213. Guthrie KM, Nguyen T, Gall CM. Insulin-like growth factor-1 mRNA is increased in deafferented hippocampus: spatiotemporal correspondence of a trophic event with axon sprouting. *J Comp Neurol*. 1995;352(1):147-160.
214. Anborgh PH, Mutrie JC, Tuck AB, Chambers AF. Pre- and post-translational regulation of osteopontin in cancer. *J Cell Commun Signal*. 2011;5(2):111-122.

Appendix A: Supplementary material for manuscript 1

Supplementary methods

ADNI-1 cohort

CSF mass spectrometry data

CSF samples from a subset of the same ADNI-1 participants (n = 86 cognitively unaffected individuals, n = 135 individuals with MCI) were also analyzed using multiple reaction monitoring (MRM) mass spectrometry. 221 proteins were examined, across 567 peptides. Two transitions per peptide were monitored. Two distinct peptides were quantified for IGFBP2: HGLYNLK and LIQGAPTIR. The results are expressed in arbitrary signal intensity units, which are on a natural log scale. A thorough discussion of the methodology has been previously reported.[1]

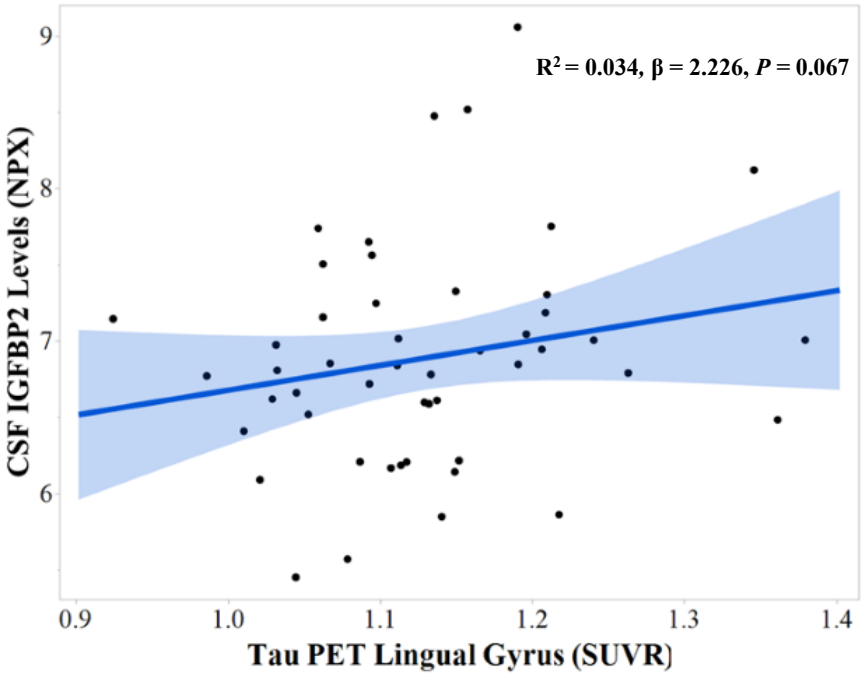
Supplementary statistical analysis

PREVENT-AD cohort

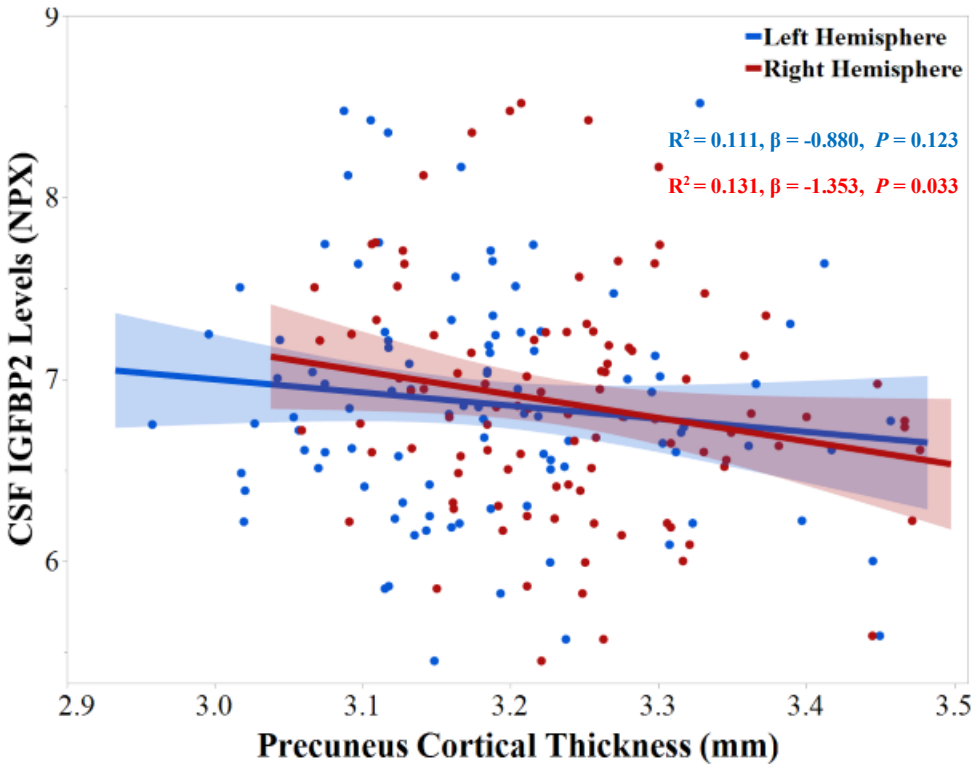
In order to assess blood-brain barrier integrity and lumbar puncture contamination, linear regression models adjusted for age, gender and *APOE ε4* carrier status, tested for associations between CSF IGFBP2 with CSF microproteins, CSF red blood cell counts and CSF white blood cell counts.

Supplementary figures

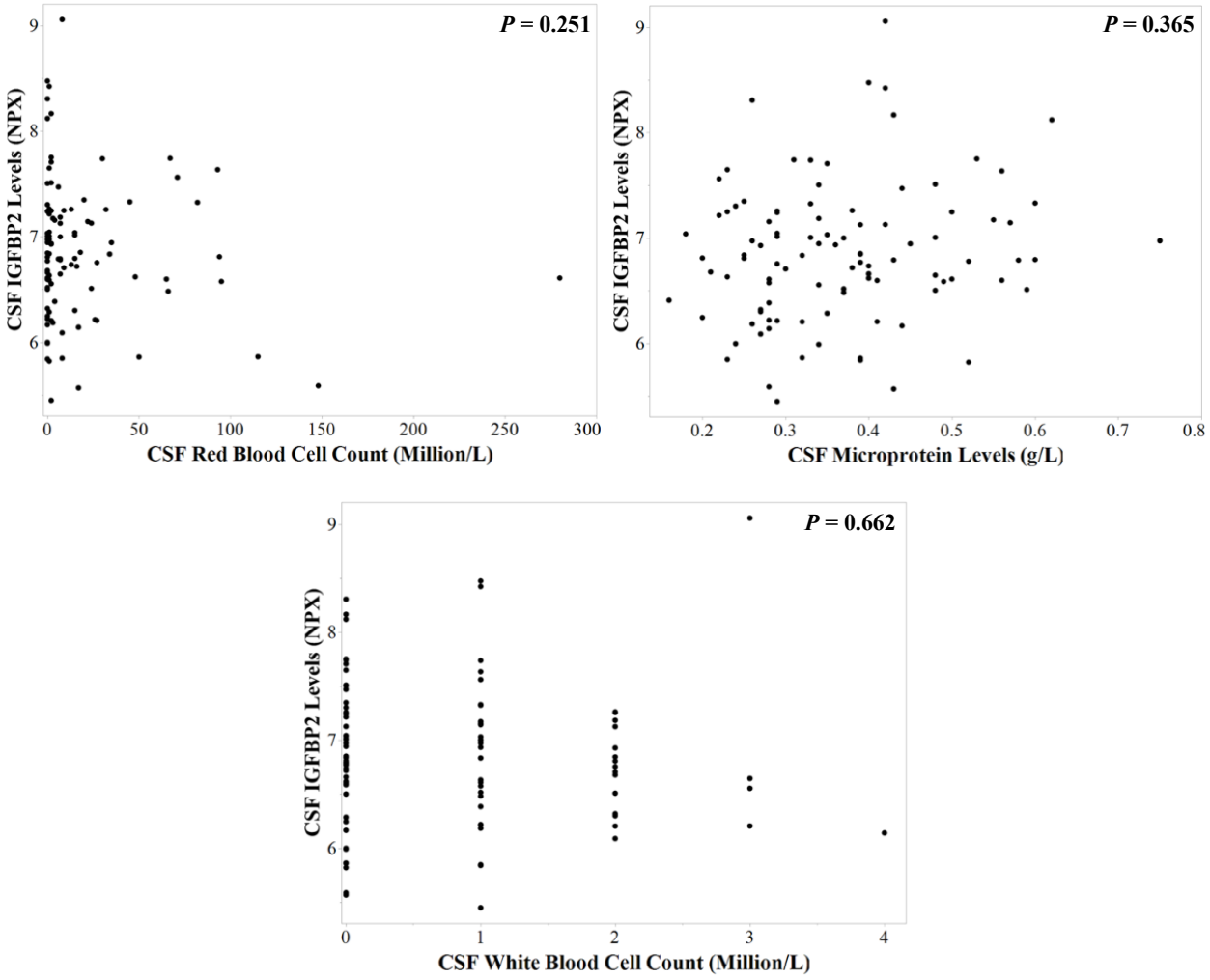
PREVENT-AD cohort



Supplementary Figure 1 CSF IGFBP2 is associated tau deposition in the lingual gyrus in a subset of PREVENT-AD participants. CSF IGFBP2 levels were measured using the Olink Proximity Extension Assay (*n* = 109). Of these individuals, *n* = 49 underwent tau PET scans, using flortaucipir. Linear regressions are represented with a confidence region of the fitted line. R^2 and *P* values are located in the top right corner. Analyses were adjusted for age, sex and *APOE* ε4 carrier status.

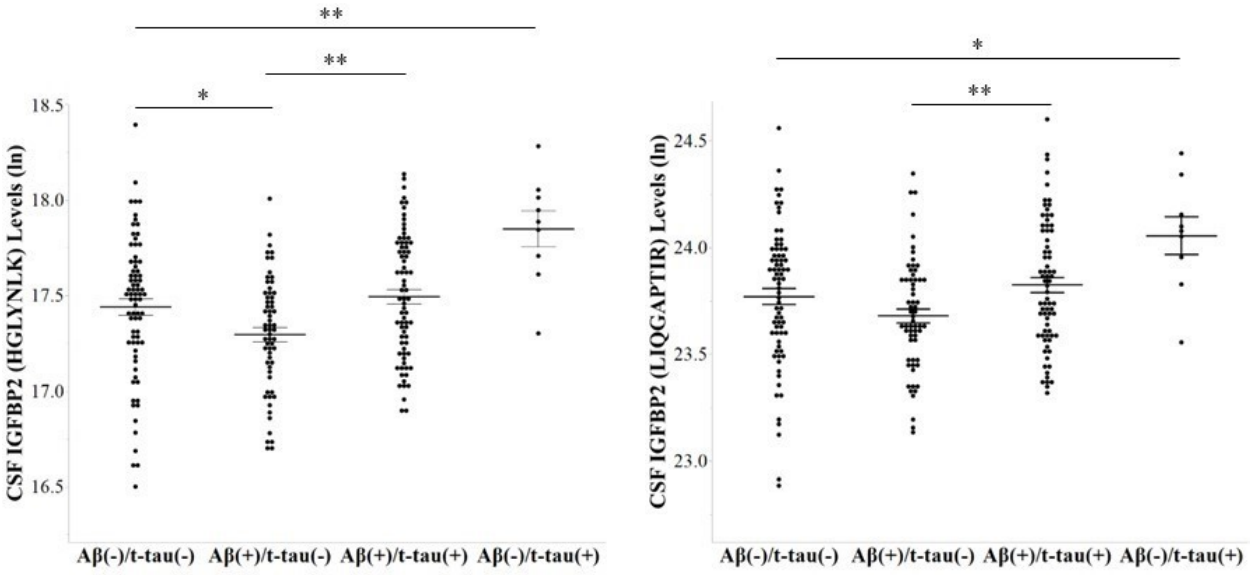


Supplementary Figure 2 CSF IGFBP2 is associated with cortical atrophy in the precuneus in PREVENT-AD participants. CSF IGFBP2 levels were measured using the Olink Proximity Extension Assay (n = 109). T1-weighted structural MRI scans were performed on a subset of PREVENT-AD participants (n = 104). The imaging processing pipeline CIVET 1.1.12 was used to analyze cortical thickness data. Linear regressions are represented with a confidence region of the fitted line (blue for left hemisphere and red for right hemisphere). R^2 and P values are located in the top right corner. Analyses were adjusted for age, sex and *APOE* ε4 carrier status.



Supplementary Figure 3 CSF IGFBP2 is not associated with blood-brain barrier and blood contamination markers in PREVENT-AD participants. In order to assess blood-barrier integrity and lumbar puncture contamination, linear regression models tested for associations between CSF IGFBP2 and CSF red blood cell counts, CSF microproteins, and CSF white blood cell counts. *P* values are located in the top right corner of each panel. Analyses were adjusted for age, sex and *APOE* ε4 carrier status. No significant associations were found.

ADNI-1 cohort



Supplementary Figure 4 CSF IGFBP2 mass spectrometry measurements across the stages of AD pathology in ADNI-1. Cognitively unaffected participants (n = 85) and participants with MCI (n = 133) from the ADNI cohort were staged as CSF amyloid and/or CSF total tau positive according to the recommended thresholds of 192 pg/mL and 93 pg/mL, respectively. Linear models, adjusted for age, sex and *APOE* ε4 carrier status were used to examine mean differences in IGFBP2 protein levels across stages. The IGFBP2 fragment HGLYNLK exhibited a reduction at Stage 1 (n = 62), relative to Stage 0 (n = 75, LIQGAPTIR at a trend-level). However, both peptides did not differ between Stage 0 and Stage 2 (n = 72). Finally, HGLYNLK and LIQGAPTIR were elevated at Stage 2 relative to Stage 1. Similarly, both peptides were elevated in SNAP (n = 9) compared to Stage 0. *P < 0.01; **P < 0.005.

Supplementary References

Kennedy JJ, Abbatiello SE, Kim K, *et al*. Demonstrating the feasibility of large-scale development of standardized assays to quantify human proteins. *Nat Methods*. 2014;11(2):149-155.