Immune mechanisms as predictors of cognitive impairment and therapeutic targets in pre-symptomatic Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is commonly known for the typical memory loss that accompanies its clinical expression. However, it is now understood that the clinical expression of the disease is most likely preceded by up to three decades of pathological changes. Thus, the lack of efficacy of drugs developed and tested in the last twenty years may owe, at least in part, to treatment being "too little too late." The period of silent pathological changes, otherwise called the pre-symptomatic phase, offers a window of opportunity to identify biological mechanisms altered in the pathogenetic process and for their modification through preventive interventions. Among important biological pathways involved in disease, immune dysfunction is increasingly recognized as an important feature of several neurological disorders. The immune component of AD was first identified in the early 1990's and has attracted considerable interest following the identification of rare genetic variants associated with immune function and AD risk. Several lines of investigation suggest that immune dysregulation may be an important mechanism in the development of AD pathology and symptoms and could therefore serve as a target for disease prevention. This thesis characterizes immune changes in relation to evolving AD pathology and symptoms and investigates typical non-steroidal anti-inflammatory drugs (NSAIDs) as agents for the prevention of asymptomatic progression of the disease. Chapter 1 provides an overview of the current situation of AD research and the objectives pursued in subsequent chapters. Chapter 2 is a review of the literature on AD and its immune hypothesis. Chapter 3 describes a study in which we characterized how markers of immune activation measured in the cerebrospinal fluid of non-demented persons related to markers of AD pathological progression. We identified a non-linear, biphasic pattern of association of immune markers with AD biomarkers. This pattern generalized to other markers of immune activation in an independent cohort. Chapter 4 describes a study utilizing markers identified in Chapter 3 to predict the severity and progression of AD symptoms for a given level of AD pathology. We identified a set of immune-related markers that portended attenuated severity of AD symptoms and slower rate of progression for a given degree of pathology. Chapters 5 and 6 discuss the results of a randomized trial of the NSAID naproxen for the prevention of the progression of an indicator of pre-symptomatic AD. We observed no benefit of the drug, possibly owing to low central nervous system permeability and absent reduction of immune activation. Chapter 7 discusses the contributions and future directions of the studies described. This thesis provides novel insights into the roles of immune activity in AD pathogenesis and symptom expression. It also describes the challenges of preventing AD at the earliest possible stage using NSAIDs.

Résumé

La maladie d'Alzheimer (MA) est généralement connue pour la perte de mémoire caractéristique de son expression clinique. Cependant, il semble désormais évident que l'apparition des symptômes cognitifs est précédée par environ trois décennies de changements pathologiques. Ainsi, l'inefficacité des traitements développés et évalués au cours des vingt dernières années peut s'expliquer, au moins en partie, par des interventions trop tardives. Cette période de changements pathologiques 'silencieux', aussi appelée 'phase asymptomatique', offre une opportunité unique pour étudier et caractériser les mécanismes biologiques altérés par la maladie qui peuvent être rétablis dans un objectif de prévention. Les dysfonctions immunitaires, mécanismes potentiellement importants, sont reconnus comme des éléments essentiels de plusieurs troubles neurologiques. La composante immunitaire de la MA a été identifiée pour la première fois dans les années 1990 et a vécu un net regain d'intérêt lors de la découverte de facteurs de risques génétiques pour la MA aussi impliqués dans la fonction immunitaire. Plusieurs recherches suggèrent que la dysfonction immunitaire est un mécanisme important dans le développement de la pathologie Alzheimer et des symptômes associés. Afin de prévenir la maladie, cette dysfonction pourrait servir de cible idéale. La présente thèse caractérise les changements immunitaires en relation à la pathologie de la MA, de ses symptômes et évalue les anti-inflammatoires non-stéroïdiens (AINS) en tant qu'agent de préventions dans la phase asymptomatique de la maladie. Le chapitre 1 est un état des lieux pointant certains manques et indiquant les objectifs de la thèse. Le chapitre 2 est une revue de littérature sur la MA et l'hypothèse immunitaire. Le chapitre 3 rapporte les résultats d'une étude dans laquelle nous avons caractérisé, dans une population de personnes non-démentes, comment certains marqueurs d'activité immunitaires sont associés aux biomarqueurs de la pathologie Alzheimer. Nous avons identifié un modèle selon lequel les marqueurs immunitaires sont associés de façon non-linéaire ou 'bi-phasique' avec les biomarqueurs Alzheimer. Ce modèle se généralise à d'autres marqueurs immunitaires dans une cohorte indépendante. Le chapitre 4 décrit une étude qui utilise les marqueurs identifiés dans le chapitre 3 afin de prédire, pour un niveau de pathologie donné, la sévérité et la progression des symptômes cognitifs. Nous avons identifié un groupe de marqueurs immunitaires qui étaient associés à une atténuation de la sévérité et la progression des symptômes. Les chapitres 5 et 6 présentent les résultats d'un essai clinique évaluant l'efficacité du naproxen, un AINS, pour la prévention de la progression d'un indicateur de la MA dans son stade pré-symptomatique. Nous n'avons mesuré aucun bénéfice du médicament sur la progression de cet indicateur. Ceci est

possiblement dû à sa faible pénétration dans le système nerveux central et à l'absence d'effet de réduction sur plusieurs marqueurs immunitaires. Le chapitre 6 discute les contributions et les suites à donner aux études présentées. Cette thèse offre ainsi de nouvelles perspectives sur le rôle de l'activité immunitaire dans le développement de la pathologie et des symptômes de la MA. Elle décrit aussi les défis rencontrés pour prévenir la MA le plus tôt possible avec les AINS.

Table of contents

Abstract i

Résumé ii

Table of contents iv

List of Figures ix

List of Tables x

List of abbreviations xi

Acknowledgements xiv

Contribution of authors xvi

Chapter 1 – Introduction 1

1.1 Alzheimer's disease dementia – Current situation 1

1.2 Objectives 1

1.3 Bibliography – Chapter 1 4

Chapter 2 – Review of the literature 5

2.1 Alzheimer disease 5

2.2 Biology of Alzheimer pathology 7

2.2.1 Amyloid pathology 7

2.2.2 Tau pathology 8

2.3 The pre-symptomatic phase 10

2.3.1 Origins and necessity of defining a 'pre-symptomatic' stage 10

2.3.2 Biomarkers of Alzheimer disease pathology 10

2.3.3 Characterization of pre-symptomatic Alzheimer disease 11

2.4 Clinico-pathological discordance 12

2.5 Risk factors for Alzheimer disease 14

2.5.1 Non-modifiable factors 14

2.5.2 Modifiable factors 15

2.6 The 'immune hypothesis' of Alzheimer disease 16

2.6.1 Immune activation in Alzheimer disease 19

2.6.2 Immune modifying treatments for Alzheimer disease 20

2.6.3 Biomarker studies of immune processes 20

2.7 Bibliography - Chapter 2 22

Chapter 3 – Bi-directional association of cerebrospinal fluid immune markers with stage of Alzheimer's disease pathogenesis 36

3.1 Preamble 36

3.2 Abstract 37

3.3 Introduction 38

3.4 Methods 39

3.4.1 Participants 39

3.4.2 Cerebrospinal fluid measurements 39

3.4.3 Pathological staging of participants 40

3.4.4 *APOE* genotyping 43

3.4.5 Statistical analyses 43

3.5 Results 44

3.5.1 Demographic characteristics 44

3.5.1 PREVENT-AD immune marker concentrations and pathological stage 46

3.5.2 PREVENT-AD immune marker concentrations and AD biomarkers 46

3.5.3 ADNI protein marker concentrations, pathological stage, and AD biomarkers 47

3.6 Discussion 49

3.7 Bibliography – Chapter 3 56

Chapter 4 – Hypothesis: CSF protein markers suggest a pathway for symptomatic resilience to AD pathology 60

4.1 Preamble 60

4.2 Abstract 61

4.3 Introduction 62

4.4 Methods 64

4.4.1 Participants 64

4.4.2 CSF measurements and classification 64

4.4.3 APOE genotyping 66

4.4.4 Analytic methods. 66

4.4.5 Data availability 68

4.5 Results 68

4.5.1 Demographic characteristics 68

4.5.2 AD biomarkers and CSF proteins relation to cognitive scores. 69

4.5.3 LASSO protein model weights predict diagnostic category 71

4.5.4 Weighted marker score predicts rate of 4-year cognitive decline 72

4.6 Discussion 73

4.7 Bibliography – Chapter 4 81

Chapter 5 – INTREPAD: a randomized trial of naproxen to slow progress of pre-symptomatic Alzheimer disease 86

5.1 Preamble 86

5.2 Abstract 87

5.3 Introduction 88

5.4 Methods 89

5.4.1 Primary research question 89

5.4.2 Standard Protocol Approvals, Registrations, and Patient Consents 89

5.4.3 Overview of Participants and Trial Regimen 89

5.4.4 Eligibility criteria. 90

5.4.5 Specification of primary efficacy outcome and initial power analysis. 91

5.4.6 Randomization, masking, and provision of study drug 92

5.4.7 Assessment methods 92

5.4.8 Primary efficacy outcome: the composite Alzheimer Progression Score 95

5.4.9 Statistical analysis 96

5.4.10 Data sharing 97

5.5 Results 97
5.5.1 Enrollment and study completion data 97
5.5.2 Baseline characteristics 97
5.5.3 Concomitant medications 98
5.5.4 Safety outcomes 98
5.5.5 Primary efficacy outcome 99
5.5.6 Secondary efficacy analyses 101
5.5.7 Exploratory efficacy analyses. 101

5.6 Discussion 102

5.7 Bibliography – Chapter 5 106

Chapter 6 – No apparent effect of naproxen on CSF markers of innate immune activation. 109

6.1 Preamble 109

6.2 Abstract 110

6.3 Introduction 111

6.4 Methods 112
6.4.1 Participants 112
6.4.2 CSF measurements 112
6.4.3 Statistical analyses 112
6.4.4 Data availability 114

6.5 Results 114
6.5.1 Summary statistics 114
6.5.2 Naproxen enters the brain of treated individuals. 115
6.5.3 Naproxen does not affect concentrations of CSF immune markers. 116

6.6 Discussion 118

6.7 Bibliography – Chapter 6 120

Chapter 7 – Discussion 122

7.1 Contributions 122
7.1.1 Immune activation – An integral part of AD pathogenesis 122
7.1.1 Immune contribution to resilience 123
7.1.3 Targeting neuro-inflammation for AD prevention and treatment 125

7.2 Future directions 126

7.3 Conclusions 129

7.4 Bibliography – Chapter 7 130

Appendix 1 – Supplementary material to Chapter 3 133

Supplementary Methods 133

CSF Apolipoprotein-E and Milliplex inflammatory marker assays 133

Supplementary Tables 134

Appendix 2 – Supplementary material to Chapter 5 144

Supplementary Methods 144

Participants – additional information 144

Cognitive evaluation 144

Neuroimaging – additional information 145

Supplementary Figures 150

Supplementary References 152

List of Figures

Figure 2.1: Hypothetical model of biomarker and cognitive changes in Alzheimer disease...6
Figure 2.2: Amyloid-β facilitates the spread of tau pathology from the temporal lobe to anatomically connected brain regions.....9
Figure 2.3: Elevated amyloid pathology is not invariably associated with AD dementia. 13
Figure 2.4: Genetic variants associated with AD.....15
Figure 2.5: Immunological mechanisms of inflammation. 18
Figure 3.1: CSF Markers and pathological staging of Alzheimer's disease. 42
Figure 3.2: Bayes factor analysis identifies 38 ADNI CSF markers potentially associated with pathological stage.....48
Figure 3.3: Markers common to both cohorts show similar association with AD pathology. 49
Figure 3.4: Overlapping theoretical biological pathways of identified ADNI and PREVENT-AD proteins. 53
Figure 4.1: LASSO regression modeling for prediction of ADASCog-11 performance.....70
Figure 4.2: Statistical comparison of models trained to predict baseline ADASCog-11 performance. 71
Figure 4.3: Weighted marker summary score predicts contrasts among clinical diagnostic categories. 72
Figure 4.4: Weighted protein marker summary score predicts 4-year cognitive trajectory. .73
Figure 5.1: Consort flow. 90
Figure 5.2: Trial timeline. 93
Figure 5.3: Treatment effects on APS.....100
Figure 5.4: Treatment effects on neuro-sensory and CSF biomarker measures. 102
Figure 6.1: Plasma and CSF concentrations of naproxen in the trial cohort. 115
Figure 6.2: Trajectory of CSF immune markers by treatment group.....117
Figure 7.1: Immune associations with chronological brain aging, AD biomarkers and cognition in persons at high risk of having pre-symptomatic AD. 128

List of Tables

Table 3.1: PREVENT-AD and ADNI demographics 45
Table 3.2: PREVENT-AD and ADNI cerebrospinal fluid markers associated with Alzheimer's Disease pathological stages.....46
Table 3.3: Coefficients of fully adjusted linear regression models evaluating the association of CSF markers with pathological stage 48
Table 4.1: List of the 23 "AD-related" markers in ADNI CSF.....65
Table 4.2: ADNI demographics overall and by assignment to Training or Validation sets ...69
Table 5.1: Variables included in the APS 96
Table 5.2: Baseline characteristics of INTREPAD participants 98
Table 5.3: Adverse Events (AEs).....99
Table 6.1: Sample characteristics.....113

List of abbreviations

AAT	a1-antitrypsin
Aβ	Amyloid-beta
ACE	Angiotensin converting enzyme
AD	Alzheimer's disease
ADAD	Autosomal Dominant Alzheimer's disease
ADAS	Alzheimer's disease assessment scale
ADNI	Alzheimer's Disease Neuroimaging initiative
AE	Adverse events
AMP	Antimicrobial peptide
APOE	Apolipoprotein E
APOJ/CLU	Apolipoprotein J / Clusterin
APP	Amyloid precursor protein
APS	Alzheimer progression score
AXL	AXL receptor tyrosine kinase
BBB	Blood brain barrier
CCL	C-C motif chemokine ligand
CD40a	CD40 antigen
CgA	Chromogranin A
CI	Confidence interval
CNS	Central nervous system
COX	Cyclooxygenase
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CU	Cognitively unimpaired
DAMP	danger-associated molecular pattern
FDG	Fluorodeoxyglucose
FDR	False discovery rate
FGF	Fibroblast growth factor
FH	Family history
GCSF	Granulocyte colony stimulating factor
GWAS	Genome wide association studies

HC	Healthy control
HB-EGFFL-GF	Heparin binding EGF-like growth factor
hFABP	Heart fatty acid binding protein
HGF	Hepatocyte Growth Factor
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IL	Interleukin
INTREPAD	Investigation of Naproxen Treatment Effects in Pre-symptomatic AD
IP10	IFNγ induced protein 10
LASSO	Least absolute shrinkage and selection operator regression
LOX-1	Lectin-like oxidized LDL-receptor-1
LP	Lumbar puncture
MAC	Membrane attack complex
MCI	Mild cognitive impairment
MCP-1	Monocyte Chemoattractant Protein-1
MCSF-1	Macrophage colony stimulating factor
Mf	Macrophage or monocyte
m-ITT	Modified intent to treat
MMP	Matrix metalloproteinase
MoCA	Montreal cognitive assessment
MRI	Magnetic resonance imaging
MTL	Medial temporal lobe
NIA-AA	National Institute of Aging and the Alzheimer's Association
NSAID	Non-steroidal anti-inflammatory drug
OR	Odds ratio
PAMP	Pathogen associated molecular pattern
PET	Positron emission tomography
PHF	Paired helical filaments
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RBANS	Repeatable battery for assessment of neuropsychological symptoms
ROS	Reactive oxygen species
SAE	Serious adverse event

S.E.	Standard error
SNAP	Suspected Non-Alzheimer Pathology
(T)-tau	Total tau
(P)-tau	Phosphorylated tau
TDP43	TAR DNA-binding protein 43
TF	Tissue Factor
TFF3	Trefoil factor 3
TGF	Transforming growth factor
TIMP-1	Tissue inhibitor of metalloproteinases
TNF	Tumor necrosis factor
TNFR-2	Tumor necrosis factor receptor-2
TREM2	Triggering receptor expressed on myeloid cells 2
TSPO	18kDa translocator protein
UPSIT	University of Pennsylvania smell identification test
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

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To those who would have been so proud,
But to whom I will never be able show this
Papys (Bernard, Robert), Véronique,
J’espère que vous pouvez voir ceci d’où vous êtes.

Contribution of authors

As the lead author on Chapters 3, 4 and 6, I designed the analytical plan, carefully considered the data for processing prior to analysis, performed statistical analyses, visualized and interpreted the results as well as wrote the manuscripts. Because INTREPAD was designed by PREVENT-AD investigators prior to the start of my degree, I did not actively participate in the experimental design described in Chapter 5. However, I led the project after the conclusion of the trial first by replicating pre-specified analyses performed by a contracted statistician and then by designing and performing exploratory secondary analyses. Again, I visualized and interpreted the results as well as wrote the manuscript. For all chapters, I received valuable help from collaborators as outlined below:

Chapter 3

- Melissa Savard: Mentoring for the statistical analysis
- Judes Poirier: Data collection
- Anne Labonté: Assay of immune markers using the Luminex Platform
- Pedro Rosa-Neto: Data collection
- Tara M. Weitz: Assay of immune markers using the MSD platform
- Terrence Town: Assay of immune markers using the MSD platform
- John Breitner: Study supervision and writing the manuscript

Chapter 4

- Melissa Savard: Statistical Analysis
- Judes Poirier: Interpretation of results, writing the manuscript
- Dave Morgan: Interpretation of results, writing the manuscript
- John Breitner: Study supervision and writing the manuscript

Chapter 5

- Jennifer Tremblay-Mercier: Study design, Supervision of data acquisition, Drafting and revising the manuscript
- Jeannie-Marie Loutsakos: Development of primary outcome Alzheimer Progression Score, Data analysis
- Cécile Madjar: Data Management, conceptualization of database, support for data analysis

- Marie-Élyse Lafaille-Magnan: Data acquisition (olfaction), Study design (randomization)
- Melissa Savard: Data analysis
- Pedro Rosa-Neto: Data acquisition (Lumbar Puncture)
- Judes Poirier: Data acquisition (laboratory methods), Study design
- Pierre Etienne: Data acquisition (supervision of safety monitoring), Data of study results, Study design, Drafting the manuscript
- John Breitner: Study conceptualization and design, Interpretation of results, Drafting and revising the manuscript, Study funding

Chapter 6

- Anne Labonté: Assay of immune markers using the Luminex Platform
- Judes Poirier: Data collection
- Pedro Rosa-Neto: Data collection
- John Breitner: Study conceptualization and design, Interpretation of results, Drafting and revising the manuscript, Study funding

Chapter 1 – Introduction

1.1 Alzheimer's disease dementia – Current situation

Dementia is a clinical syndrome that can be provoked by any of several brain disorders. The syndrome is characterized by global decline in cognitive ability that is not attributable to clouding of consciousness but is of sufficient severity to result in impaired performance in activities of daily life. It is estimated that 40 million people currently live with dementia worldwide, a number expected to multiply dramatically by 2050.[1] Alzheimer disease (AD), in particular, is the most common cause of dementia, representing an estimated 60-80% of cases. In the United States alone, there are currently 5.7 million people living with AD dementia, >95% of whom are aged 65 and older. In this latter age group, it is estimated that at least 487,000 new cases will be diagnosed in 2019, a figure projected to double by 2050 with the aging population.[2] As a result, the annual cost of care for U.S. patients living with dementia is expected to rise from $290 billion in 2019 to $1.1 trillion by 2050 if the current trend remains unchanged. AD therefore represents a tremendous public health challenge with devastating social and financial consequences which requires rapid solutions.

However, there currently exists no disease-modifying treatment for AD. No new drug has been approved for the treatment of AD dementia since memantine in 2003 and the field has since suffered major clinical trial failures.[3-6] Several reasons have been suggested for these failures among which the lack of efficacy of antibodies tested, the enrollment of populations without the pathology targeted[7] and more, prominently, timing of treatment.[8,9] In fact, it is now generally accepted that AD pathological changes occur silently over several decades. This period of insidious changes, often called the 'pre-symptomatic' phase of the disease, provides a 'window of opportunity' for disease modifying treatments. It is therefore critical to study and characterize more precisely the changes that occur during this phase in order to identify important disease mechanisms that may both accurately predict disease progression and serve as viable targets for prevention.

1.2 Objectives

In recent years, the availability of whole genome sequencing and techniques to analyze large datasets have allowed the field to characterize further the genetics of AD. The nature of the set of risk genes make it clear that there are important pathways other than those involved in

'typical' AD pathogenesis. Many studies have now shown that immune pathways feature prominently among elements that may modify disease risk. While these are currently being studied extensively in AD animal models, there has been much less work of this sort in humans. Therefore, we used a number of assays of immune markers to attempt to understand how specific immune activities change over the disease course in humans, whether they predict symptom severity and progression and whether they may be targeted for disease prevention. These important objectives represent original research and are described below for each chapter.

Chapter 3 objectives

The study of brain immune markers, like several other modalities, has long been limited to case-control studies comparing the levels of several analytes between healthy and diseased participants. These studies yielded largely conflicting results such that an individual marker could be reported as decreased, increased or unchanged in persons with dementia. Chapter 3 of this thesis describes our investigation of whether cerebrospinal fluid markers of immune activity could be more closely related to the evidence of accruing of AD pathology rather than the clinical diagnostic category.

Chapter 4 objectives

Immune activation in AD is generally regarded as dysregulated and a possible effector by which AD pathology damages the brain and leads to cognitive decline. Having first identified a set of markers closely related to AD pathological change (Chapter 3), we investigated specifically whether a "signature" of these markers could predict symptom severity and progression beyond what was typically explained by AD pathology.

Chapter 5 objectives

Earlier epidemiological studies suggested that non-steroidal anti-inflammatory drugs (NSAIDs) held promise for AD, but clinical trials that enrolled AD patients suggested that their benefits might only be apparent at the earliest stages of pre-symptomatic illness. Chapter 5 describes the results of a double-blind placebo-controlled randomized trial of the NSAID naproxen for the prevention of progression in early pre-symptomatic AD.

Chapter 6 objectives

Chapter 6 describes a follow-up study to Chapter 5 in which we investigated the ability of Naproxen to enter the central nervous system and modify concentrations of various CSF immune markers.

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Chapter 2 – Review of the literature

2.1 Alzheimer disease

Alzheimer disease (AD) is mostly an age associated neurodegenerative disorder causing the larger part of dementia cases. AD is more commonly (in)famous for its clinical expression characterized by an amnestic syndrome with a signature impairment in episodic memory.[1] The latter deficit can manifest in several cognitive faculties (*e.g.*, free recall, recognition) and sensory modalities (*e.g.*, auditory, visual, olfactory).[2-4] AD also affects language ability, semantic knowledge as well as working memory, attention and visuospatial abilities.[5] Pathologically, it is characterized by the accumulation of amyloid-β (Aβ) protein into extracellular senile plaques, the formation of intracellular neurofibrillary tangles of hyper-phosphorylated *tau* protein and, in the later stages, by widespread neuronal and synaptic loss. The 'amyloid hypothesis' is presently the most widely accepted model of AD pathogenesis.[6] It posits that initial accumulation of Aβ, most likely resulting from reduced clearance from the brain,[7] is the causative agent of AD, triggering *tau* hyper-phosphorylation, vascular injury, neuronal death and consequent dementia. While the diagnosis of AD was historically based on the presence of dementia symptoms,[8] it is now evident that several decades of brain and subtle cognitive changes precede their appearance. Thus, AD is now defined as having three phases: a pre-symptomatic stage of clinically silent pathological changes, a stage of mild cognitive impairment (MCI) in which there is clinically measurable decline in cognitive function that does not impair one's ability to perform activities of daily living and the dementia stage.[9-11] A hypothetical model depicting the AD sequence is shown in Figure 2.1.

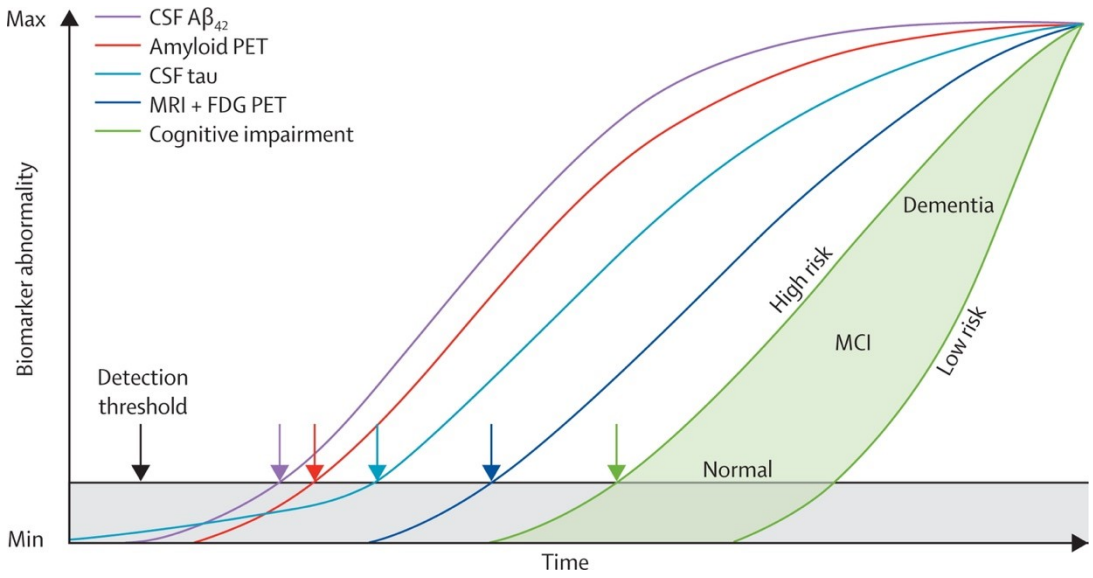

Figure 2.1: Hypothetical model of biomarker and cognitive changes in Alzheimer disease.

The pre-symptomatic phase of AD spans several decades during which several biomarkers may become abnormal (black horizontal line). Early *tau* accumulation in aging (light blue) occurs prior to Aβ accumulation (purple and red) but below detection limits. Aβ abnormality occurs first, most likely detectable earlier using cerebrospinal fluid (CSF) biomarkers (purple) and then with positron emission tomography (PET). *Tau* biomarkers then become abnormal. Biomarkers of neurodegeneration (structural magnetic resonance imaging [MRI] and fluorodeoxyglucose [FDG-PET]) become abnormal last (both markers represent conjointly in blue). By the time cognitive impairment becomes measurable, biomarkers of Aβ pathology have already plateaued. This model leaves the possibility that cognitive impairment may initiate and progress differently based on each individual's risk profile (light green-filled area). Aβ=amyloid-β. MCI=mild cognitive impairment. Figure and caption adapted from [12].

2.2 Biology of Alzheimer pathology

2.2.1 Amyloid pathology

Alois Alzheimer described the neuropathological hallmarks of AD in the brain of the first-described patient in 1906. However, the components of senile plaques were only elucidated eight decades later when Glenner and Wong identified the Aβ peptide as the main component of plaques in 1984.[13,14] The Aβ peptide results from the sequential cleavage of the amyloid precursor protein (APP) protein by β- and γ-secretase at the C- and N-terminal of the Aβ region. Alternatively, APP can also be cleaved by a so-called α-secretase but, because α-secretase cuts within the Aβ region, the resulting Aβ peptides are generally considered to be non-pathogenic.[15] In the pathological pathway, the APP protein is first cleaved by β-secretase and consequently releases a large soluble APP-β fragment. The remaining membrane-bound portion is then cleaved by γ-secretase. This process is imprecise and yields Aβ peptides of varying length of which the most abundant are those ending at amino acid 40 ($A\beta_{40}$) and 42 ($A\beta_{42}$).[16] These Aβ monomers, most particularly the $A\beta_{42}$ fragments, can spontaneously aggregate into oligomers, eventually leading to the formation of fibrillar Aβ that makes up senile plaques.[17-19] While the latter constitute a principal AD pathological hallmark, they may only indicate a final, inert, stage of Aβ accumulation since the soluble oligomeric forms of $A\beta_{42}$ are likely those exerting the strongest neurotoxic effects.[20-23]

Some families carry disease-causing mutations. These are invariably involved in Aβ processing affecting the APP gene[24,25] or presenilins (PSEN)[26-28] which form the γ-secretase catalytic subunit. Several mutations in the APP, PSEN-1 and PSEN-2 genes have been identified and are associated with increased production of $A\beta_{42}$ in addition to AD causation.[29,30] Similarly, individuals with Down's syndrome, who have three copies of chromosome 21 which harbors the APP gene, have increased incidence of AD dementia with age (~66% at 60 years of age),[31] evident amyloid pathology as early as the second decade of life and in virtually all individuals by age 40[32] as well as AD biomarker abnormality.[33,34] These observations provide strong support for the amyloid hypothesis of AD etiology, while suggesting that Aβ accumulation may be an early event in the AD cascade. They have also encouraged several initiatives to study these populations to gain further understanding of early disease mechanisms.[35,36]

Aβ pathology does not seem to accumulate evenly in the brain. Several investigators have attempted to map its stereotypical progression in *post-mortem* studies and, more recently, using Aβ tracers developed for positron emission tomography (PET) imaging. In their original attempt to characterize plaque deposition, Braak and Braak suggested that amyloidosis may start in the basal neocortex.[37] However, later investigations by Thal *et al.* observed amyloid plaques *throughout* the neocortex even at the earliest stages of plaque pathology.[38] PET studies have yielded equally contrasting results with some suggestions that amyloid accumulation may start in temporal and frontotemporal areas,[39-41] the orbitofrontal-amygdala-hippocampus axes[42] or the entire neocortex.[43] Several studies suggest that Aβ deposition in the frontotemporal areas may be an early but non-specific event,[39-41] while accumulation in the precuneus and frontal medial regions may be more specific to the AD process[44,45] and, therefore, predictive of future global amyloid accumulation.

2.2.2 Tau pathology

While amyloid deposits are extracellular, neurofibrillary lesions are their intraneuronal counterparts. These lesions are present in the cell bodies and apical dendrites as neurofibrillary tangles, in distal dendrites as neuropil threads and in neurites associated with some amyloid plaques thus forming neuritic plaques.[46] Paired helical filaments (PHFs),[47] of which *tau* protein is a principal component,[48-50] is the major fibrous component of all three types of neurofibrillary lesions.

Unlike Aβ pathology, however, *tau* pathology deposits do occur in a stereotypical fashion, first being found in transentorhinal regions (Braak I-II stages), then extending to the limbic cortices (Braak Stages III-IV) and finally spreading to isocortical regions.[51] This particular pattern of *tau* deposition has recently been confirmed *in vivo* through PET imaging of the human brain,[52] and these observations have led many to speculate that this pathology may spread through the brain following anatomical connections.[53,54] However, *tau* accumulation in temporal areas has also been characterized as a relatively common and apparently harmless phenomenon of aging.[55,56] Recent data support the notion that the presence of amyloid pathology favors the spread and the accrual of *tau* outside of the medial temporal lobe and triggering cognitive decline thereby making AD an amyloid-facilitated tauopathy (Figure 2.2).[57,58]

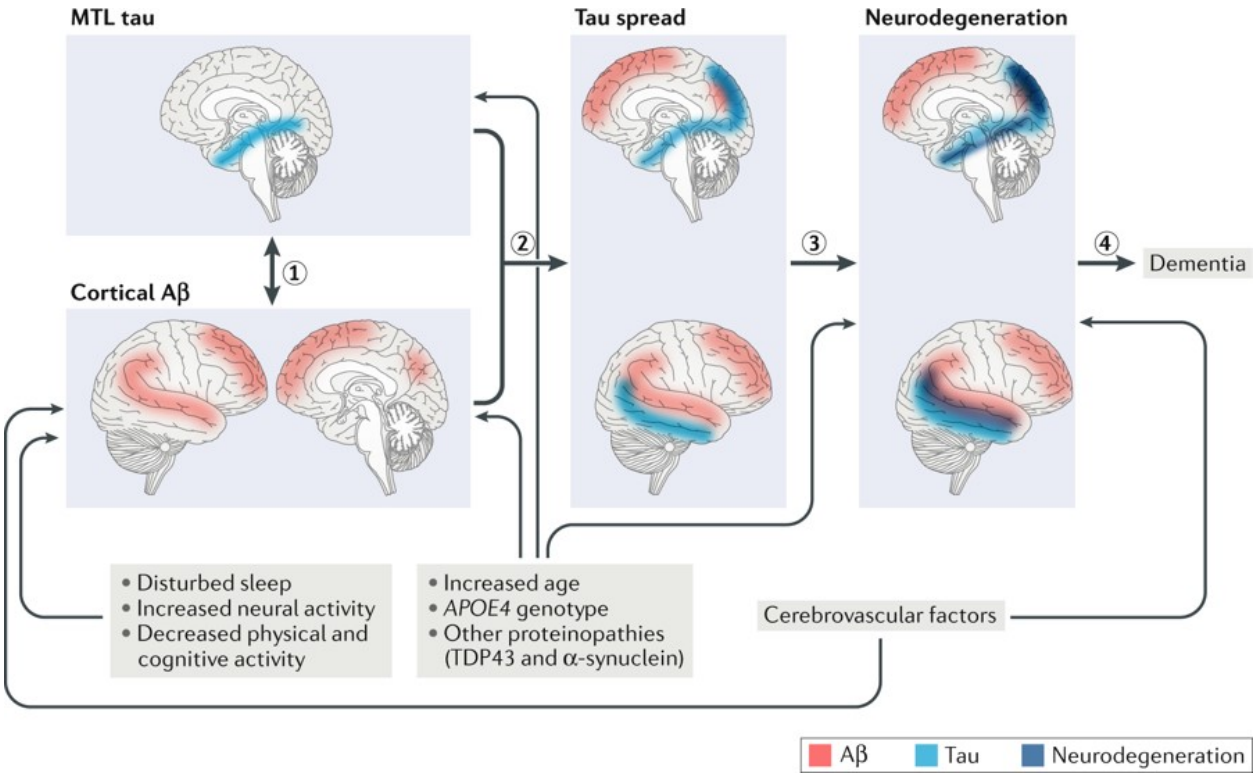

Figure 2.2: Amyloid-β facilitates the spread of tau pathology from the temporal lobe to anatomically connected brain regions.
The initial stages of Alzheimer disease (AD) development reflect relationships between cortical amyloid-β (Aβ; red) and *tau* (blue) in the medial temporal lobe (MTL) (1). This process most likely begins with cortical Aβ deposition. This favors the spread of naturally occurring *tau* deposition in the MTL into the medial parietal, lateral parietal and temporal cortices (2). *Tau* spread is associated with neurodegeneration (dark blue) following a similar topography to tau deposition (3), which eventually leads to cognitive decline and dementia (4). Disturbed or diminished sleep, increased neural activity, reduced physical and cognitive activity and cerebrovascular disease may drive Aβ deposition. Old age, the apolipoprotein E gene ε4 allele (*APOE*4) and other proteinopathies such TDP43 (TAR DNA-binding protein 43) or α-synuclein may drive Aβ accumulation, *tau* deposition in the MTL and neurodegeneration. Figure and caption adapted with permission from [59].

2.3 The pre-symptomatic phase

2.3.1 Origins and necessity of defining a 'pre-symptomatic' stage

The AD field has focused its drug development efforts on Aβ pathology as the principal target. However, no Aβ-targeting drug has been approved for AD treatment. Worse, many Aβ-lowering drugs have suffered major setbacks, even when they clearly demonstrated the ability to interact with amyloid and remove plaques from the brain.[60-63] The failure of these clinical trials has been attributed, at least in part, to the idea that Aβ pathology develops over several decades and that plaques represent an inert form. In reality, by the time patients are diagnosed with AD dementia, they present with extensive Aβ and *tau* pathologies, severe executive and memory deficits,[64] cortical and hippocampal atrophy[65,66] as well as altered brain connectivity.[67] It is frequently conjectured that this damage may be too severe to be reversed. Therefore, efforts have been made to identify the disease at earlier phases. First, researchers identified MCI, a clinical phase of subtle cognitive impairment believed commonly to be a prodrome of dementia.[68] However, evidence suggests that amyloid and *tau* pathologies most likely start accumulating even before there is measurable cognitive impairment. This earlier period of insidious pathological changes termed the 'pre-symptomatic' (or sometimes 'pre-clinical') phase of AD may represent a 'window of opportunity' at which time disease prevention and treatment may be more feasible. For several obvious reason, prevention is preferred, in part because delaying the symptom onset by as little as one year is estimated to reduce the number of new AD cases in the US by five million between now and 2030.[69]

2.3.2 Biomarkers of Alzheimer disease pathology

For many years, a clinical diagnosis of AD could only be defined as 'probable', while a definite diagnosis was made from neuropathological evaluation of biopsied or *post mortem* brain. Today several biomarkers of *tau* and Aβ pathology have been developed that may track the progression of AD pathological changes *in vivo*. The first biomarkers to successfully demonstrate differences between clinically diagnosed patients and controls, and to be confirmed to correlate with severity of brain pathology, were cerebrospinal fluid assays of Aβ and *tau* proteins.[70-72] While phosphorylated-*tau* (a marker of neurofibrillary tangle pathology) and total-*tau* (a broader marker of neurodegeneration) levels increase in CSF with AD

pathological changes, $A\beta_{42}$ levels tend to decrease, possibly reflecting the peptide's retention in plaques.[73] It wasn't until the early 2000's that PET tracers with high affinity for fibrillar Aβ were successfully used for imaging of Aβ plaques *in vivo*.[74,75] Tracers binding to *tau* protein were only recently made available,[76] but both Aβ and *tau* tracers have now shown good associations with CSF biomarkers[45,77-82] and brain pathology measured *post mortem*.[83-87] More recently, several groups have developed experimental assays for biomarkers of Aβ and, although far less advanced in their development, *tau* pathologies in blood. While these remain experimental, they would clearly open the possibility for less invasive and easier measures for assessment of AD pathology.[88-90]

2.3.3 Characterization of pre-symptomatic Alzheimer disease

As AD pathology accumulates, fluid and imaging biomarkers become increasingly abnormal. Accordingly, since 2011 the National Institute of Aging and the Alzheimer's Association (NIA-AA) working group has proposed an evolving definition of pre-symptomatic AD based on biomarker abnormality.[11,91,92] An initial definition proposed that measurable Aβ biomarker abnormality alone without evidence of cognitive impairment made up the initial stage of pre-symptomatic AD. When Aβ abnormality was accompanied by evidently abnormal *tau* biomarkers (considered to be markers of ongoing neurodegenerative processes), individuals were considered as being at Stage 2. Finally, Stage 3 was proposed as abnormality in Aβ and *tau* biomarkers as well as subtle cognitive impairment.[11] This original classification appears in our work described in Chapter 3.[93] However, a recent update, benefitting notably from the availability of imaging biomarkers of *tau* pathology, suggests instead the adoption of the 'ATN' classification.[91,92] This approach allows the characterization of each individual purely on pathological grounds depending on the measured level of Aβ **(A)** and *tau* **(T)** pathologies as well as other evidence of neurodegeneration **(N)**. Because there exist several other neurodegenerative disorders which are characterized by *tau* accumulation (otherwise known as tauopathies), this definition only considers one to be on the AD spectrum if they have abnormal Aβ biomarkers. Individuals with *tau* pathology in the absence of brain Aβ deposition are generally suggested to be on a non-AD pathway – although this remains subject to debate[94] – and are classified as having Suspected Non-Alzheimer Pathology, or SNAP.[95] Notably, therefore, this classification relies on a model positing that Aβ abnormality is an *is an initiating event, or is at least necessary, as part of the AD cascade*.[96] However, more recent multivariate

models suggest a strong interaction between several pathological changes early on in pathogenetic processes.[97] A notable exception to this approach may consider striking weaknesses that may occur in correlations between AD pathology and symptoms. Specifically, the ATN method appears to abandon a classical concept of AD as a clinical-pathological entity. As we show in the following section, such an approach may overlook the possibility of interventions that could modify the *symptomatic expression* of a given pathological state, thereby ignoring a genuine possibility of interventions that might alter symptoms, *i.e.,* reduce disease morbidity, without direct effect on pathology.

2.4 Clinico-pathological discordance

While Aβ pathology is a defining characteristic of AD, it is only weakly – if at all—associated with the severity and progression of symptoms at the dementia stage.[98-101] Aβ pathology is therefore necessary but insufficient to develop clinical AD. Meta-analyses suggest that the association of evident Aβ pathology with signs of cognitive impairment is similarly weak in asymptomatic older adults.[102,103] In fact, it has long been noted that some 20-30% of older individuals have evidence of Aβ plaque pathology without cognitive impairment. [104-108] More importantly, however, there is no apparent dose-response relating symptoms to Aβ pathology, such that symptoms will invariably become apparent once there is a sufficient pathological change. Figure 2.3, for instance, shows Aβ-PET data from the Alzheimer's disease neuroimaging initiative (ADNI; http://adni.loni.usc.edu/). On the left is represented the amount of brain Aβ measured in cognitively unimpaired older adults while on the right is the same information from clinically diagnosed AD patients. It is therefore evident that some older adults can have as much if not more evidence of Aβ pathology than some AD patients, and yet remain cognitively unimpaired.

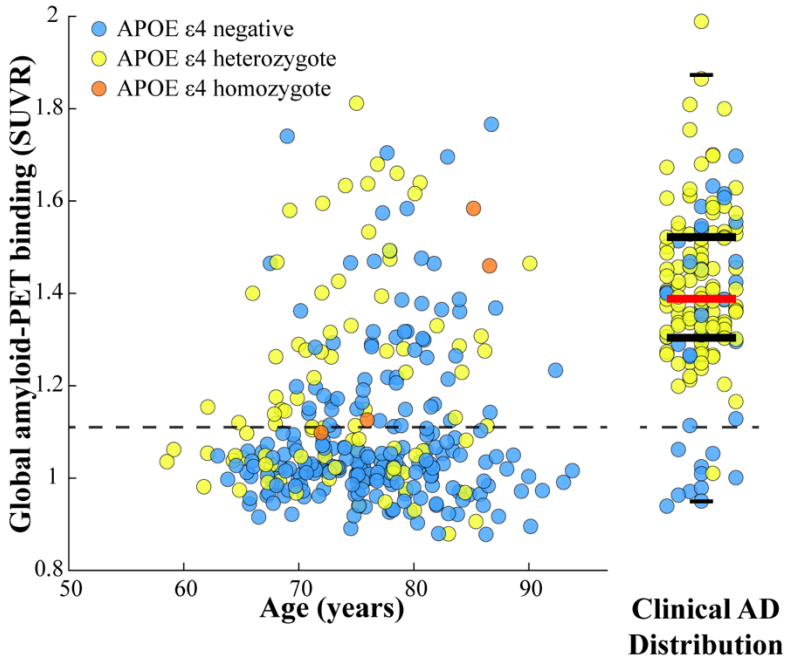

Figure 2.3: Elevated amyloid pathology is not invariably associated with AD dementia. Global Aβ-PET binding reflects the severity of Aβ pathology in the brain. The left part of the graph shows the distribution global Aβ-PET tracer retention by age in **cognitively unimpaired** older adults from the ADNI. The right part of the graph shows the global Aβ-PET tracer retention in clinically diagnosed AD patients from the ADNI (red bar is the group mean, thick black lines are 25th and 75th percentile and small black lines are 1st and 99th percentile). The horizontal dotted line indicates the threshold used to define Aβ positivity. A good proportion of cognitively unimpaired older individuals have substantial evidence of brain Aβ deposition without any evidence of cognitive impairment. SUVR: Standard Uptake value ratio.

This apparent 'disconnect' between degree of AD pathology and the severity of AD symptoms may have important implications for prevention strategies. While degree of Aβ pathology is not related to cognitive symptoms, it is associated with risk of subsequent cognitive decline[109-114] and risk of AD dementia[115,116], particularly so when there is also evident neurodegeneration or *tau* pathology.[117-120] It is evident, however, that some individuals can cope with the pathology thereby avoiding or delaying the onset of AD symptoms. Such observations have given rise to the concepts of *resistance* and *resilience*.[121] In this schema, resistance refers to one's ability to develop less pathology than expected for age; by contrast resilience refers the the development of fewer or less severe symptoms than expected given the amount of AD pathology. The mechanisms that may foster either are not well understood, but it appears possible that resilience may be promoted by certain lifestyle[122,123] or psychiatric characteristics,[124,125] as well as through diverse biological pathways.[126-129]

2.5 Risk factors for Alzheimer disease

2.5.1 Non-modifiable factors

2.5.1.1 Autosomal dominant Alzheimer disease

A small proportion (<1%) of AD cases occur as a result of fully penetrant autosomal dominant mutations. The initial observation that aged individuals with Down syndrome (characterized by trisomy of chromosome 21) had AD-like pathology spearheaded the discovery of genetic loci associated with autosomal dominant AD (ADAD) on chromosome 21[130] and particularly in the APP gene.[24] Additional mutations in genes on chromosome 14[26,131-133] and chromosome 1[27] encoding Presenilin-1 and Presenilin 2 proteins were identified subsequently. The fact that these genetic variants modify the expression of proteins involved in Aβ peptide production and processing in ADAD was taken as strong evidence in support of the amyloid hypothesis of AD pathogenesis. Although ADAD has strikingly similar pathological features to sporadic disease and may therefore promote understanding of AD, it is also free of age-related brain changes and comorbidities that accompany the disease at older age. Thus, there may be important mechanistic distinctions in the development of the two disease forms. ADAD is not central to this thesis' topic and will not be covered in depth.

2.5.1.2 'Sporadic' Alzheimer disease

ADAD or the genetic form of AD is often contrasted with more common (>95% of cases) late-onset or sporadic forms of AD that occur most often after the age of 65 years. However, the now-traditional term sporadic to describe later-onset AD may be questioned given its strong hereditary component.[134,135] In fact, having a first-degree family history (FH) of sporadic AD is associated with a 1 to 14-fold increased risk of dementia.[136,137] This increase in risk may partly be driven by elevated co-occurrence with carriage of the ε4 allele of the *APOE* gene.[138,139] Polymorphisms in this gene, encoding the lipid transport protein apolipoprotein E, is the second most important risk factor after age, portending 4 to 16-fold increased risk of AD dementia.[140,141] However, the advent of Genome Wide Association Studies (GWAS) has also helped identify several more common genes that modify the risk of developing AD more weakly (for review see [142], Figure 2.4). In combination, however, these variants having rather

small individual effects can produce substantial modification in risk and age of onset of dementia.[141]

Another possible congenital risk factor for AD may be biological sex. In fact, most of the individuals living with AD are women.[143] While it has long been debated that this observation may stem solely from increased female longevity in conjunction with increased disease incidence with age, recent work suggests that females may be at increased risk owing to accrued vulnerability particularly to AD-related *tau* pathology.[144-146]

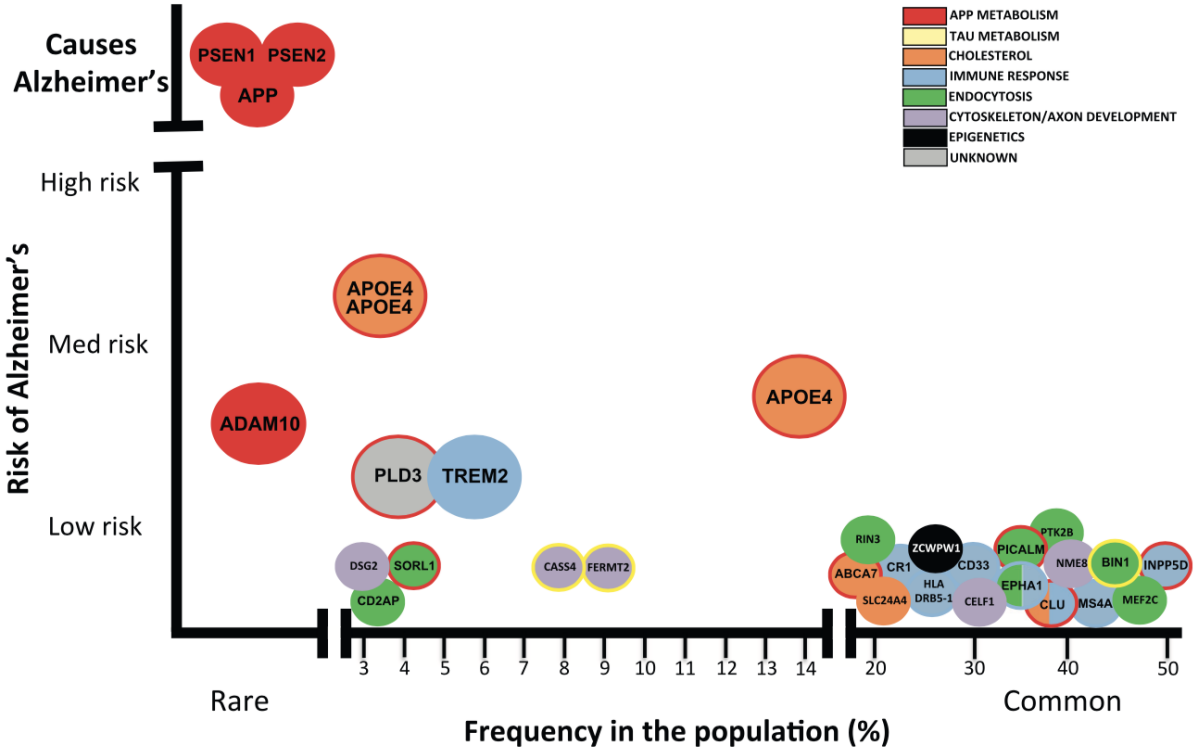

Figure 2.4: Genetic variants associated with AD.
Rare and common variants contribute to Alzheimer's disease risk. GWAS, genome-wide associated studies. Figure and caption adapted with permission from [142].

2.5.2 Modifiable factors

While most of AD risk may be inherited, it has recently been suggested that up to 35% is potentially modifiable.[147] As discussed previously in this Chapter (see *2.4 Clinico-pathological discordance*), some factors may promote cognitive resilience to the presence of AD pathology. These factors therefore also appear to increase 'cognitive reserve' a term intended to denote

increased ability of the brain to remain relatively symptom-free in the presence of AD pathology – perhaps by using compensatory mechanisms.[148] Possible contributors to cognitive reserve have been identified as measures of education, social and cognitive activity as well as stimulating work environment, most likely throughout the lifespan.[149,150] These predictors of cognitive reserve have been associated with attenuated AD risk.[122,123] Similarly, maintained physical activity in middle and late adulthood may also reduce the risk of developing dementia and cognitive decline.[151,152] It is unclear, however, whether this approach to risk reduction may involve reduction in accrual of AD pathology.[153,154] Other health conditions that have been shown to modify AD risk include cardiovascular diseases,[155,156] obesity and a sedentary lifestyle.[157] In fact, cardiovascular risk factors are associated with increased cognitive decline and accruing evidence of AD pathologies[156,158,159] while aggressively treating hypertension has been suggested to reduce incidence of MCI and AD.[160,161]

Among other health conditions associated with modified AD risk, inflammatory diseases such as osteoarthritis have been found to be associated with reduced incidence of AD.[162] This finding in particular was a probably progenitor of the 'immune hypothesis' of AD that emphasizes the importance of inflammatory mechanisms in the pathogenesis of the disease (next section). Although somewhat neglected, this hypothesis was recently brought back following genetic studies linking immune pathways and increased AD risk.[163-166]

2.6 The 'immune hypothesis' of Alzheimer disease

The immune system is generally divided into innate and adaptive components. The adaptive immune system provides a highly specialized and specific response to insult. It is acquired through the presentation of unknown pathogens throughout life. In contrast, the innate immune component is composed of all defense mechanisms present from birth such as physical barriers (*e.g.* skin) or basic mechanisms that recognize evolutionarily conserved structures on pathogens or endogenous stress signals (*e.g.* pattern recognition receptors [PRR]).[167] The recognition of insults by the immune system yields a normal immune cascade otherwise known as inflammation[168] which is mediated by chemical markers such as cytokines and chemokines which activate (*e.g*, interferons[IFN]-γ and interleukin[IL]-1β) and guide (*e.g*, IL-8) immune cells.[169] This process acts locally – by activating tissue resident cells such as macrophages–

and distantly by recruiting and facilitating their entrance to the affected tissue by increasing vascular permeability (Figure 2.5 gives an example of tissue inflammation).

A key aspect of inflammation is that it evolved to remove noxious agents invading the organism, in part by creating a toxic environment for it. If an inflammatory state is prolonged, however, there is a risk of developing inflammatory changes thought to damage host tissues and lead to chronic disease or death (*e.g*, as in osteoarthritis or sepsis).[170,171] Thus, the resolution of inflammation is necessary to guarantee the return to homeostasis. Resolution is an active process that occurs through diverse regulatory pathways.[172] First, a turnover of neutrophils is assured by an increased susceptibility of these cells to undergo apoptosis as they age thereby facilitating their clearance by macrophages.[173,174] The inflammatory state is further reduced when this turnover happens in conjunction with attenuated recruitment of cells by inhibition of pro-inflammatory cytokine production (*e.g*, through the activity of anti-inflammatory mediators such as IL-10)[175] or through competition by receptor antagonists (*e.g*, IL-1RA inhibits biological activity of IL-1α and β).[176] To attain total resolution of the inflammatory response, the clearance of infiltrating immune cells and the termination of further recruitment needs to be followed by the restoration of normal tissue function. In this last step, macrophages play an apparently important role by contributing to regeneration and repair processes.[177] There is some evidence suggesting that apparent lack of resolution is an integral part of AD.[178] Thus, there is growing interest for the role that inflammation may hold in the pathogenesis and progression of the disease.

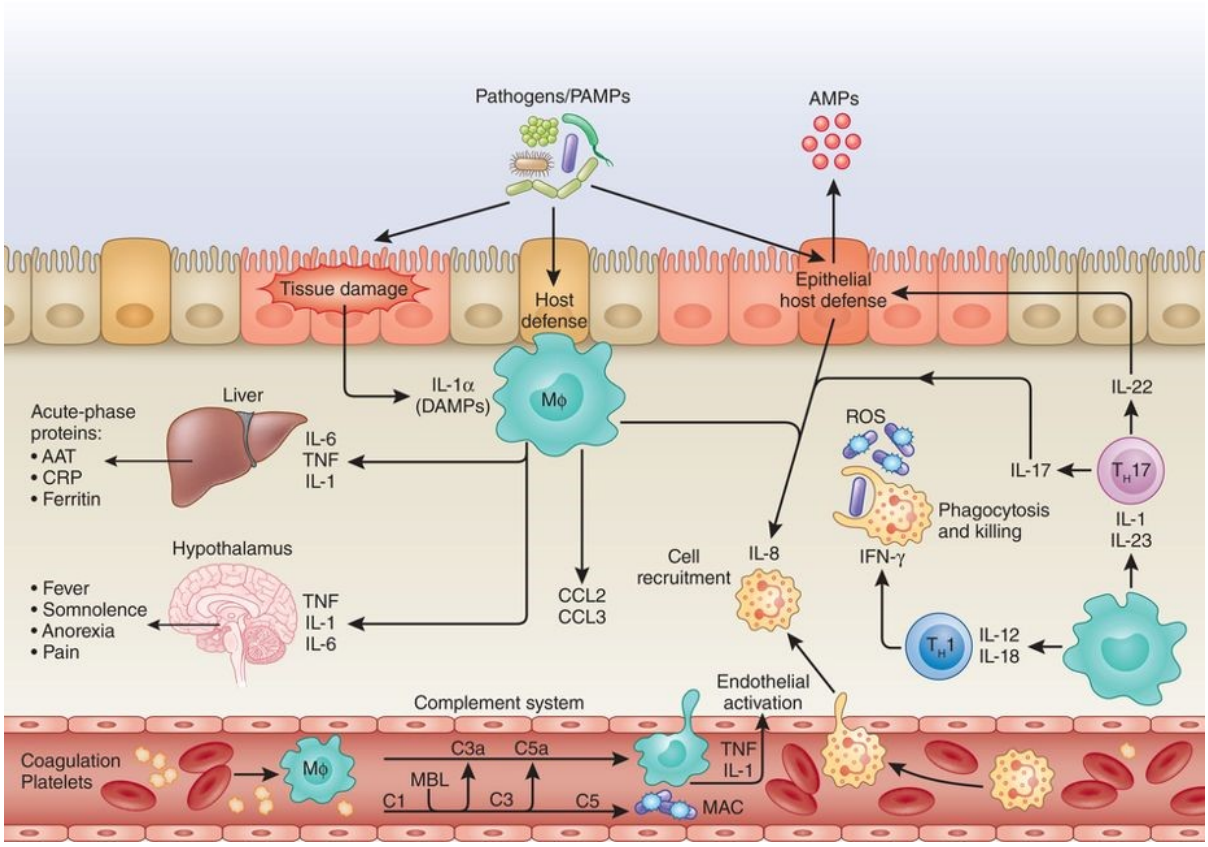

Figure 2.5: Immunological mechanisms of inflammation.
The organism senses pathogens or PAMPs through PRRs thereby initiating an inflammatory response signaling through pro-inflammatory cytokines and chemokines. IL1 and TNF have local and distant effects through activation of macrophages and neutrophils as well as triggering production of acute phase reactants by the liver and physiological symptoms of infection (*e.g.*, fever, pain). They also activate endothelial cells to increase vascular permeability so that cells can be more easily recruited to the site of infection. Additional recruited cells include myeloid (*e.g*, neutrophils, macrophages) and lymphoid (*e.g*, T and B cells, natural killer) cells. AAT, a1-antitrypsin; PAMPs, pathogen-associated molecular patterns; AMPs, antimicrobial peptides; DAMPs, danger-associated molecular patterns; MAC, membrane attack complex; ROS, reactive oxygen species; CRP, C-reactive protein; MBL, mannose-binding lectin; Mf, macrophage or monocyte; CCL, C-C motif chemokine ligand. Figure and caption adapted with permission from [168].

2.6.1 Immune activation in Alzheimer disease

It has long been believed that the brain benefitted from 'an immune privilege'. It is therefore uncertain how much the peripheral immune system is involved in central nervous system (CNS) immune responses.[179] Clearly, however, microglia (the resident macrophages of the brain originating from primitive macrophages in the yolk sac[180-182]) and astrocytes are believed to be primarily involved in CNS immune responses. The initial observation that activated microglia and astrocytes co-localize with AD neuropathological hallmarks led to the hypothesis that 'raging inflammation' contributes to the pathological cascade by inducing a neurotoxic environment.[183-186] This idea was further supported by the observation that aggregates of misfolded proteins may induce microglial responses similar to other pathogens.[187-190] However, microglia and astrocytes function not only as immune cells but are involved also in active housekeeping roles and provide trophic support to neurons that helps maintain CNS homeostasis.[191] The responses they elicit may therefore have both beneficial and deleterious consequences on the functional units of the brain (*i.e.*, neurons).[192]

As illustrated in Figure 2.4, the importance of immune contributions to AD has been reinforced by the discovery of several immune-related genetic polymorphisms associated with disease risk.[163-166,193,194] Among these genetic variants, the triggering receptor expressed on myeloid cells 2 (TREM2) and CD33 have attracted considerable attention. TREM-2 is a trans-membrane receptor expressed by microglia in the CNS that may be implicated in the development of neurodegenerative diseases in general and AD in particular.[195] Studies in animal models of AD suggest that TREM-2 may facilitate microglial migration and activation to better deal with accruing AD pathology.[196-205] Accordingly, the R47H mutation at the *TREM*-2 locus encodes a loss-of-function variant that (while it is relatively rare) is associated when present with an increase in human AD incidence comparable to that of *APOE*-ε4.[206] Similarly, disease causing CD33 mutations are associated with impaired microglia ability to clear Aβ plaques.[207-209] Nonetheless, an overactive or maladaptive immune response to pathology may yield dramatic damage as demonstrated particularly through activation of the complement system,[210-213] inflammasomes,[214] or microglia-induced reactive astrocytes.[215] Importantly, even in the well-controlled setting of animal model studies, there is no consensus. For instance, TREM-2 expression has been shown to aggravate *tau* pathology in tauopathy models.[216-218]

2.6.2 Immune modifying treatments for Alzheimer disease

In the early 1990's, Patrick McGeer and colleagues observed that co-existence of AD and rheumatoid arthritis (RA) in autopsy data was surprisingly rare.[162] Because McGeer had earlier observed that inflammatory processes were vigorous near AD lesions, and because RA is often treated with anti-inflammatory drugs, he suggested that these drugs could be responsible for low prevalence of AD changes. Subsequent epidemiological studies from the 1990's and early 2000's identified an inverse relationship between NSAID use and incidence of AD.[219-223] These findings were not replicated, however, in studies that used improved assessment of exposure classification or considered older populations. These suggested little or no benefits of NSAID use.[224-227] This apparent lack of benefit was reinforced by results from randomized controlled trials in persons with AD dementia or MCI. These showed either negative results,[228] or acceleration of disease progression.[229,230] Prevention trials using similar drugs yielded comparable results, varying between no or deleterious effects of NSAID use,[231,232] although the 2011 ADAPT paper did suggest a possible protective effect of naproxen several years after treatments had been started (and then stopped). These observations led some to conjecture that the effects of NSAIDs might depend on timing of administration, possibly having a mixed effect with acceleration of symptom onset in those with more advanced pathology.[233,234]

2.6.3 Biomarker studies of immune processes

Inflammatory processes in AD have been less extensively studied in humans than in animal models. One reason for this may be the lack of a validated human biomarker of AD-related neuro-inflammation. Experimental approaches using plasma and CSF assays of typical immune mediators to compare inflammatory states of AD patients and matched controls have yielded conflicting results.[235] However, the recent renewed interest in immune involvement in AD has yielded an increasing number of studies. Several recent studies have shown that multiple inflammatory mediators in CSF (and plasma) are strongly associated with biomarkers of AD progression.[236-243] However, there may be some non-linearity in the association between CSF biomarkers of AD progression and immune activation, possibly owing to a stronger association of immune activation with *tau* biomarkers than with evidence of Aβ plaques.[244-246]

Recently, positron emission tomography (PET) tracers have also been developed to attempt imaging of microglial activation *in vivo*.[247] These tracers bind the 18kDa translocator protein (TSPO), a transmembrane protein expressed by activated microglia and astrocytes.[248] Unfortunately, these tracers have the important limitation that about a third of individuals have a genetic mutation such that they do not express TSPO.[249] This mutation does not, however, seem to affect the expression of AD dementia,[250] and studies using these tracers confirm CSF observations that AD subjects have increased microglial activation compared to healthy controls.[251,252] Even so, this increase may not occur completely linearly.[253] Given the relative novelty of *tau* PET ligands, only one study to date has considered *both* Aβ and *tau* burden in relation to microglial activation and it tends to confirm CSF findings that microglial activation may occur concurrently with local *tau* deposition.[254] Interestingly, increased PET TSPO tracer uptake is associated with disease severity, but higher binding in AD patients is associated with attenuated subsequent cognitive decline.[255,256] The presence of TSPO signal in two different cell types makes the interpretation of findings difficult. Nonetheless, they seem to support that immune activation is an integral part of the AD process even if some immune responses to pathology may promote improved clinical outcomes while others aggravate the clinical phenotype.

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Chapter 3 – Bi-directional association of cerebrospinal fluid immune markers with stage of Alzheimer's disease pathogenesis

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3.1 Preamble

Prior to this work, mostly case-control studies had investigated differences in immune marker levels between cognitively unimpaired controls and patients with either mild cognitive impairment or AD dementia. This approach seemed unreliable given that results were largely inconsistent across studies.[1] However, one interesting result from Alcoolea *et al*. was that some immune markers seemed not to so much to be associated with biomarker evidence of Aβ pathology but rather were increased in relation to increasing evidence of *tau* pathology.[2] In the published work that is Chapter 3, we investigated specifically whether such a biphasic association existed between markers of immune activation and AD biomarkers in cerebrospinal fluid, independent of diagnosis. We first observed an unexpected pattern in a cohort of healthy elderly at high risk of AD owing to a family history of the disease. To confirm that it was not artifactual, we searched for a similar *pattern* in a larger group of individuals without dementia (healthy and with MCI) from the ADNI, whose CSF had been successfully assayed for 83 proteins. The observed "chevron" pattern was recently replicated at all stages of the disease (healthy, MCI and AD) for CSF sTREM2, an important marker of immune activation in AD.[3] The work of this Chapter is published in the *Journal of Alzheimer's disease.* 2018;63(2):577-590. doi: 10.3233/JAD-170887.

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3.2 Abstract

Immune mechanisms may be important in the pathogenesis of Alzheimer disease (AD). Yet, studies comparing healthy and demented individuals' cerebrospinal fluid (CSF) and plasma immune marker levels have yielded conflicting results. We analyzed CSF from 101 members of the parental history-positive PREVENT-AD cohort of healthy aging adults, and 237 participants in the initial cohort of the Alzheimer's Disease Neuroimaging Initiative (ADNI-1). Following recent practice, we used the biomarkers total-*tau* and amyloid-$\beta_{1\text{-}42}$ to allocate participants from each study into four stages of AD pathogenesis: Stage 0 (no abnormality), Stage 1 (reduced amyloid-$\beta_{1\text{-}42}$), Stage 2 (reduced amyloid-$\beta_{1\text{-}42}$ and increased total-*tau*), or "Suspected Non-Alzheimer Pathology" (elevated total-*tau* only). Investigating the PREVENT-AD participants' CSF assay results for 19 immune/inflammatory markers, we found six that showed a distinct bi-directional relationship with pathogenetic stage. Relative to Stage 0, these were diminished at Stage 1 but strongly increased at Stage 2. Among 237 ADNI participants without dementia (90 healthy controls and 147 with mild cognitive impairment), we found that 23 of 83 available CSF markers also showed this distinct pattern. These results support recent observations that immune activation may become apparent only after the onset of both amyloid and *tau* pathologies. Unexpectedly, they also suggest that immune marker activity may *diminish* along with earliest appearance of amyloid-β plaque pathology. These findings may explain discordant results from past studies and suggest the importance of characterizing the extent of AD pathology when comparing clinical groups.

3.3 Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized by extracellular amyloid-beta (Aβ) plaques, intracellular phosphorylated *tau* neurofibrillary tangles, and widespread synapse loss. It is widely acknowledged that a decades-long period of pathogenetic change precedes the appearance of dementia.[1-3] Immune mechanisms are thought to be important in this process.[4] In the early 1990's, McGeer, McGeer, Rogers, Sibley [5] observed that co-existence of AD and rheumatoid arthritis (RA) in autopsy data was rare. Because these investigators had earlier observed that inflammatory processes were vigorous in the vicinity of AD lesions,[6] and because RA is often treated with anti-inflammatory drugs, they suggested that the latter might explain the low prevalence of AD changes in RA patients. Ensuing epidemiological studies identified an inverse relationship between the use of non-steroidal anti-inflammatory drugs and incidence of AD For review see, [4]. More recently, genetic studies have identified immune/inflammatory pathways as modifiers of AD dementia risk.[7-10] Inflammatory changes are therefore widely thought to represent a response to appearance of AD pathology, increasing linearly with disease progression. It is commonly assumed that such inflammatory activity is harmful.

In fact, this notion has not been verified *in vivo* by measures in plasma or cerebrospinal fluid (CSF).[11] Instead, investigations in fully penetrant autosomal dominant familial AD suggest that significant increases in immune signaling may only occur *after* the appearance of both Aβ and *tau* pathologies.[12] Similarly, other studies investigating the association of single CSF immune markers and Alzheimer pathology have suggested that these markers *decline* alongside the reduced $A\beta_{1-42}$ concentration that typifies early AD pathogenesis.[13-15] Here, we describe multi-analyte investigations of the association between AD pathogenesis, as revealed by CSF biomarkers, and several immune/inflammatory proteins. We report initial observations in the high-risk PREVENT-AD cohort of healthy older persons, followed up with analyses of CSF from the Alzheimer's Disease Neuroimaging Initiative (ADNI-1). Our objective was to determine whether variations in AD pathological "stage" [16,17] related to CSF immune and other protein levels, and could therefore explain conflicting results from past studies.

3.4 Methods

3.4.1 Participants

PREVENT-AD participants (Table 3.1) were 101 cognitively intact volunteers with a parental or multiple-sibling history of "sporadic" AD.[18] They scored an education-adjusted ≥23/30 on the Montreal Cognitive Assessment,[19] and had little if any difficulty with subsequent cognitive testing. Most were 60 years of age or older, but persons aged 55-59 years were eligible if their age was within 15 years of their youngest-affected relative's onset. Each participant and study partner provided written informed consent. All procedures were approved by the McGill University Faculty of Medicine Institutional Review Board. All research complied with ethical principles of the Declaration of Helsinki.

ADNI-1 data were downloaded in February 2016 from http://adni.loni.usc.edu. ADNI was launched in 2003 as a public-private partnership led by Principal Investigator Michael W. Weiner. Its primary goal has been to test whether serial magnetic resonance imaging, positron emission tomography (PET), and various clinical, biological and neuropsychological markers can be combined to measure progression of MCI and early AD. Given our primary interest in analyses of ADNI participants *before* onset of dementia, we limited analyses to 237 ADNI-1 participants with available CSF data in the Healthy Control (n = 90) and MCI groups (n = 147), the latter to enrich for persons who might have Aβ or *tau* pathology (Table 3.1).

3.4.2 Cerebrospinal fluid measurements

Lumbar punctures (LPs) in PREVENT-AD volunteers were performed following an overnight fast using the Sprotte 24-gauge atraumatic needle. Samples of 20-30 mL were aliquoted (500µL) into propylene cryotubes and stored at -80°. We used procedures from the BIOMARK-APD consortium of the EU Joint Program in Neurodegenerative Disease, to measure CSF concentrations of the AD biomarkers $A\beta_{1\text{-}42}$, total-*tau* (t-*tau*) and P_{181}-*tau* (P-*tau*) using the Innotest enzyme-linked immunosorbent assay kit (Fujirebio, Ghent, Belgium). CSF apolipoprotein-E (apoE) levels were assayed using the Milliplex APOMAG-62k human apolipoprotein cardiovascular disease multiplex kit (EMD-Millipore, Billerica, MA, USA).

To characterize immune/inflammatory status, we attempted to assay CSF concentrations of 45 cytokines and chemokines using a combination of the Milliplex HCYTMAG60PMX29BK xMap kit (EMD-Millipore; Appendix 1 Methods) and the Mesoscale V-plex neuro-inflammation panel-1 (Mesoscale Discovery, Rockville, MD). Detailed procedures for the latter have been described elsewhere.[20] We excluded measures that fell outside the useful assay range. We also excluded markers with excessive assay variation, as measured by a mean coefficient of variation exceeding 15%. We then performed an outlier analysis to identify and remove from consideration assay values above the third or below the first quartile ± 1.5 times the interquartile distance.[21] After applying these QC restrictions, there remained 10 markers (out of 21 initially) that were assayed using both Mesoscale and Luminex technologies. We compared the two techniques' agreement and range of values for each analyte. When necessary, we chose the marker assay with more quality-control acceptable readings or without ceiling or floor effects. We excluded analytes having ≤50 quality-control-acceptable readings from at least one assay technique. Nineteen immune marker assays met these standards.

The ADNI investigators had measured CSF $A\beta_{1-42}$ and t-*tau* concentrations using Research Use Only INNOBIA AlzBio3 immunoassay reagents (Fujirebio) on an xMap Luminex platform (http://adni.loni.usc.edu/methods/biomarker-analysis/). CSF levels of 83 other proteins had been assayed via a multiplex x-Map kit from Rules Based Medicine (MyriadRBM, Austin, TX). Rigorous quality control standards excluded markers with >10% missing data. Results were then normalized using boxcox transformation, etc. (for details see http://adni.loni.usc.edu/wp-content/uploads/2012/01/2011Dec28-Biomarkers-Consortium-Data-Primer-FINAL1.pdf.)

3.4.3 Pathological staging of participants

Following recent convention,[16,17] we used CSF biomarker data to characterize extent of AD pathology in both PREVENT-AD and ADNI. Disease progress was classified as Stage 0 (normal CSF $A\beta_{1-42}$ and t-*tau*), Stage 1 (low $A\beta_{1-42}$, low t-*tau*), Stage 2 (low $A\beta_{1-42}$ and elevated t-*tau*), and Suspected Non-Alzheimer Pathology (SNAP, high t-*tau* only). Very few PREVENT-AD participants met the typical "below-normal amyloid" threshold of 550 pg/mL. However, that threshold was intended to discriminate healthy controls from subjects with AD *dementia*, and may be too stringent for identification of persons with early Aβ pathology.[22]

Given recent evidence that increasing amyloid burden (reflected by decreasing CSF amyloid concentration) portends poorer clinical outcomes even before typical thresholds are crossed,[23-25] we chose a more sensitive cut-off value for $A\beta_{1-42}$ abnormality at the 25th percentile value or <870 pg/mL. Similarly, we specified a t-*tau* abnormality threshold at the 75th percentile, or >335 pg/mL (Fig. 3.1A). Since ADNI analyses, which used different assay methods, had included individuals with MCI we adopted the recommended Aβ and *tau* thresholds of <192 pg/mL and >93 pg/ml (Fig. 3.1B).[26] Because our PREVENT-AD thresholds were somewhat arbitrary, and because we wished if possible to increase contrasts between stages, we decided *a priori* to exclude data from 19 PREVENT-AD individuals whose marker concentrations were within ±5% of either the Aβ or *tau* threshold.

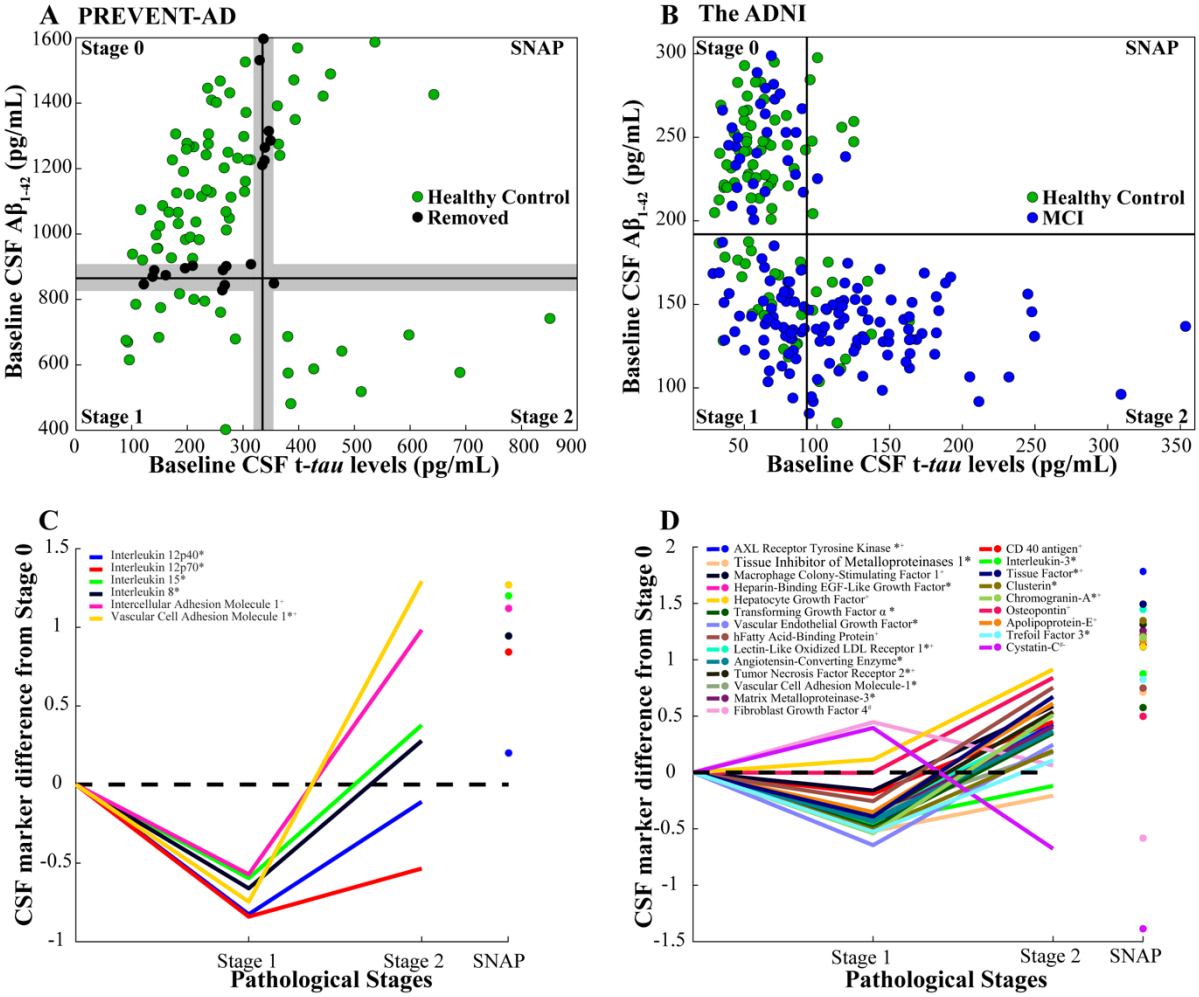

Figure 3.1: CSF Markers and pathological staging of Alzheimer's disease.
(A) 101 healthy PREVENT-AD participants were classified as Stage 0, 1, 2 or SNAP based on $A\beta_{1\text{-}42}$ (869.75 pg/mL) and t-tau (334.6 pg/mL) thresholds. Among them, to enhance contrasts, nineteen (black) with CSF Aβ and t-tau levels within ±5% of inter-stage thresholds were removed a priori from consideration. (B) ADNI healthy-control (green) and MCI (blue) participants were similarly classified using the project's recommended thresholds for $A\beta_{1\text{-}42}$ (192 pg/mL) and t-tau (93 pg/mL). (C) and (D) Linear models, adjusted for participants' age, gender, APOEε4 carrier status and (for ADNI only) clinical diagnostic category, were used to assess CSF protein marker level differences by stage. CSF marker data were standardized using z-scores. The β coefficients for stage differences from these models are represented. In PREVENT-AD (C), six CSF markers were associated bi-directionally with pathological stage. Five were lower (*) in Stage 1 vs. Stage 0, and two were greater ($^{+}$) in Stage 2 vs. Stage 0 ($P \leq 0.05$ uncorrected). In ADNI (D) 23 CSF markers were either increased ($^{\#}$) or decreased (*) at Stage 1 and at Stage 2 ($^{+}$ or $^{-}$) vs Stage 0. All 23 were significantly different at Stage 2 vs. Stage 1 ($P_{FDR} \leq 0.05$).

3.4.4 *APOE* genotyping

In PREVENT-AD, *APOE* genotype was determined using the PyroMark Q96 pyrosequencer (Qiagen, Toronto, ON, Canada). DNA was amplified using RT-PCR with primers rs429358 amplification forward 5'-ACGGCTGTCCAAGGAGCT G-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'-CGATGACCTGCAGAAG-3'. In ADNI, *APOE* genotypes had been determined using DNA extracted by Cogenics (Beckman-Coulter, Pasadena, California) [27].

3.4.5 Statistical analyses

In PREVENT-AD, we used multiple linear regression to examine the association of standardized (z-score) concentrations of the 19 immune markers or apoE with pathological stage. To examine agreement of our results with prior studies, we assessed association of these same marker scores with the individual CSF AD biomarkers $A\beta_{1-42}$, t-*tau*, P_{181}-*tau* and the t-*tau*/$A\beta_{1-42}$ ratio. To meet model assumptions, we log-transformed P-*tau* and t-*tau,* and used boxcox transformation to normalize the t-*tau*/Aβ ratio. Models were adjusted for participant age, gender and *APOE* ε4 carrier status. P-values were corrected, as elsewhere, for multiple comparisons using the false discovery rate (FDR) method [28] specifying two-sided $\alpha = 0.05$. Analyses relied on Matlab (Mathworks inc.; Natick, Massachusetts), SPSS24 (IBM Corp.; Armonk, NY) or R v3.2.2.

We then tested the robustness of our PREVENT-AD results in the ADNI-1 cohort. To deal with the multiplicity of available ADNI protein markers, we used Bayes factor analysis, employing Matlab's CGBayesNets,[29] to identify and remove molecular species unlikely to be associated with pathological stage. The Bayes' Factor is the ratio of posterior likelihoods of the data being associated with vs. independent of pathological stage.[30] We kept only individual markers with a (logarithmic) Bayes' Factor > 0, thus providing any evidence in favor of an association between marker levels and pathological stage. Multiple linear regression models then identified markers that differed by stage after adjustment for participant age, gender, *APOE* ε4 carrier status and clinical diagnostic group. As in PREVENT-AD, we also used

regression models to assess association of these markers with $A\beta_{1-42}$, t-*tau*, P_{181}-*tau* and the t-*tau*/$A\beta_{1-42}$ ratio.

3.5 Results

3.5.1 Demographic characteristics

Demographic characteristics of both the PREVENT-AD and ADNI-1 participants are summarized in Table 3.1. Whereas PREVENT-AD included 70% women, ADNI-1 had a majority of men. ADNI participants were also more than a decade older on average. The table indicates the two groups' distributions by pathological stage. As expected, ADNI included larger proportions of persons at Stages 1 or 2.

Table 3.1: PREVENT-AD and ADNI demographics

	PREVENT-AD					ADNI-1				
	All participants	Stage 0	Stage 1	Stage 2	SNAP	All participants	Stage 0	Stage 1	Stage 2	SNAP
Sample	101‡	51	12	9	10	237	78	71	79	9
Age Mean (sd)	62.90 (5.56)	62.80 (5.31)	60.91 (5.52)	63.99 (4.39)	64.52 (8.66)	75.26 (6.68)	75.24 (6.71)	75.48 (6.09)	74.77 (7.40)	77.89 (4.04)
% Male	30.69	35.29	25	55.56	30	61.60	64.10	61.97	62.03	33.33
% *APOE4*-positive	36.63	31.37	50	88.89	20	42.19	10.26	52.11	68.35	11.11
Clinical Diagnostic Category (HC: MCI)	101: 0	51: 0	12: 0	9: 0	10: 0	90: 147	49: 29	23: 48	11: 68	7: 2
Education (y) Mean (sd)	14.88 (2.93)	15.00 (2.89)	14.92 (2.35)	13.67 (3.32)	15.1 (1.59)	15.83 (2.94)	15.82 (2.76)	15.76 (3.15)	15.73 (2.94)	17.33 (2.79)
CSF $A\beta_{1\text{-}42}$ (pg/mL) Mean (sd)**	1062.91 (280.65)	1161.58 (158.54)	705.02 (115.67)	611.35 (85.68)	1422.15 (113.63)	178.50 (56.06)	244.41 (24.71)	146.44 (22.31)	133.95 (21.90)	251.20 (28.32)
CSF t-tau (pg/mL) Mean (sd)**	273.09 (129.97)	221.94 (56.14)	177.59 (73.16)	522.45 (162.64)	435.48 (90.79)	92.17 (49.85)	58.08 (15.30)	67.47 (17.38)	146.15 (47.97)	108.58 (12.95)
CSF P-tau (pg/mL) Mean (sd)**	46.83 (18.00)	40.25 (10.37)	32.70 (10.71)	75.47 (19.89)	72.29 (11.45)	31.91 (15.94)	20.14 (7.20)	27.20 (13.00)	47.83 (11.95)	31.33 (8.92)

pg/mL, picograms per milliliter; sd, standard deviation; %APOE, proportion of *APOE* ε4 carriers; SNAP, Suspected Non-Alzheimer Pathology; HC, healthy controls; MCI, mild cognitive impairment; **ADNI and PREVENT-AD used different assays to measure AD biomarkers. ‡ To enhance contrast, 19 individuals within $\pm$ 5% of inter-stage thresholds were not assigned to a stage, except as noted in text. See text also for definition of the several stages.

3.5.1 PREVENT-AD immune marker concentrations and pathological stage

After categorizing PREVENT-AD participants by pathological stage, FDR-adjusted models revealed only trend-level differences in the 20 analyzed markers when comparing Stage 0 *vs*. Stage 1 or Stage 2. Inference here was limited, however, by the small number of individuals at Stages 1 and 2. Omitting FDR adjustment, we observed a consistent bi-directional pattern of association between six marker scores and pathological stage, with reduced concentrations at Stage 1 and/or increased levels at Stage 2 (Table 3.2, Figure 3.1C). Because a *post hoc* power analysis indicated that we had less than the desired 90% power to observe a statistically significant effect for IL-12p40, however, results with this latter marker should be interpreted cautiously. As well, IL-15, IL-8, ICAM-1 and VCAM-1 showed significant elevation at Stage 2 *vs.* Stage 1 (Fig. 3.1C). Despite the small size of the SNAP sample, this group showed elevation after FDR adjustment in all of the above-mentioned markers as well as Granulocyte Colony-Stimulating Factor (GCSF), Interferon Gamma-Induced Protein 10 (IP-10) and IL-16 (Appendix 1 Table 1).

Table 3.2: PREVENT-AD and ADNI cerebrospinal fluid markers associated with Alzheimer's Disease pathological stages

PREVENT-AD	ADNI	
IL-12p40	AXL Receptor Tyrosine Kinase	Chromogranin A
IL-12p70	CD40 antigen	Cystatin-C
IL-8	IL-3	Fibroblast Growth Factor 4
IL-15	Macrophage Colony Stimulating Factor-1	Matrix Metalloproteinase-3
Soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1)	Heparin-Binding EGF-Like Growth Factor	Osteopontin
Soluble Intercellular Cell Adhesion Molecule-1 (sICAM-1)	Hepatocyte Growth Factor	Tissue Inhibitor of Metalloproteinases-1
	Transforming Growth Factor α	Tumor Necrosis Factor Receptor-2
	Vascular Endothelial Growth Factor	Vascular Cell Adhesion Molecule-1
	Heart Fatty Acid Binding Protein	Apolipoprotein-E
	Lectin like Oxidized LDL-Receptor-1	Clusterin (Apolipoprotein J)
	Angiotensin Converting Enzyme	Trefoil Factor-3
	Tissue Factor	

3.5.2 PREVENT-AD immune marker concentrations and AD biomarkers

In corroborative analyses, we found that apoE and seven of the 19 CSF immune markers in PREVENT-AD increased with t-*tau*/A$\beta_{1\text{-}42}$ ratio ($P_{FDR} \leq 0.05$, Appendix 1 Table 2). Levels of these markers increased especially with t-*tau* and P-*tau*. Interestingly, four markers (apoE, Interleukin[IL]-15, IL-8 and IL-12p70) also *increased* with Aβ concentration, suggesting

reduced plaque burden.[31,32] Although initially unexpected, this association between increasing levels of immune markers and Aβ has been observed previously.[12-15] It provides one possible explanation for the bidirectional pattern in Fig. 3.1C.

3.5.3 ADNI protein marker concentrations, pathological stage, and AD biomarkers

Given the limited size of the PREVENT-AD sample, we wished to verify whether this bi-phasic pattern generalized to other proteins assayed in CSF. Thus, we pursued similar analyses in ADNI-1, including participants with MCI. Bayes factor analysis reduced the 83 ADNI CSF protein markers to 38 showing any likelihood of association with pathological stage (Fig. 3.2). Multiple linear regression modeling then showed that 23 of these differed significantly between Stage 0 and Stage 1 or Stage 2 ($P_{FDR} \leq 0.05$; Fig. 3.1D, Appendix 1 Table 3). Marker results by stage again revealed bi-directional patterns, with all but two markers showing reduction at Stage 1 and/or increase at Stage 2. Exceptions were Cystatin-C and Fibroblast Growth Factor-4 (FGF-4), which showed the opposite pattern. Importantly, all remaining markers were increased at Stage 2 *vs.* Stage 1. As in PREVENT-AD, all but three of these (Interleukin-3, Tissue Inhibitor of Metalloproteinase-1 and FGF-4) correlated overall with t-*tau*/$A\beta_{1-42}$ ratio (Appendix 1 Table 4).

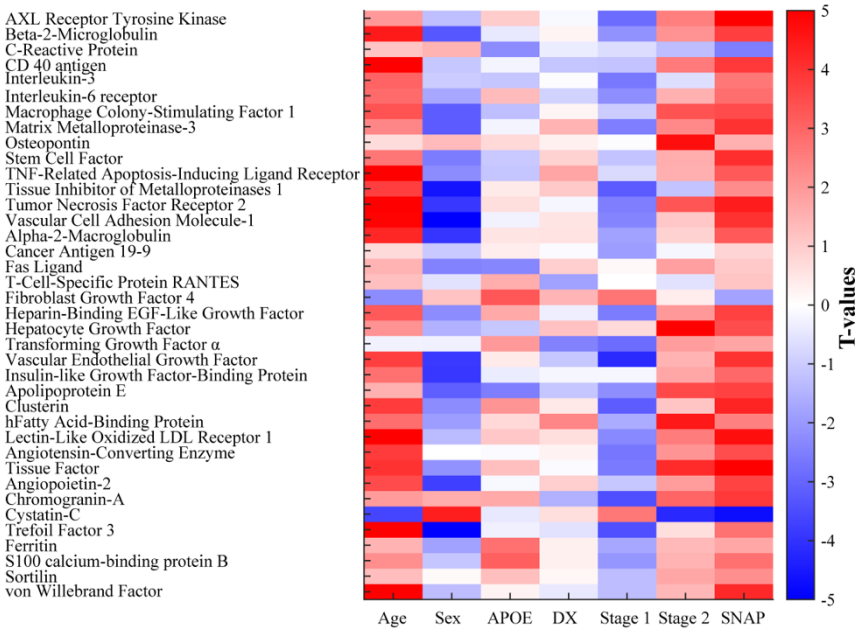

Figure 3.2: Bayes factor analysis identifies 38 ADNI CSF markers potentially associated with pathological stage.
Shown is a matrix representation of linear regression models predicting association of 38 CSF marker levels with pathological stage with adjustment for age, gender, clinical diagnostic category and *APOE* status. T-values for each association are reported. For categorical variables, the results are shown as comparisons against a reference. For gender, the reference category is male; for APOE it is non-carriage of ε4; for diagnosis (Dx), healthy control; and for pathological stage, Stage 0 (no abnormality).

Only three markers (apoE, VCAM-1 and Vascular Endothelial Growth Factor [VEGF]) were common to analyses of the PREVENT-AD and ADNI samples. In both groups, VCAM-1 and apoE correlated well with t-*tau*/ $A\beta_{1\text{-}42}$ ratio (Fig. 3.3) and showed similar patterns of association with pathological stages, although results were less robust in PREVENT-AD. These associations failed to generalize for VEGF (Appendix 1 Tables 1 and 3). *APOE* carrier status and clinical diagnostic group did not contribute in our models, as illustrated in Table 3.3 and Figure 3.2.

Table 3.3: Coefficients of fully adjusted linear regression models evaluating the association of CSF markers with pathological stage

	Age (y)	Gender	*APOE* ε4 carrier status	Clinical Diagnostic Group	Pathological Stage
PREVENT-AD					
apoE	β: 0.00 p: 0.29	β: 0.08 p: 0.71	β: –0.08 p: 0.74		**β: 0.32 p: 0.002**
VCAM-1	β: 0.00 p: 0.20	β: 0.15 p: 0.58	β: –0.28 p: 0.29		**β: 0.40 p: 0.002**
VEGF	β: 0.00 p: 0.23	β: –0.27 p: 0.29	β: –0.16 p: 0.56		β: 0.03 p: 0.77
ADNI					
apoE	β: 0.01 p: 0.22	**β: –0.39 p: 0.002**	**β: –0.47 p: 0.0006**	β: –0.22 p: 0.10	**β: 0.38 p: 5.38E-7**
VCAM-1	**β: 0.04 p: 5.37E-6**	**β: –0.68 p: 7.08E-8**	β: –0.20 p: 0.12	β: –0.06 p: 0.63	**β: 0.24 p: 7.19E-4**
VEGF	**β: 0.03 p: 1.39E-3**	**β: –0.47 p: 3.26E-4**	β: –0.16 p: 0.24	**β: –0.28 p: 0.04**	**β: 0.29 p: 1.54E-4**

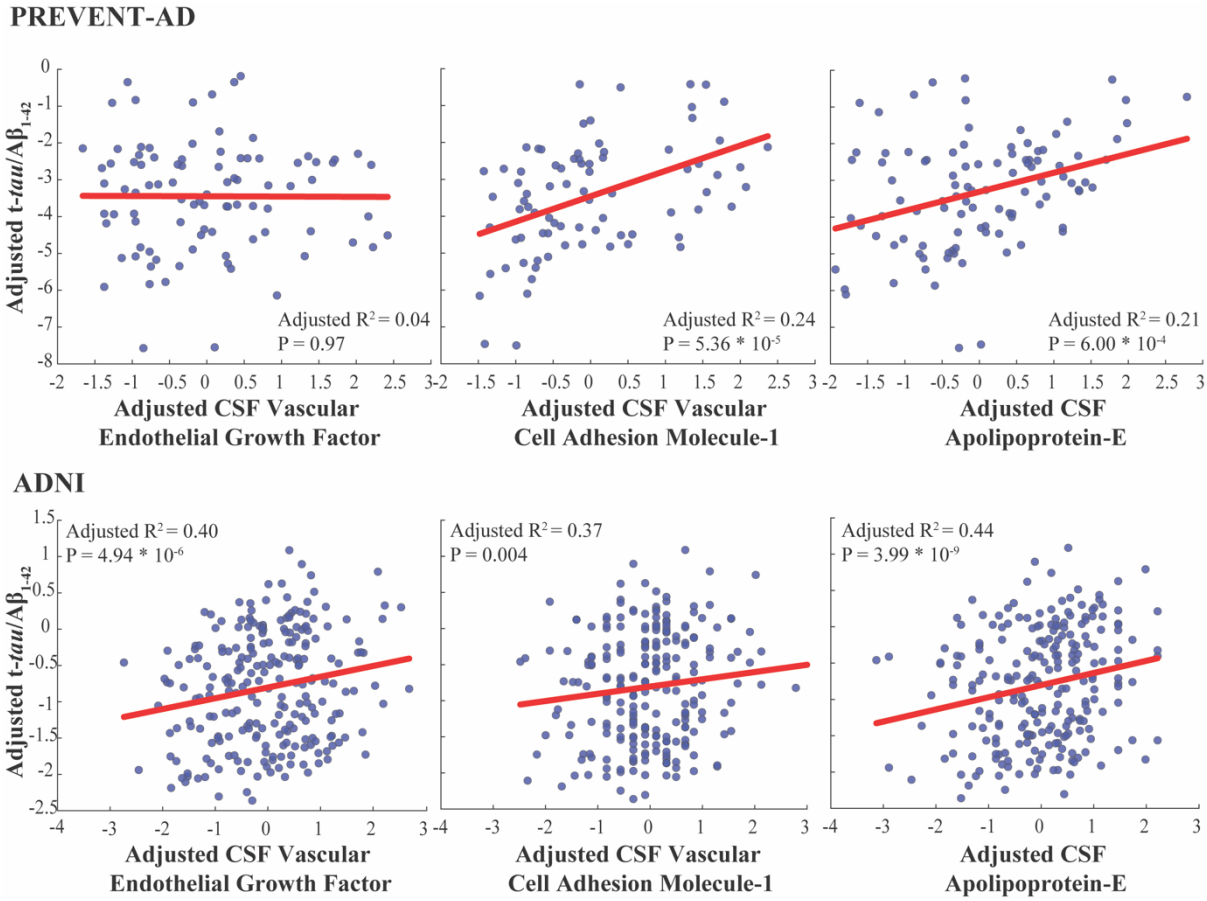

Figure 3.3: Markers common to both cohorts show similar association with AD pathology.
Adjusted response plots show the association of apolipoprotein-E level, VEGF and VCAM-1 with t-*tau*/A$\beta_{1\text{-}42}$ ratio. R^2 values are given for the fully adjusted model, and P-values are reported for the given CSF marker in this model.

3.6 Discussion

Immune processes are important in the pathogenesis of AD.[4] For example, activated microglia and astrocytes co-localize with amyloid plaques in the brains of AD animal models and individuals with dementia.[6]

In the high-risk but cognitively unimpaired PREVENT-AD cohort, we observed a distinct bi-directional relationship between six immune marker levels and pathological stage, an approximate indicator of disease progression. This non-linear pattern appeared despite a direct overall correlation between four of these markers with t-*tau*/A$\beta_{1\text{-}42}$ ratio. In a corroborative analysis of the ADNI-1 cohort including 90 HCs and 147 individuals with MCI having CSF measurements for 83 proteins, we identified a similar pattern of change with advancing AD

pathology. As described previously by others,[12-15] CSF immune marker levels increased overall with advancing AD pathology in both cohorts. However, an important refinement of this trend suggested that such marker levels increased strongly only after appearance of *tau* abnormality. Indeed, a number of CSF proteins, including immune markers, appeared to *decrease* with decline in $A\beta_{1-42}$ (*i.e.*, with increasing plaque burden).

PREVENT-AD: Increased CSF immune activity with advancing AD pathology

Studies in the PREVENT-AD cohort are of particular interest because they focus on changes in the pre-symptomatic or very early-symptomatic evolution of AD pathology. This characteristic of the sample enabled us to examine closely the stages of transition between absence of AD-related changes through early evidence of *tau* pathology. In findings similar to those of others,[20] increases in the t-*tau*/$A\beta_{1-42}$ ratio were accompanied by higher concentrations of IL-15, MCP-1, ICAM-1 and VCAM-1. Observations in this cohort that immune markers tended to increase only after the appearance of *tau* abnormality are consistent also with recent observations in autosomal dominant AD.[12] Furthermore, the pattern is reminiscent of that observed for YKL-40 in pre-clinical "sporadic" AD.[14]

ADNI: Bi-directional pattern of association between CSF proteins and pathological stage

Studies in the ADNI sample provided complementary evidence to PREVENT-AD. While ADNI included healthy individuals, these were on average a decade older. In addition, ADNI considered individuals with more severe cognitive symptoms who were accordingly more likely to have advanced pathology. This feature provided a notable opportunity to verify the bi-directional pattern observed in PREVENT-AD. Some 25% of the markers analyzed showed the expected pattern. Interestingly, again most of these proteins also showed a correlation with the t-*tau*/$A\beta_{1-42}$ ratio.

These results suggest that, independent of clinical diagnosis, there exists a moderate early decrease in CSF immune markers that is not captured by t-*tau*/$A\beta_{1-42}$ ratio. This pattern appears primarily related to decreasing CSF $A\beta_{1-42}$. Because, in both samples, CSF t-*tau* accounts for more variance in the ratio than does $A\beta_{1-42}$, this effect may ordinarily be hidden by the stronger—opposite– association with *tau*.

Nature of markers showing the bi-directional pattern

PREVENT-AD markers that exhibit a bi-directional pattern of association with stage are readily recognized as markers of neuro-immune processes. The IL-12/23 signaling pathway is thought to be important in AD, as its regulation may mitigate pathological and cognitive expression of disease in mice.[33] Both IL-12 and IL-23 promote immune responses by stimulating T-cells.[34] IL-12 does so by inducing production of interferon-γ while IL-23 favors the production of IL-17.[35] It is unclear, however, whether IL-12 levels are elevated in AD.[36-38] IL-8 plays an important role in the chemotaxis of neutrophils[39,40] which have been recently identified as possible actors in the immune response to AD pathology.[41] IL-8 is a useful marker of intrathecal inflammation and is produced by a variety of activated innate immune cells.[42] Again, however, it is unclear whether it is upregulated in AD.[11,43] IL-15 is a general stimulator of immunity as it activates natural killer, CD8, and T-cells.[44,45] Its levels have been reported as being lower in plasma of AD patients[46] but higher in the CSF of AD *vs.* other, non-inflammatory neurological disorders.[47]

ICAM and VCAM are cell adhesion molecules. While they play roles in the vascular system, they are also important in immune responses. For instance, endothelial cell VCAM-1 expression is induced by cytokines, promoting adhesion of lymphocytes.[48] In inflammatory reactions, production of VCAM-1 by endothelial cells was initially believed to limit inflammation.[49] Recent work however, suggests that this peptide may play an active role in blood brain barrier (BBB) disruption.[50] In the inflamed brain, VCAM is also produced by activated astrocytes.[51] ICAM-1, produced by fibroblast and endothelial cells, is potentiated by IL-1 signaling.[52] ICAM-1 is involved in leukocyte recruitment and migration through the BBB.[53] In all, therefore, these markers appear closely related to the potentiation of inflammation through recruitment of immune cells via chemotactic signaling or BBB disruption.

Although they are individually different, ADNI markers showing the same bi-directional relation to pathological stage appear to have overlapping mechanisms of action (Fig. 3.4). For instance, AXL receptor tyrosine kinase,[54,55] IL-3,[56] CD40 antigen,[57] Macrophage colony stimulating factor,[58] Lectin-like oxidized LDL receptor,[59] and Chromogranin-A[60] among others

have been implicated in activation and recruitment of immune cells in CNS inflammatory processes. Similarly, other markers such as VEGF,[61] VCAM-1 and the Matrix-Metalloproteinase 3[62] are associated with vascular function but may facilitate the entrance of immune cells into the CNS through either chemotaxis or BBB breakdown. All of this suggests that several immune and vascular changes may occur concurrently with the appearance of AD pathology, as suggested previously.[3]

It is presently unclear why most of these markers remain unchanged or are diminished with appearance of early plaque pathology. Speculative explanations include a lack of inflammatory response to earliest-stage AD pathology.[12,13,15] However, animal work has shown that Aβ plaques are surrounded by activated microglia with associated cytokine production (reviewed by [4]). An interesting observation in both cohorts is that, despite their small numbers, participants in the SNAP group have even higher immune marker levels than the Stage 2 group. This observation is relatively weak in PREVENT-AD, where it reaches statistical significance only for IL12-P70 ($p = 0.001$), but is stronger in ADNI where it is apparent for 17/23 proteins (data not shown, all $p < 0.05$). These findings raise the possibility that Aβ plaques initially buffer or diminish the normal brain immune response to insult. Additional studies, including longitudinal observations will be necessary to evaluate this idea.

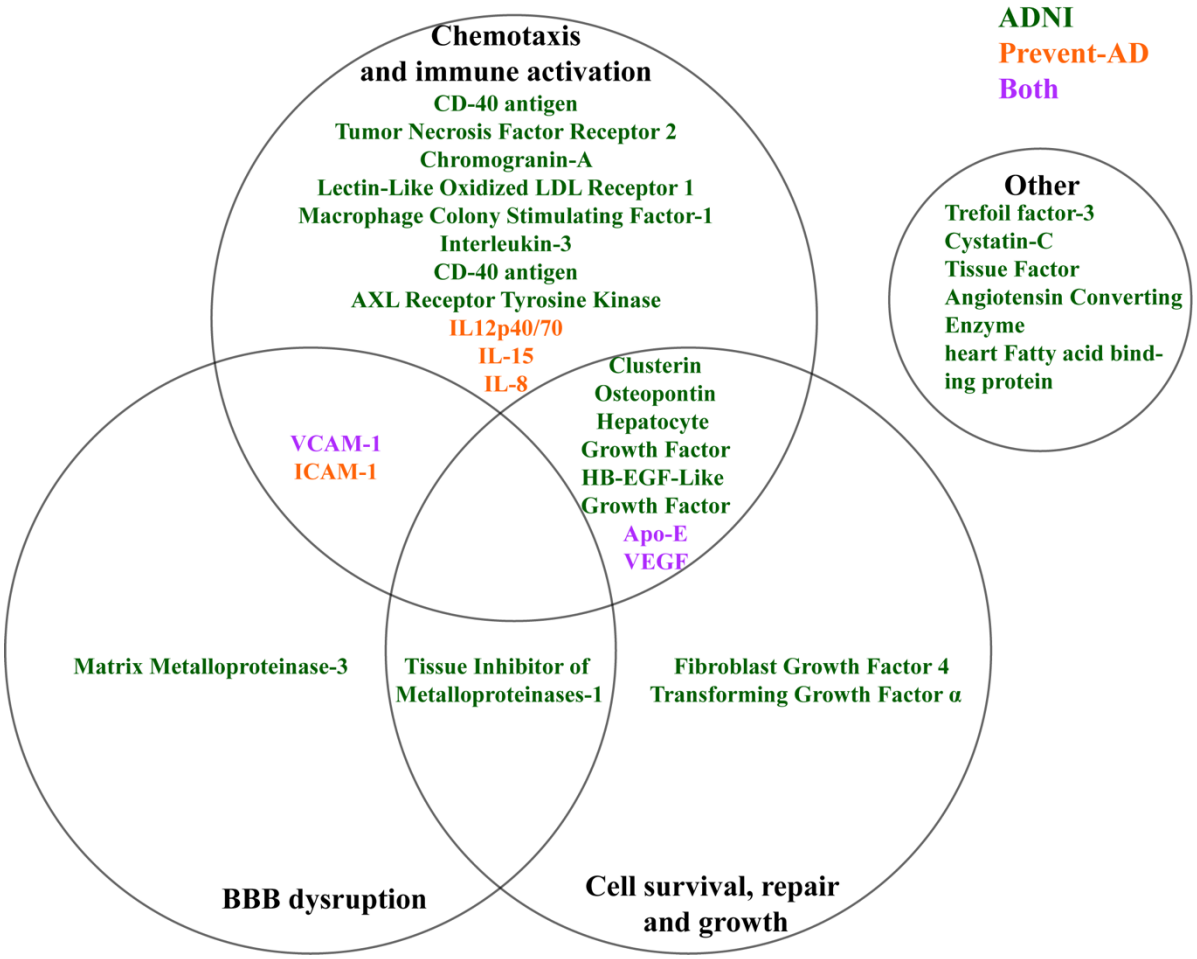

Figure 3.4: Overlapping theoretical biological pathways of identified ADNI and PREVENT-AD proteins.

Strengths and limitations

A clear limitation in PREVENT-AD is its small numbers of individuals at pathological Stages 1 and, especially, 2. Another caveat is that only VCAM-1 remained significantly reduced at Stage 1 or increased at Stage 2 when we included the 19 participants with AD biomarkers in the ±5% "no man's land" around inter-stage thresholds. Even so, an association with stage for the remaining four immune markers remained evident at a trend level of $P < 0.1$ (unadjusted). Furthermore, the differences in PREVENT-AD and ADNI assays for measurement of CSF AD and protein markers pose some difficulty for the interpretation of these results. In all, three markers assayed in both cohorts showed similar results, thereby supporting our conclusions. At the very least, therefore, studies of immune mechanisms in AD should consider *stage* and extent of pathological change beyond clinical diagnosis.

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Conflict of Interest/Disclosure Statement

The authors declare no conflicts of interest.

3.7 Bibliography – Chapter 3

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Chapter 4 – Hypothesis: CSF protein markers suggest a pathway for symptomatic resilience to AD pathology

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4.1 Preamble

This study follows up on the findings from Chapter 3. After having identified a set of markers related to biomarker evidence of AD pathology independent of clinical diagnosis, we questioned whether the levels of these markers were related to symptom severity and progression for a given level of AD biomarkers. In particular, we were interested to know how markers involved in immune activation were related to cognitive outcomes. This study chose an agnostic, unbiased data-driven approach to identify markers that predicted cognitive performance and trajectory in separate 'training' and independent 'validation' sets of persons. Given an abundant literature on harmful associations of immune activation with clinical outcomes we expected markers of immune activation to be associated with worse cognitive performance. Surprisingly, we identified a set of markers typically associated with immune activation that were strongly related to *better* outcomes and slower symptom progression. These associations remained even after adjusting for measure of Aβ and *tau* pathologies, thereby suggesting pathways for symptomatic resilience to AD pathology. These results presented an opportunity to suggest a hypothesis proposing that some component of immune activation may help individuals remain cognitively normal in the presence of AD pathology.

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4.2 Abstract

Introduction: We sought biological pathways that explained discordance between AD pathology and symptoms.

Methods: In 306 ADNI-1 participants across the AD clinical spectrum we investigated association between cognitive outcomes and 23 cerebrospinal fluid (CSF) analytes associated with abnormalities in the AD biomarkers $A\beta_{1\text{-}42}$ and total-*tau*. In a 200-person 'training' set, LASSO regression estimated model weights for the 23 proteins, and for the AD biomarkers themselves, as predictors of ADAS-Cog_{11} scores. In the remaining 106 participants ('validation' set), fully adjusted regression models then tested the LASSO-derived models and a related protein marker summary score as predictors of ADAS-Cog_{11}, ADNI diagnostic category, and longitudinal cognitive trajectory.

Results: AD biomarkers alone explained 26% of the variance in validation set cognitive scores. Surprisingly, the 23 AD-related proteins explained 31% of this variance. The biomarkers and protein markers appeared independent in this respect, jointly explaining 42% of test score variance. The composite protein marker score also predicted ADNI diagnosis and subsequent cognitive trajectory. Cognitive outcome prediction redounded principally to ten markers related to lipid or vascular functions, or to microglial activation or chemotaxis. In each analysis, apoE protein and four markers in the latter immune-activation group portended *better* outcomes.

Discussion: CSF markers of vascular, lipid-metabolic and immune-related functions may explain much of the disjunction between AD biomarker abnormality and symptom severity. In particular, our results suggest the *hypothesis* that innate immune activation improves cognitive outcomes in persons with AD pathology. This hypothesis should be tested by further study of cognitive outcomes related to CSF markers of innate immune activation.

4.3 Introduction

Forty years after the "re-discovery" of Alzheimer's disease (AD) as the chief cause of old-age dementia,[1] we still lack interventions that substantially reduce its morbidity. The last treatment for AD *symptoms* was introduced in 2003. Prevention of such symptoms is preferable, but no pharmacologic interventions for this purpose are available. The resulting AD pandemic creates an imperative to search anew for mechanisms that provoke AD symptoms.[2] Identification of such mechanisms can suggest the development of rational treatments.

Despite enormous information gained, the prevailing hypotheses regarding AD pathogenesis have failed to deliver strategies for mitigation of symptoms. The disappointment is perhaps greatest with respect to the amyloid cascade hypothesis.[3] Substantial evidence supports this conception that oligomerization or aggregation of neurotoxic Aβ peptides provoke neurodegenerative change including intraneuronal deposits of hyperphosphorylated and mis-folded *tau* protein, leading in turn to synaptic dysfunction.[4] Hopes here were bolstered, but as yet to no avail, by the demonstration that much of this change occurs *before* the onset of formal cognitive symptoms,[5,6] the ideal time for preventive interventions. Especially frustrating has been the demonstration that anti-amyloid therapies have succeeded in "target engagement," removing or reducing amyloid burden, with little or no symptomatic benefit and even the potential for harm.[7-9]

Also popular has been the idea that neurodegeneration in AD results from the deleterious consequences of an innate immune response that accompanies its pathogenesis.[10] This theory was buttressed by epidemiologic findings of reduced AD occurrence in persons with chronic inflammatory disease,[11] and in long-term users of non-steroidal anti-inflammatory drugs.[12] Later observational studies in older patients failed to confirm any advantage of NSAID use, however,[13] and large scale treatment and prevention trials showed that anti-inflammatory drugs brought no benefit,[14] or even possible harm.[15-17]

More recently, the genetics of later-onset AD have pointed again to immune as well as lipid metabolic pathways,[18] while additional observations have linked vascular health and AD risk.[19,20] For instance, central nervous system (CNS) vascular and blood brain barrier dysfunction may lead to abnormal accumulation of the AD pathological hallmarks.[21] Altered innate immune functions appear to provoke related changes,[22-24] and lipid dysmetabolism is implied by the strong association between AD risk and polymorphisms at apolipoprotein-

encoding genes such as *APOE* (especially) and *CLU (*Apo J).[25,26] Recent data-driven analyses further suggest that changes in some of these pathways occur early in the disease process, probably before accumulation of Aβ abnormality.[27,28] Thus, AD appears to represent a multi-factorial failure of several inter-related biological systems. To date, however, investigation of these inter-twined pathways has not suggested a route to prevention.

Often overlooked in the search for causality of AD has been a poorly understood *divergence between biological pathogenesis and symptomatic expression*. This "disconnect" is best exemplified by observations that some 20%-30% older persons whose autopsy results reveal extensive AD pathology were cognitively unimpaired before death.[29-31] Such findings have since been corroborated *in vivo* through PET imaging.[32-34] These and related observations of symptomatic 'resilience'[35] suggest that one might suppress the expression of AD dementia and related cognitive symptoms despite the presence of the disease's pathological hallmarks. We suggest that this phenomenon of resilience presents a potential pathway to symptom prevention. Some psychosocial and behavioral antecedents of symptomatic resilience are known,[36,37] and other recent work has identified biological factors that may exacerbate or reduce cognitive decline.[38-40] Nonetheless, further elucidation of biological determinants of symptomatic resilience may offer more practical potential pathways for pharmacologic intervention.

To undertake a fresh examination of the last topic, we conducted a series of unbiased searches for *biological* correlates of symptomatic resilience among 306 participants in the Alzheimer's Disease Neuroimaging Initiative (ADNI-1) who had donated cerebrospinal fluid (CSF). This work relied on an expectation that relevant biological pathways should typically be associated with CSF protein markers of their activity. In previous studies among a subset of this sample, we had identified 23 CSF proteins associated with the AD pathological process .[41] Here, we investigated the latter markers' possible role in symptomatic resilience by assessing their ability to predict cognitive performance and trajectory in relation to a given level of apparent AD pathology.

4.4 Methods

4.4.1 Participants

We downloaded data from http://adni.loni.usc.edu. ADNI was launched in 2003 as a public-private partnership led by Principal Investigator Michael W. Weiner. Its primary goal has been to test whether serial magnetic resonance imaging, positron emission tomography (PET), and various clinical, biological and neuropsychological markers can be combined to measure progression of mild cognitive impairment (MCI) and early AD dementia. We studied 306 ADNI-1 participants with available CSF data across the broader AD clinical spectrum. These included 90 healthy controls (HC), 147 persons with mild cognitive impairment (MCI), and 69 with AD dementia. ADNI assessed the cognitive status of these persons annually using the 11 and 13 point versions of the Alzheimer's Disease Assessment Scale ($ADAS\text{-}Cog_{11}$, $ADAS\text{-}Cog_{13}$)[42] as well as the Mini-Mental State Exam.[43] Each ADNI site had received approval from its institutional ethical standards committee on human experimentation. Written informed consent was obtained from all research participants, and from collateral informants when applicable. All research complied with ethical principles of the Declaration of Helsinki.

4.4.2 CSF measurements and classification

The ADNI investigators measured CSF $A\beta_{1\text{-}42}$ and total (t)-*tau* concentrations with Research Use Only INNOBIA AlzBio3 immunoassay reagents (Fujirebio, Ghent, Belgium) on an xMap Luminex platform (http://adni.loni.usc.edu/methods/biomarker-analysis/). We assigned the 306 ADNI participants to groups according to their CSF $A\beta_{1\text{-}42}$ and t-*tau* levels, considering them to be "amyloid-positive" if their $A\beta_{1\text{-}42}$ concentrations were below the ADNI-recommended threshold value of 192 pg/mL. Similarly, "*tau*-positive" individuals had t-*tau* values exceeding 93 pg/mL.[44] The ADNI also attempted to assay CSF levels for 159 other proteins using a multiplex X-Map kit from Rules Based Medicine (MyriadRBM, Austin, TX). Rigorous quality control standards led to exclusion of markers with unacceptable variability in assay results or >10% missing data, yielding only 83 acceptable measures. Results for these were normalized when indicated using boxcox transformation, etc. (http://adni.loni.usc.edu/wp-content/uploads/2012/01/2011Dec28-Biomarkers-Consortium-Data-Primer-FINAL1.pdf.) Our previous studies interrogated these proteins' relation to AD

pathology after scoring participants as 0 for no biomarker evidence of such pathology (A-/T-), 1 if positive for amyloid only (A+/T-), or 2 for presence of both amyloid and *tau* abnormalities (A+/T+).[45] We used Bayes factor analysis to reduce the 83 proteins to 38 showing positive likelihood of association (direct or inverse) with AD biomarker pathology score. Linear regression modeling (with false discovery rate correction, $q \leq 0.05$) then identified the aforementioned 23 "AD-related" proteins (Table 4.1) that showed a statistically significant association with AD pathology.

Table 4.1: List of the 23 "AD-related" markers in ADNI CSF

Markers (with abbreviations)
AXL receptor tyrosine kinase (AXL)
CD40 antigen (CD40a)
Interleukin-3 (IL-3)
Macrophage colony stimulating factor-1 (MCSF-1)
Heparin-binding EGF-like growth factor (HB-EGFL-GF)
Hepatocyte growth factor (HGF)
Transforming growth factor α (TGF-α)
Vascular endothelial growth factor (VEGF)
Heart fatty acid–binding protein (hFABP)
Lectin like oxidized LDL-receptor-1 (LOX-1)
Angiotensin-converting enzyme (ACE)
Tissue factor (TF)
Chromogranin-A (Cg-A)
Cystatin-C
Fibroblast growth factor-4 (FGF-4)
Matrix metalloproteinase-3 (MMP3)
Osteopontin
Tissue inhibitor of metalloproteinases-1 (TIMP-1)
Tumor necrosis factor receptor-2 (TNFR-2)
Vascular cell adhesion molecule-1 (VCAM-1)
Apolipoprotein E (apoE)
Clusterin/apolipoprotein-J (apoJ))
Trefoil factor-3 (TFF-3)

4.4.3 APOE genotyping

ADNI *APOE* genotypes had been determined using DNA extracted by Cogenics (Beckman-Coulter, Pasadena, California).[46]

4.4.4 Analytic methods.

4.4.4.1 Training and validation sets

For purposes of internal validation, we randomly assigned 200 of the noted ADNI participants to a 'training' set and the remaining 106 persons to a separate 'validation' set. We compared characteristics of the training and validation sets using the Mann-Whitney U test for continuous non-normally distributed variables or the χ^2 test for discrete data when appropriate. Of note, our earlier search for markers associated with AD pathology relied on 74 (70%) of validation set participants (as well as 163, or 63%, of training set participants) who were either healthy controls (HC) or had mild cognitive impairment (MCI). To verify lack of circularity in our approach, we therefore identified an alternate, more restricted set of "AD-related" proteins as before,[41] only now omitting consideration of persons in the validation set. The resulting 21 proteins included 19 of the 23 described above. Substitution of the 21 proteins in the modeling analyses described below produced essentially no change in results.

4.4.4.2 LASSO regression modeling of baseline cognitive performance

To identify and evaluate markers associated with cognitive performance, we used Least Absolute Shrinkage and Selection Operator (LASSO) regression.[47] This multivariable technique identifies specific variables (items) that predict a given outcome in the context of all others, and assigns optimal item weights for this prediction. Working exclusively in the training set, we developed three LASSO models for prediction of ADNI baseline cognitive impairment score (ADAS-Cog_{11}). Model 1 used only the "classic" AD biomarkers $A\beta_{1\text{-}42}$ and total (t-)*tau*; Model 2 used the 23 AD-related protein markers as previously described; while Model 3 used the combination of markers in Models 1 and 2. We used a 10-fold cross-validation procedure to optimize penalization and model weight parameters after dividing the training data randomly into ten equal cross-validation sets. On each of ten iterations, we omitted one such set and

optimized model weights and penalization parameters for the remaining nine. Averaging marker weights across the ten cross-validation folds then yielded an optimal consensus model. We estimated the predictive capacity of this model in the training set by examining predicted *vs.* observed ADAS-Cog scores. Finally, we tested the generalizability of the model by applying it to the never-before-seen validation set. Using a bootstrapping procedure (5000 iterations), we estimated 95% confidence intervals for the proportion of variance (R^2) explained by each model, thereby enabling comparisons of their performance. As a control measure, we tested the specificity of the findings from the 23 AD-related markers (Model 2) by comparing their performance with similar "models" obtained using 100 randomly chosen protein marker sets of 23 species from the 60 remaining ADNI-1 CSF proteins.

4.4.4.3 Applying model weights to predict clinical diagnostic category

We next tested whether the marker weights obtained from Model 2 could predict differential expression of symptoms and functional disability, as reflected by ADNI diagnostic category. To do this, we calculated a weighted summary score of marker levels for each participant by multiplying his/her standardized (z-scored) marker levels times the *inverse* of the corresponding marker weights from Model 2 (inverse weighting so that higher score predicted improved clinical outcomes rather than increased ADAS score). We tested this marker summary score as a predictor of contrasting ADNI diagnostic categories (HC *vs.* MCI, MCI *vs.* AD dementia, or HC *vs.* AD dementia) using a multinomial logistic regression model. Importantly, we adjusted this model not only for age, sex, education, and *APOE* ε4 carrier status but also for CSF $A\beta_{1\text{-}42}$ and t-*tau* levels. In effect, this adjustment therefore assessed the association of marker summary scores with the various diagnostic categories independent of these covariates. The logistic approach allowed calculation of odds ratios (ORs) for each standardized unit of the weighted protein marker score as a predictor of the three diagnostic contrasts. Upon observing the results (below), we evaluated further whether the association of markers with diagnostic assignment was a trivial consequence of cognitive test score variation. To do this, we re-ran the logistic models, now including $ADAS\text{-}Cog_{11}$ score as an additional covariate.

4.4.4.4 Using weighted marker scores to predict 4-year cognitive trajectory

Finally, we investigated whether the above-described inverse-weighted protein marker score predicted subsequent 4-year cognitive trajectory. For this analysis, we used a linear mixed effects model to assess the interaction of time with (baseline) weighted marker score as a predictor of ADAS-Cog change. This model was adjusted not only for participant age, sex, *APOE* ε4 carrier status, years of education, and CSF $A\beta_{1-42}$ and t-*tau,* but also for diagnosis and baseline cognitive performance.

All analyses used a two-sided $\alpha = 0.05$ and relied on Matlab (Mathworks inc.; Natick, Massachusetts).

4.4.5 Data availability

All data used for this work are available at the ADNI website (http://adni.loni.usc.edu/) subject to a data usage agreement with the ADNI investigators. Full details can be found at http://adni.loni.usc.edu/data-samples/access-data/

4.5 Results

4.5.1 Demographic characteristics

Demographic characteristics of participants are summarized in Table 4.2. The training and validation sets were comparable in age, sex ratio, years of education, MMSE as well as CSF total (t)-*tau* and $A\beta_{1-42}$. However, there was some disparity in distribution of clinical diagnostic categories, the training set having disproportionate numbers of HCs and fewer AD participants ($\chi^2 = 9.41$, $P = 0.01$). Presumably owing to its larger proportion of subjects with dementia, the validation set also had somewhat higher (worse) $ADAS\text{-}Cog_{11}$ scores than the training set ($P = 0.02$). Of note, 34 (38%) of HC participants had CSF evidence of Aβ pathology, making them especially suitable candidates for the investigation of symptomatic resilience.

Table 4.2: ADNI demographics overall and by assignment to Training or Validation sets

	All participants			Training			Validation			
	HC	MCI	AD	HC	MCI	AD	HC	MCI	AD	P
Sample	90	147	69	69	94	37	21	53	32	**0.01**
Age, mean (s.d.)	75.69 (5.46)	74.99 (7.34)	75.16 (7.60)	76.21 (4.82)	74.90 (7.68)	74.35 (7.99)	73.95 (7.06)	75.15 (6.76)	76.09 (7.13)	0.95
Sex, male:female	46:44	100:47	39:30	34:35	63:31	20:17	12:9	37:16	19:13	0.34
APOE-ε4, Carriers:Non-carriers	68:22	69:78	20:49	54:15	45:49	11:26	14:7	24:29	9:23	0.08
Education years, mean (s.d.)	15.64 (2.94)	15.95 (2.94)	15.16 (2.98)	15.77 (2.94)	15.44 (2.91)	15.03 (3.05)	15.24 (3.00)	16.85 (2.79)	15.31 (2.95)	0.10
CSF $A\beta_{1-42}$ (pg/mL), mean (s.d.)	207.9 (53.2)	160.5 (50.0)	141.3 (35.6)	205.3 (54.4)	154.3 (44.1)	135.0 (29.3)	216.4 (49.1)	171.5 (57.9)	148.6 (41.0)	0.43
CSF t-*tau* (pg/mL), mean (s.d.)	69.8 (27.7)	105.9 (55.2)	122.9 (60.0)	70.9 (29.1)	110.9 (60.1)	133.2 (67.8)	66.2 (22.9)	97.0 (44.5)	110.9 (47.8)	0.35
Aβ status, negative:positive	56:34	31:116	4:65	42:27	15:79	1:36	14:7	16:37	3:29	0.70
Baseline $ADAS\text{-}Cog_{11}$	6.14 (2.85)	11.90 (4.50)	18.43 (6.78)	6.22 (2.82)	11.91 (4.66)	17.85 (7.92)	5.90 (3.02)	11.84 (4.24)	19.10 (5.21)	**0.02**

NOTE. *P* values are given for overall difference between training and validation sets.
Abbreviations: HC, Healthy controls; MCI, mild cognitive impairment; AD, Alzheimer's dementia; s.d., standard deviation; pg/mL, picograms per milliliter; *APOE-ε4* C:NC, number of *APOE ε4* carriers and noncarriers; CSF, cerebrospinal fluid.

4.5.2 AD biomarkers and CSF proteins relation to cognitive scores.

Figure 4.1 shows the results of the LASSO modeling approach. Model 1 (AD biomarkers only) demonstrated good predictive accuracy in the training set and generalizability in the never-before-seen validation set, explaining 26% of ADAS score variance in the latter ($P < 10^{-7}$). As expected, t-*tau* had a positive weight (increasing *tau* levels predicting increasing ADAS-Cog score, *i.e.,* greater cognitive deficit), while $A\beta_{1-42}$ had a negative weight. Model 2, relying exclusively on the 23 AD-related protein markers, explained 31% of variance in validation set cognitive performance ($P < 10^{-9}$), but its apparent improvement over Model 1 was uncertain ($P = 0.19$, Figure 2). Here, the largest positive weights (strongest association with ADAS score, suggesting *diminished* cognitive abilities) were observed for heart Fatty Acid Binding Protein (hFABP), Clusterin (apo-J), and Hepatocyte Growth factor (HGF). The strongest negative weights (lower scores associated with ADAS score or, equivalently, higher scores associated with improved cognition) were apparent for Chromogranin-A (Cg-A), Apolipoprotein-E (apoE), Vascular Endothelial Growth Factor (VEGF) and CD-40 antigen (CD-40a). The combination of CSF protein markers and AD biomarkers (Model 3) best predicted $ADAS\text{-}Cog_{11}$ scores, explaining 41% of their variance ($P < 10^{-13}$), a significant improvement over both Model 1 and Model 2 ($P \sim 0.01$ and 0.05; Figure 4.2). Importantly, excepting HGF, all the key protein markers in Model 2 retained similar relevance after addition of the AD biomarkers (Model 3), suggesting substantial independence from the latter.

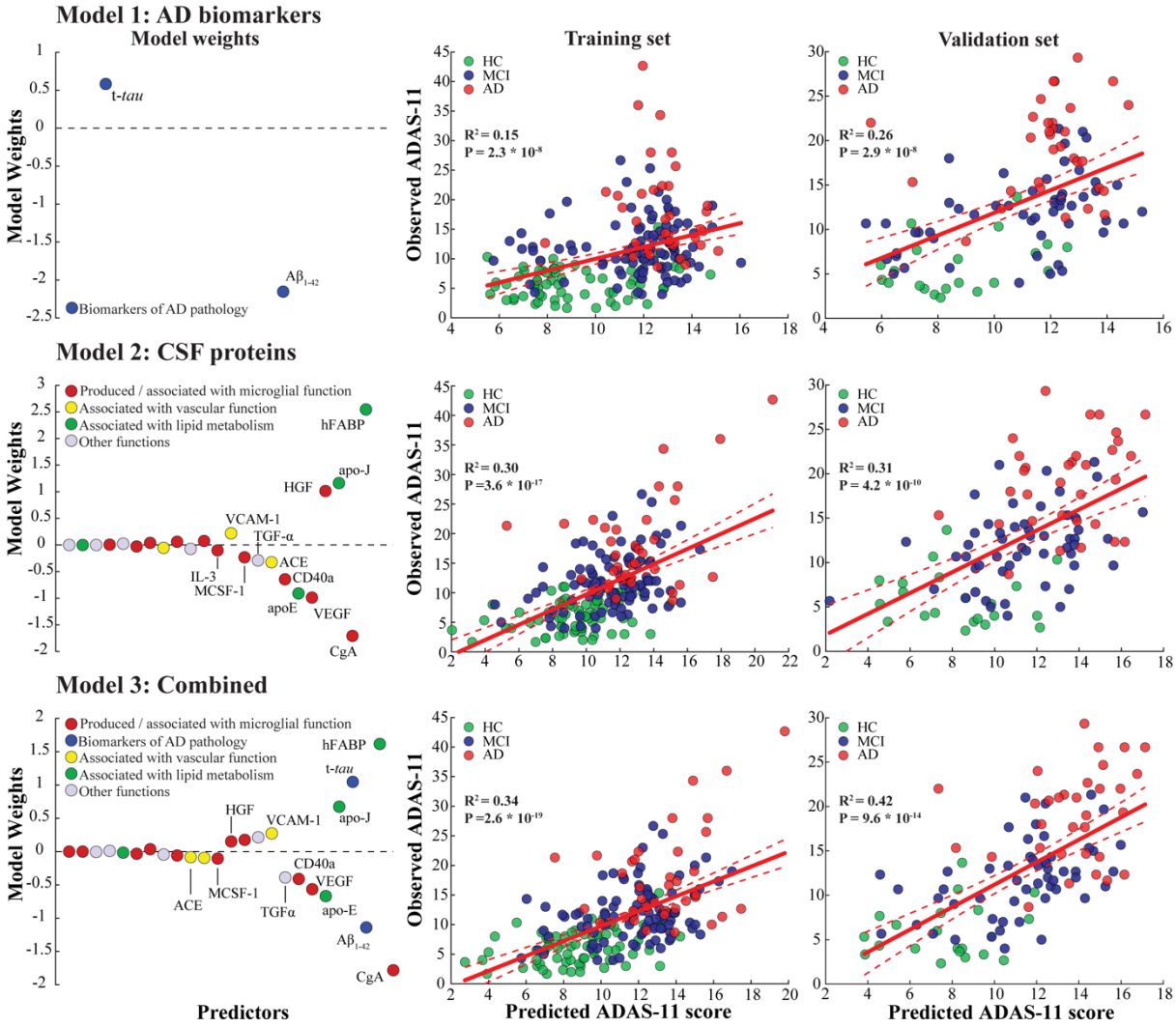

Figure 4.1: LASSO regression modeling for prediction of ADASCog-11 performance. Three models were trained to predict baseline $ADAS_{Cog}$-11 performance. Model 1 (Top row) considered typical AD biomarkers (CSF $A\beta_{1\text{-}42}$ and t-tau) only. This model's predictions explained only a fraction of the variance in training set $ADAS_{Cog}$-11scores (R^2 = 0.15), but generalized well to the validation set (R^2 = 0.26). Model 2 (Middle row) considered the 23 CSF AD-related proteins of interest here. This model relied strongly on hFABP, apoJ, HGF, Cg-A, apoE, VEFG and CD-40a (color-coded with relevant functions annotated in the figure), which apparently accounted for >90% of its predictive abilities. It provided robust predictions in both the training (R^2 = 0.30) and validation sets (R^2 = 0.31). Model 3 (Bottom row) used the combination of AD biomarkers and the 23 CSF proteins. It provided good to excellent predictions in the training (R^2 = 0.36) and validation sets (R^2 = 0.42). Dots in the left column represent marker weights in each model. The middle column shows correlations in the training set between observed $ADAS_{Cog}$-11 scores and those predicted by each model, with color-coded dots indicating each participant's ADNI diagnosis. The right hand column shows equivalent correlations for the validation set.

Control "models" were derived from 100 samples of 23 proteins each, drawn randomly from the residue of 60 ADNI proteins unrelated to AD pathology. These failed to converge on an optimal solution in 31% of instances. In the remaining analyses, the randomly chosen protein

species typically predicted only a trivial amount of cognitive performance variance in the training set (median $R^2 = 0.02$). Even the best-performing of these control models (training set R^2 up to 0.16) failed to generalize to the validation set (median $R^2 = 0.01$; range = 0 – 0.10), indicating that these "control protein" models may have reached an "overfitted" state in the training set.

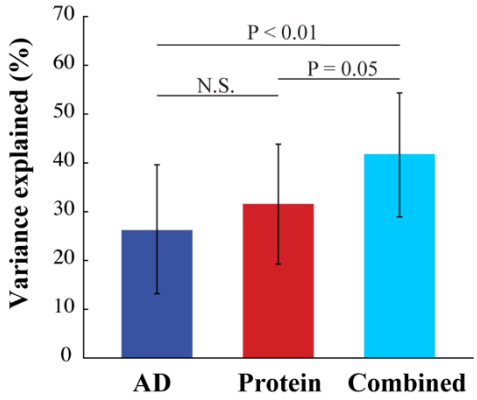

Figure 4.2: Statistical comparison of models trained to predict baseline ADASCog-11 performance.
Each model's performance was evaluated in the unseen validation set. Bars represent proportion of variance explained (R^2 * 100) for each model. We generated 95% confidence intervals using bootstrapping (5000 iterations) and used a bootstrap test to compare models. Model 1: typical AD biomarkers; Model 2: 23 AD-related proteins; Model 3: combined AD biomarkers and AD-related protein markers. N.S: Not Significant.

4.5.3 LASSO protein model weights predict diagnostic category

Results from the adjusted multinomial logistic model for prediction of diagnostic contrasts (HC vs MCI, etc.) are presented in the Forest plot of Fig. 4.3. The figure indicates that a 1 standard-unit increase in the inverse-weighted marker summary score was associated in the validation set with a ~3.5-fold *decrease* in probability of MCI *vs* HC (OR = 0.29; 95% CI [0.12-0.68], P < 0.005). Similarly, each unit increase in marker score implied a 4.1-fold decrease in the probability of AD dementia *vs.* MCI (OR = 0.25; 95% CI [0.11-0.55], P < 0.001) and a 14.2-fold decrease in the probability of AD dementia *vs* HC (OR = 0.07; 95% CI [0.02-0.22], P < 0.001). These results were only partly mitigated by inclusion of cognitive scores in the model (all P remaining < 0.01), suggesting that the markers predicted *functional capacity* (important to the described diagnoses) beyond pure cognitive deficit.

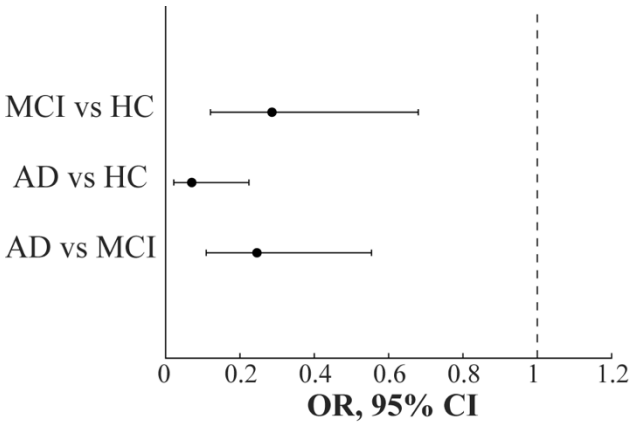

Figure 4.3: Weighted marker summary score predicts contrasts among clinical diagnostic categories.
A multinomial logistic regression model evaluated association of weighted protein marker summary scores with contrasts between clinical diagnostic groups in the validation set. The odds ratios (ORs, point estimate indicated by dots, with 95% CI shown by horizontal lines) show decreased likelihood of a more severe diagnosis (AD dementia vs. MCI vs. HC) after adjustment for age, sex, APOE ε4 carrier status, and years of education. OR's suggest change associated with each standardized unit increase in weighted marker score. Importantly, the model was also adjusted for CSF levels of $A\beta_{1\text{-}42}$ and t-tau, indicating distinction in clinical severity for a given level of AD pathology.

4.5.4 Weighted marker score predicts rate of 4-year cognitive decline

Here, we tested the association between weighted protein marker score and subsequent four-year cognitive trajectory, verifying that such association would survive adjustment not only for biomarkers and AD risk factors but also for baseline cognitive performance and clinical diagnostic category. The fully adjusted model indicated that each standard unit increase in inverse-weighted marker score was associated with a *decrease* in slope of ADAS-Cog_{11} performance of $\beta = 0.87$ points / year, (s.e. = 0.39; P = 0.04; Figure 4.4). The limited sample size of the validation set precluded evaluation of weighted marker score prediction of 4-year cognitive trajectory among individual diagnostic categories.

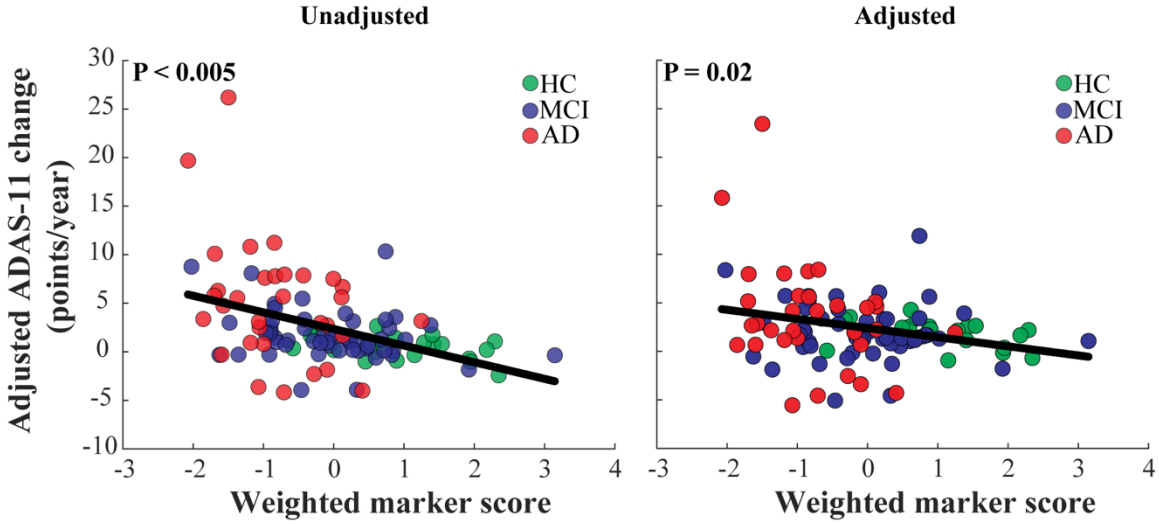

Figure 4.4: Weighted protein marker summary score predicts 4-year cognitive trajectory.
Linear mixed effects models were used to evaluate association of weighted protein marker summary scores with 4-year trajectory on the $ADAS_{Cog}$-11 scale. These models were adjusted for age, sex, APOE ε4 carrier status, years of education, CSF t-tau and Aβ levels (left). Higher summary scores were associated with a decrease in $ADAS_{Cog}$-11 scores ($P < 0.005$). As expected, this apparent relationship was diminished moderately with adjustment for baseline cognitive performance (right), but summary scores remained robustly associated with longitudinal cognitive decline ($P < 0.02$).

4.6 Discussion

We examined CSF AD-related protein markers alongside biomarkers of AD for their relevance to cognitive symptom expression. To do this, we used a 200-person training set of participants from ADNI-1, applying LASSO regression with a ten-fold cross-validation procedure to assign predictor variable weights. The resulting models were then tested in a validation set of the remaining 106 ADNI participants. We found that the AD biomarker and CSF protein models appeared to be additive, with the contributions of their constituent variables being mostly independent. Lack of collinearity between the two sets of markers was further suggested by the relative invariance of protein marker weights in the conjoint model vs. that derived from the proteins only.

In particular, the protein marker model predicted disjunction between degree of AD pathology and symptom expression. Not only did it predict (cross-sectional) cognitive performance in the never-before-seen validation set, but a summary score derived from its constituent marker weights strongly predicted ADNI clinical diagnostic category. Importantly, the latter prediction survived statistical adjustment for CSF levels of Aβ and *tau* as well as AD risk

factors and, notably, cognitive score. Otherwise stated, the protein markers alone predicted variation in diagnosis *for a given level of AD pathology* and cognitive score. In fully adjusted models that also included a term for baseline cognitive score and diagnosis, the inverse-weighted protein marker summary score also predicted rate of cognitive change over the succeeding four years. Together, these observations suggest that AD-related protein markers are strongly associated with baseline cognition, with cognitive diagnosis, and with cognitive decline associated with a given level of AD biomarker "pathology." Limitation of this effect to the 23 AD-associated markers was suggested by absence of any similar prediction of cognitive outcomes in models constructed from multiple sets of 23 "control" markers from the remaining 60 ADNI-1 protein species.

Function of the predictive CSF proteins

While these findings await independent confirmation, we suggest it is reasonable here to consider mechanistic explanations that might advance our understanding of symptomatic resilience to AD pathology. Notably, most of the predictive capability of the 23 AD-related proteins resided in ten marker species that were responsible for >>95% of the observed effect (data not shown). In models that included (were adjusted for) Aβ and *tau* levels, these were: hFABP, apoJ, apoE, ACE, Cg-A, CD-40a, VEGF, HGF, TGF-α and M-CSF-1. Among these, only HGF appeared to be collinear with the AD biomarkers, becoming inapparent as a predictor of cognitive outcomes when analyses included the biomarkers. A gene ontology analysis suggested that the nine remaining proteins are involved in a variety of overlapping mechanisms involved in lipid metabolic, immune and vascular pathways.

The functions of several of these proteins recall findings from the genetics of late-onset AD. For example, apoE protein (determined by *APOE* genotype, the strongest genetic risk factor for late-onset AD after age) was among the nine key protein markers. ApoJ (clusterin), conditioned by the *CLU* risk polymorphism,[26] was another key "predictor." Both apoE and apoJ appear to be involved in a dynamic equilibrium between Aβ plaques and soluble Aβ species, possibly owing to their role in cholesterol transport[48] (for apoE) or in breakdown of protein aggregates (for apoJ).[49] ApoE also has an important role in astrocyte-mediated clearance of Aβ.[50] Recent data suggest it may also be important in microglial activation in neurodegenerative diseases via coupling with the TREM-2 pathway,[51] the product of another

important AD risk gene. FABP is also implicated in the transport of lipids and may be elevated in the CSF of AD patients and individuals with progressive MCI.[52]

FABP-mediated lipid metabolism may also be linked to inflammatory processes,[53] in keeping with a broader notion that CNS lipid metabolism is important to innate immune activation. It is probably not surprising, therefore, that the above three proteins conjoin with four other evidently *immune-related* markers among the "short list" of nine proteins that appear to modify clinical outcomes. Specifically, CD-40a is a key mediator of immune activation believed to hold an early role in the pathogenesis of AD.[54,55] It activates antigen presenting T-cells while it regulates the deposition of Aβ. Cg-A is similarly associated with CNS microglial activation.[56,57] Although important to vascular homeostasis,[58] VEGF has been shown also to induce microglial chemotaxis and proliferation,[59,60] and to be an important mediator of the immune response to tumors.[61] M-CSF-1 causes proliferation and differentiation of macrophages, regulates number of microglial cells and their state of differentiation, and promotes neuronal survival.[62,63]

Notably, each of the above four "immune-related" markers was *inversely* related to ADAS-Cog score; *i.e.,* higher levels predicted improved cognition. Because there has been a widespread assumption that "inflammatory" processes exert a deleterious influence on AD symptom development (see below), we had expected higher levels of these four markers to be associated with *increasing* symptom severity, but we found the opposite to be true. This observation appears to converge with other evidence that enhanced immune responses can be beneficial for individuals who are older,[64] or who have established AD dementia.[65] Therefore, the described functions of the predictive markers may suggest that immune activation occurs as *a response* to insults such as AD pathology, presumably involving activation and recruitment of immune cells. Such activation could, in turn, enhance clearance of toxic proteins and promotion of cell survival, thereby resulting in improved cognitive outcomes for a given level of AD pathology.

Strengths and limitations

The principal strength of this work appears to be its reliance on an unbiased data-driven, hypothesis-free approach to identify markers associated with both AD pathology and symptom expression. Our analytical plan was based on rigorous cross-validation and internal validation procedures to build models from important predictors that generalized well to unseen data.

However, several limitations should also be noted. Most importantly, these analyses relied on data from a protein screen of ADNI CSF that was assayed using multiplex technology. The ADNI investigators had initially attempted to assay 159 proteins, only 83 of which passed QC criteria. Such results occur typically because of insufficient assay sensitivity for many analytes, here including several key markers of immune activation. Furthermore, despite their apparent links to immune activation, the predictive proteins act at the interface of several processes thought to be key in the AD cascade. It remains possible, therefore, that other pathways may act independently or in concert with immune activation to produce benefits on cognitive function. A further limitation of our analyses was their reliance principally on cross-sectional data, limiting our ability to infer ordinality or causality of the observed associations. Finally, we relied on CSF Aβ and *tau* as indicators of the extent of AD pathology (an important component in the phenomenon of resilience). Unfortunately, we could not include PET measures of Aβ or *tau* pathology because few participants had these measures available (96 for Aβ, 19 for *tau*), many with an excessive interval between baseline and PET imaging (median lag from LP to scan: 60 months for Aβ, 132 months for *tau*). Similarly, cortical thickness data from a region of interest as described by Jack et al.,[66] or hippocampal volume as measures of AD progression also resulted in significant missing data (~20%), although these yielded similar results in reduced samples (data not shown). Before their significance can be fully evaluated, therefore, our results require corroboration in other data sets, preferably using assay methods with improved sensitivity as well as other indicators of AD pathology.
Nevertheless, the available data suggest an important pathway for amelioration of symptomatic expression of AD, and therefore *a hypothesis for future investigation*:

A hypothesis and experimental approaches to its assessment

Hypothesis: ***Immune activation modulates the cognitive and functional deficits that otherwise accompany the accrual of "classical" AD pathology.***

It has long been understood that immune activation accompanies evidence of the AD process.[67] Early epidemiological studies showed an inverse association between anti-inflammatory drug use and AD risk.[12,68] These findings led to widespread speculation that AD pathology induced immune activation, which in turn generated a neurotoxic environment, neuronal death, and eventually dementia.[69] This formulation recalls other instances in which uncontrolled CNS

immune responses lead to nervous system damage and cognitive impairment (*e.g.,* in Lyme disease).[70] In neurodegenerative disease models, maladaptive immune responses appear to provoke neuronal death through the generation of reactive astrocytes.[71] The present results are at odds with these ideas, however, suggesting instead that some component of innate immune activation may be associated with *improved* cognitive outcomes inn AD. This improvement might result from increased clearance of Aβ species,[72] a process in which astrocytes appear also to play an important role.[50]

Importantly, the majority of the CNS immune responses are provided by microglia and astrocytes. These cells have multiple functions and roles at the interplay of pathways thought to be involved in AD pathogenesis. For instance, astrocytes are the main producers of apoE protein in the CNS, and are clearly involved in blood brain barrier (BBB) function and in the clearance of Aβ and neurotoxic neurotransmitters.[73] In addition, both astrocytes and microglia help maintain CNS homeostasis and provide trophic support to neurons. While malfunction of these important cells may result from accumulating AD pathology, they may also be the cause of such pathology by provoking either accumulation of pathological proteins, BBB dysfunction, or deterioration and loss of trophic support. Any of these may promote neurodegeneration. In this context it appears noteworthy that risk alleles at the polymorphic TREM-2 and CD-33 genetic loci are associated with *reduced* microglial clearance of Aβ plaques.[22,74,75] These loss-of-function mutations result in reduced microglial activation and clustering around plaques.[76]

An important limitation of the latter observations, however, is that they derive mainly from animal models. We know of no present evidence of similar mechanisms in human studies, notably because these efforts have encountered difficulties in reliable measurement of the most important markers of immune activation in CSF.[77,78] Thus, to date, human studies of association between disease and fluid markers of inflammation have yielded contrasting results.[79]

While we and others have observed reduced CSF immune marker levels in individuals with evidence of "pure" amyloid pathology,[41,80] (thereby suggesting that a maladaptive immune response might result in brain accrual of Aβ), the noted limitations in measurement sensitivity restricted our analysis to only 23 markers. Measurement of many other protein markers, particularly other markers of immune activity, could render a far more complete analysis of biological networks involved. Future efforts to test the proposed hypothesis and identify its related pathways will therefore require newer immune marker assay methods having improved

sensitivity. Although costly, these techniques should allow identification and measurement of many more relevant immune marker proteins.

As here, an expanded set of envisioned assay results would benefit from use of unbiased, data-driven feature selection approaches. Importantly, these techniques identify and assess the relevance of individual markers in the context of all others. An important objective of the proposed work should be to identify which among many available markers of immune activity have greatest apparent "effect" on cognitive outcomes – a topic not readily studied in animal models of preclinical AD. These markers, or families of them, can in turn suggest specific immune pathways relevant to the phenomenon of resilience, thereby prompting additional focused biological investigation using modern, high-resolution techniques.

Finally, we would note that our current or proposed analyses relate to cross-sectional data, and therefore cannot assess important questions about the ordinality and potential causality of discovered associations. To answer these last questions, longitudinal data will be needed. Such data could in turn be analyzed using recently introduced machine-learning algorithms that can provide a virtual "motion picture" of sequential events in the biological pathways underlying resilience. Such work may also identify immune pathways involved in accumulation or clearance of pathological proteins and symptomatic resilience thereby pointing more directly to timing at which pathways could be either up- or down-regulated to achieve symptom mitigation. Together with other biological or mechanistic experimentation mentioned above, results from such studies may suggest promising new targets for novel prevention strategies.

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Author Contributions

P.F.M and J.B contributed to the design of the study. P.F.M and M.S. contributed to the analysis of the data. P.F.M., J.P., D.M. and J.B contributed to data interpretation and critical revision of the manuscript for intellectual content. J.B. supervised the study.

Potential Conflicts of Interest

The authors declare no conflict of interest.

4.7 Bibliography – Chapter 4

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Chapter 5 – INTREPAD: a randomized trial of naproxen to slow progress of pre-symptomatic Alzheimer disease

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5.1 Preamble

While Chapters 3 and 4 explored gaps in the study of CSF markers of immune activity in relation to AD biomarkers and symptoms, Chapter 5 continues a line of work initiated in the early 1990's. Initial studies of NSAIDs in the epidemiology of AD had suggested attenuated incidence with long-term use of these drugs. However, the epidemiological findings did not lead to successful intervention trials. In fact, most showed no benefit[1] or even harm,[2,3] prompting investigators to start treatment at the earliest stages of disease. However, results of ADAPT, the first NSAID prevention trial again suggested harm,[4] even though follow-up analyses suggested that individuals further removed from their onset might have benefitted from treatment.[5,6] Chapter 5 presents the results of a more recent prevention trial of the NSAID naproxen. This trial (INTREPAD) enrolled high-risk participants who had been meticulously screened for apparent cognitive disorder. This work has been published in Neurology. 2019 Apr 30;92(18):e2070-e2080. doi: 10.1212/WNL.0000000000007232.

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5.2 Abstract

Objective: Evaluate the safety and efficacy of low-dose naproxen for prevention of progression in pre-symptomatic AD among cognitively intact persons at-risk.

Methods: INTREPAD, a two-year double-masked pharmaco-prevention trial, enrolled 195 AD family history-positive elderly (mean age 63 years) screened carefully to exclude cognitive disorder (NCT-02702817). These were randomized 1:1 to naproxen sodium 220mg twice-daily or placebo. Multimodal imaging, neurosensory, cognitive and (in ~50%) CSF biomarker evaluations were performed at Baseline, 3, 12, and 24 months. A modified intent-to-treat analysis considered 160 participants who remained on-treatment through their first follow-up examination. The primary outcome was rate of change in a multimodal composite pre-symptomatic Alzheimer Progression Score (APS).

Results: Naproxen-treated individuals showed a clear excess of adverse events. Among treatment groups combined, the APS increased by 0.102 points/year (S.E = 0.014; $P < 10^{-12}$), but rate of change showed little difference by treatment assignment (0.019 points/year). The treatment-related rate ratio of 1.16 (95% Confidence Interval 0.64–1.96) suggested that naproxen does not reduce the rate of APS progression by more than 36%. Secondary analyses revealed no notable treatment effects on individual CSF, cognitive, or neurosensory biomarker indicators of progressive pre-symptomatic AD.

Conclusions: In cognitively intact individuals at-risk, sustained treatment with naproxen sodium 220 mg twice-daily increases frequency of adverse health effects but does not reduce apparent progression of pre-symptomatic AD.

Classification of Evidence: This study provides Class I evidence that, for people who are cognitively intact, low-dose naproxen does not significantly reduce progression of a composite indicator of pre-symptomatic AD.

5.3 Introduction

Alzheimer's disease (AD) includes a decades-long period of pre-symptomatic biochemical, imaging, neuro-sensory, and subtle cognitive changes.[1,2] Because cognitive changes emerge gradually, AD prevention trials using cognitive endpoints typically follow thousands of individuals for several years. Improved efficiency may result from use of multimodal composite indicators of early AD pathogenesis as well as subtle cognitive decline. One such indicator, the "Alzheimer Progression Score" (APS),[3] assesses pre-symptomatic AD progression using Item Response Theory modeling. Predictive validity of the APS method has recently been demonstrated.[3]

Among potential preventive interventions in pre-symptomatic AD, non-steroidal anti-inflammatory drugs (NSAIDs) have retained interest. Numerous observational studies have shown reduced incidence of AD in users of these drugs, at least in relatively young elderly.[4] However, NSAID prevention trials have failed to show benefit, and instead have caused harm to older individuals,[5] or those with imminent symptoms.[6,7] These findings suggest that NSAID prevention trials must screen (preferably younger-old) participants carefully for cognitive or other prodromal AD symptoms.[8] Such principles were applied when designing INTREPAD (Investigation of Naproxen Treatment Effects in Pre-symptomatic Alzheimer's Disease; NCT-02702817), a two-year double-masked randomized trial of oral naproxen-sodium 220 mg. twice-daily vs. placebo for safety and efficacy against progression of AD-related change.

5.4 Methods

5.4.1 Primary research question

INTREPAD was designed to test the safety and efficacy of low-dose naproxen in reducing the rate of change of a composite indicator of pre-symptomatic AD. To that end, Class I evidence is provided here. An important additional objective of the work was to assess the practicality and utility of a more efficient approach to AD prevention trials using novel methods of recruitment, data collection, and analysis. A notable feature was the use of a composite primary efficacy outcome derived from a parallel observational program of cognitive, structural and functional magnetic resonance imaging, and neurosensory measures, along with cerebrospinal fluid (CSF) biomarkers.

5.4.2 Standard Protocol Approvals, Registrations, and Patient Consents

INTREPAD (NCT-02702817) was approved by the institutional review board of McGill University. Recruitment occurred between November 2011 and March 2015. Data gathering ended March 28, 2017. All participants provided written informed consent for each trial procedure. Data were collected at the Douglas Mental Health University Institute, an affiliate of McGill University (Montréal). All research procedures complied with the ethical principles of the Declaration of Helsinki. A Data Monitoring Committee reviewed all safety and efficacy data prepared by a contract (unmasked) statistician on October 20, 2016, and upon completion (June 26, 2017).

5.4.3 Overview of Participants and Trial Regimen

We recruited 462 healthy older individuals with a parental or multiple-sibling history of AD for participation in PREVENT-AD, an observational cohort study of healthy persons aged 55+ without evidence of cognitive deficit.[9] Among these, 195 eligible volunteers were randomized in INTREPAD to receive either low-dose naproxen sodium or placebo (Figure 5.1). The primary efficacy analysis considered a modified Intent-to-Treat (m-ITT) group of 160 persons who remained on assigned treatments until their first follow-up evaluation 90 days after Baseline. In all, 166 (85%) completed their participation per protocol (154 m-ITT and 12

others in the ITT group with two years of follow-up and biomarker assessment). Participants who completed the trial on study treatments numbered 124.

Initial integrity of participants' cognitive abilities was evaluated by telephone interview, followed by in-person assessment using the Montréal Cognitive Assessment (MoCA) and the Clinical Dementia Rating scale (CDR).[10,11] In an early protocol change, we further verified intact cognitive status after Baseline testing using the trial's principal cognitive assessment measure, the Repeatable Battery for Assessment of Neuropsychological Status (RBANS).[12] As a consequence, 14 enrollees (3 originally assigned to naproxen and 11 to placebo) were considered unsuitable for further participation because of notable cognitive deficits that had escaped detection at Baseline suggesting early mild cognitive impairment (MCI). Final determination of cognitive eligibility relied for some on full neuropsychological evaluation.

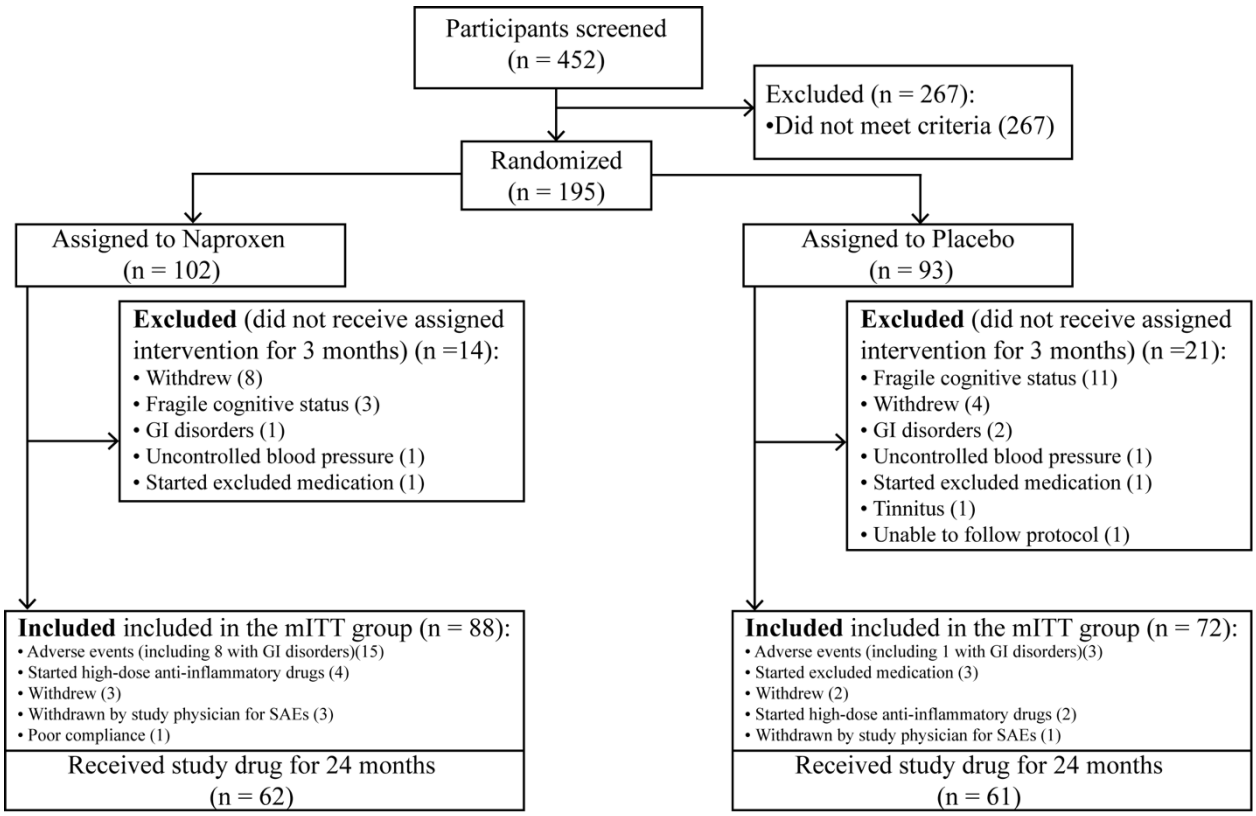

Figure 5.1: Consort flow.
Fragile cognitive status indicates individuals with cognitive deficits suggestive of early mild cognitive impairment. GI = gastrointestinal; mITT = modified intent-to-treat; SAE = serious adverse event

5.4.4 Eligibility criteria.

Other eligibility criteria included 1) at least one parent or two siblings with AD, 2) age ≥60 years, or ≥55 years if within 15 years of youngest-affected relative's onset, 3) health and social stability sufficient to enable participation for five years of longitudinal study; and 4) no contraindications to sustained treatment with naproxen sodium. Family history was ascertained from an expert's diagnosis of AD or, when necessary, via a brief, structured questionnaire (see eMethods). Exclusion criteria included regular use (more than 4 doses per week) of corticosteroids, NSAIDs, other anti-inflammatory/immunosuppressant agents or aspirin. A complete list of inclusion/exclusion criteria is available in the eMethods.

5.4.5 Specification of primary efficacy outcome and initial power analysis.

The original INTREPAD analysis plan called for a primary efficacy determination based on a composite of data that was not then specified. This composite was under development in data from the parallel (non-trial) parent PREVENT-AD Cohort, with near-identical characteristics.[9] Initial power analyses considered a subset of data from the public use ADNI database. We simulated an attempt to demonstrate a "true" 25% reduction in the slope of the CSF concentration of total-*tau* over one year, specifying 85% statistical power. This analysis suggested that the needed power could be provided by a sample of 228 aged cognitively normal participants assigned 1:1 to active drug vs. placebo. We assumed that greater power would be provided by two (rather than one) years of observation, and thus specified a target enrollment of 200 with an assumption that ~80% would remain in an m-ITT primary analysis pool of persons who remained on study treatments through their first follow-up examination (but note below that results belied these assumptions).

When the primary efficacy outcome (APS)[3] was fully specified, we performed a similar simulation in the parallel, non-trial PREVENT-AD Cohort. This simulation now relied on the Cohort's observed slope, random intercept variance, and error variance using a longitudinal random effects model. It suggested that 160 participants would afford 85% power to detect only a 50% difference in slope between two study arms, or 68% power to detect a 40% difference. Because the PREVENT-AD data did not include CSF biomarker measures, however, we expected that the trial's CSF assay results would provide a substantial increase in power, as had been the case in the observational BIOCARD study,[13] (see *Discussion*.)

5.4.6 Randomization, masking, and provision of study drug

Using www.randomization.com, participants were randomized 1:1 in 34 permuted blocks of six to identical-appearing tablets of naproxen sodium 220 mg or placebo, both generously donated by Pharmascience, Inc. (Montréal), for administration twice-daily with meals. The Douglas Hospital pharmacy stored and prepared study drug in sealed dosage packets for each participant visit. Participants, the principal investigator, study staff and all clinicians responsible for assessments or marker measures remained masked to treatment assignment until safety and efficacy analyses were complete. A protocol for interim unmasking was available but not needed. Only an external statistician (DM) had advance access to treatment assignment.

5.4.7 Assessment methods

Figure 5.2 shows the timeline for data gathering. Complete evaluations were performed at Baseline (BL) and after 3, 12, and 24 months (3M, 12M, 24 M) of treatment.

5.4.7.1 Safety

Follow-up interviews (on-site or by telephone) for adverse events (AEs) were administered *ad hoc* or, at a minimum, every 3 months using structured medical history and review-of-system questionnaires. On-site safety evaluation included routine labs, electrocardiogram, and a brief physical and neurological examination. Potentially important incidental magnetic resonance imaging (MRI) and other newly discovered health risks were referred for expert review. Research nurses rated Adverse Events (AEs) using the Cancer Therapy Evaluation Program Common Terminology Criteria, version 3.0. AEs were graded as mild, moderate or severe after physician review, and each AE was also assigned a Preferred Term (PT) and a System Organ Class (SOC) using the Medical Dictionary for Regulatory Activities (MedDRA) classification system, version 19.1. Serious AEs (SAEs) that were life-threatening or required hospitalization were reported in real-time to the McGill Research Ethics Board. Relationship of AEs and SAEs to treatment (assuming assignment to naproxen) was assessed by a study physician. Elective surgeries were not considered SAEs.

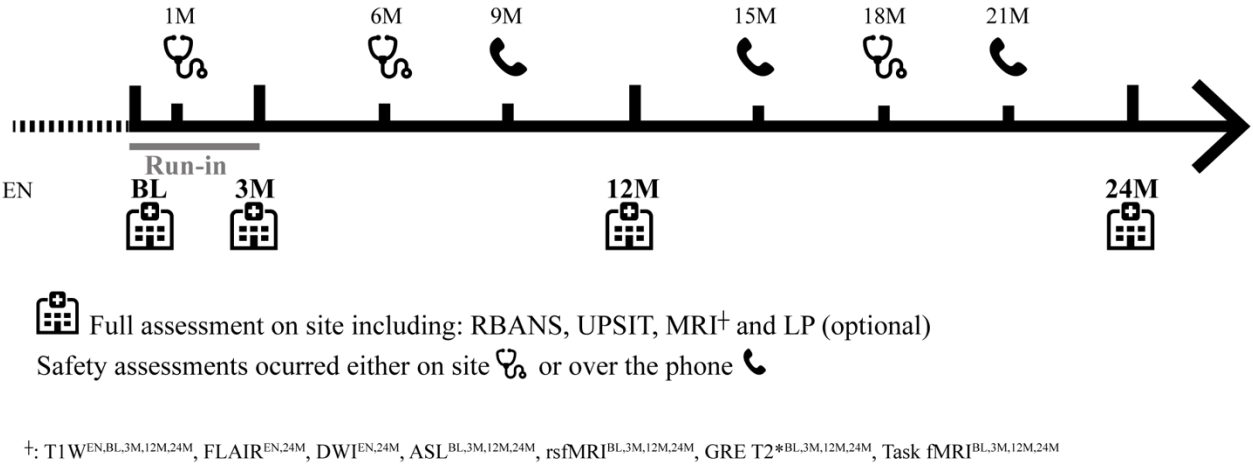

Figure 5.2: Trial timeline.
EN: Enrollment; M: months; RBANS: Repeatable Battery for Assessment of Neuropsychological Status; UPSIT: University of Pennsylvania Smell Identification Test; MRI: Magnetic Resonance Imaging; LP: lumbar puncture; T1W: T1 Weighted - FLAIR: FLuid Attenuated Inversion Recovery - DWI: Diffusion Weighted Imaging - ASL: Arterial Spin Labeling - RS-fMRI: Resting State Functional MRI - GRE T2*: GRadient Echo quantitative T2* - Task: episodic memory task. Full assessment (on site): BL (Baseline), 3M, 12M, 24M. Safety follow-up (on site): 1M, 6M, 18M. Safety follow-up (telephone): 9M, 15M, 21M.

5.4.7.2 Compliance

Participants were asked to bring unused supplies of drug to trial visits. Research nurses evaluated compliance at each visit, inquiring when indicated into reasons for missed doses.

5.4.7.3 Cognitive and neuro-sensory performance.

At BL, 3M, 12M and 24M, neuropsychological performance was measured using the RBANS, which evaluates five cognitive domains (immediate memory, delayed memory, attention, language and visuospatial abilities) and a total summary score. The RBANS is available in 4 equivalent versions (for longitudinal assessment) in both French and English. Version "A" was administered at Baseline, and alternate forms were used in random order at follow-up visits. We developed correction factors to improve version equivalence and scored results without correction for age (often used clinically; see Appendix 2 Methods for details).
At each visit's functional MRI session, participants were also given alternate versions of an episodic memory task (encoding and retrieval of objects).[14] They were asked to identify whether items had been observed during the encoding period or were new at the retrieval session (details in Appendix 2 Methods). A correct response required a "hit" or correct

rejection. Odor identification, a neuro-sensory faculty, was also tested using the 40-item "scratch and sniff" University of Pennsylvania Smell Identification Test (UPSIT).[15,16] The latter, comprising 40 items administered in four randomly ordered booklets, was available in both French and English.

5.4.7.4 Neuroimaging markers.

Brain structural and functional MRI were performed at BL, 3M, 12M and 24M visits on a Siemens TIM Trio 3 Tesla MRI system (Siemens Medical Solutions, Erlangen, Germany) using the Siemens standard 12-channel head coil (see Figure 5.2 and Appendix 2 Methods). Using conventional pipelines, averages of grey matter density were calculated for 78 regions based on T1-weighted images using the SPM12 toolbox to define probabilistic grey matter maps. Cortical thickness was estimated from T1-weighted images using version 1.12 of the CIVET pipeline.[17] Brain volumes were computed from the same images using a volumetric pipeline.[18] Regional cerebral blood flow (CBF) was evaluated using quantitative pipelines from Single-Echo Pseudo-Continuous Arterial Spin Labeling (pCASL) acquisitions.[19]

5.4.7.5 CSF biomarkers.

A subset of 93 participants in the m-ITT pool volunteered for up to four serial lumbar punctures (LPs) over the two-year trial interval following their clinical and imaging evaluations. After an overnight fast, LPs were performed by PR-N using an introducer and the Sprotte 24-gauge atraumatic needle. Samples of 20-30 mL were withdrawn by syringe and aliquoted (500µL) into propylene cryotubes for storage at -80°. We followed procedures specified by the BIOMARK-APD consortium of the EU Joint Program in Neurodegenerative Disease to measure CSF concentrations of the AD biomarkers $A\beta_{1\text{-}42}$, total-*tau* (t-*tau*) and Phosphorylated-*tau* (^{181}P-*tau*) with the Innotest ELISA assay kit (Fujirebio, Ghent, Belgium).

5.4.7.6 APOE Genotype.

This was determined using RT-PCR amplified DNA and the PyroMark Q96 pyrosequencer (Qiagen, Toronto, ON, Canada), as described previously.[16]

5.4.8 Primary efficacy outcome: the composite Alzheimer Progression Score

The primary efficacy outcome was annual rate of change in the Alzheimer Progression Score (APS) using marker weights estimated beforehand in the non-trial PREVENT-AD Cohort. The APS is a composite that incorporates multi-modal imaging, neuro-sensory, cognitive and CSF markers, based on an assumption that change in each of these arises from a single underlying latent process (AD pathogenesis). Its scores are scaled as a standard normal-distribution, with higher scores denoting increasing severity. Constituent measures are summarized in Table 5.1. At each measurement, a uniform scheme of weightings for individual markers yielded a composite summary score. All available data were used to estimate individual scores, and missing data were accommodated in a process that essentially "averaged over" missing values. To verify robustness to missing data, Gross *et al*.[20] had used iterative "leave-one-out" analyses in a similar outcome measure comprising six markers. Leoutsakos *et al.*[3] also examined effects of missing CSF data on the APS, noting similarly satisfactory findings. The APS approach had been validated using data from the BIOCARD study,[13] before being incorporated into INTREPAD efficacy analyses. In BIOCARD, the APS approach showed substantial abilities to predict subsequent "conversion" to Mild Cognitive Impairment or AD dementia.[3] Analyses there also showed temporal measurement invariance. Before applying the PREVENT-AD cohort-derived marker weights to the analysis of INTREPAD, we demonstrated that they provided excellent performance in the trial sample.[3] However, the trial data (Table 1) included two variables (CSF total-*tau* and $A\beta_{1\text{-}42}$ levels) that were not available from non-trial participants. We incorporated these measures only after verifying that doing so did not materially alter the weights for the remaining variables. Full specification of variables and APS parameters preceded unmasking of treatment assignment.

Table 5.1: Variables included in the APS

Measures	Variables
Cognitive measures	RBANS attention index score
	RBANS immediate memory index score
	Item recognition task
Neurosensory measure	UPSIT total score
Gray matter density measures	Bilateral entorhinal cortex
	Bilateral lingual cortex
	Bilateral putamen
Gray matter cortical thickness measures	Right superior parietal gyrus
	Right superior dorsal frontal gyrus
Cerebral blood flow measures	Right rostral anterior cingulate cortex
Brain volume variables	Bilateral hippocampus volumes
	Lateral ventricular volume
CSF measures	Total tau concentrations (pg/mL)
	β-amyloid$_{1-42}$ concentrations (pg/mL)

Abbreviations: RBANS = Repeatable Battery for Assessment of Neuropsychological Status; UPSIT = University of Pennsylvania Smell Identification Test.

5.4.9 Statistical analysis

We followed a statistical analysis plan finalized on June 8, 2017 by JL and the PREVENT-AD Research Group. To assure consideration to potentially important AEs occurring during the first three months of the trial, safety analyses were based on the full intention-to-treat population (n = 195). These analyses were based on summary listings of AEs, using a χ^2 test for pairwise comparisons. The Baseline characteristics of the treatment groups were compared *pro forma* using Fisher's exact (for sex), χ^2 (for number of *APOE* ε4 alleles) and two-sample t-tests (for age, education, parental age of AD onset, MoCA score and APS).

The primary efficacy analysis was based on the m-ITT sample of participants who had remained on treatment through at least one follow-up (3-month) assessment (n = 160). Secondary outcomes were rate of change in cognition (RBANS total score), olfaction (UPSIT Score) and CSF biomarkers of AD (Aβ_{1-42}, t-*tau*, P-*tau*, t-*tau*/Aβ_{1-42} and P-*tau*/Aβ_{1-42} ratio) extending over the 24 months of treatment. For both primary and secondary efficacy analyses, we used longitudinal linear random effects models (random intercepts) to assess between-group differences in rates of change. We performed an additional *post hoc* analysis that included Baseline APS score as a covariate. We also constructed additional exploratory models

to test treatment effects on APS rate of change after first assigning participants into tertiles based on their Baseline CSF $A\beta_{1-42}$.

Version 9.4 of the SAS statistical package and R (Core Team 2014) were used for analyses except for *post hoc* exploratory analyses, which were performed using Matlab. APS scores were calculated using STAT, R and MPLUS.[21] All statistical tests were two-sided. A P-value ≤ 0.05 was considered to indicate statistical significance.

5.4.10 Data sharing

All de-identified data and related documentation from this trial are available upon request to qualified researchers without limit of time, subject to a standard Data Sharing Agreement. The PREVENT-AD program is currently developing a less restrictive approach to data sharing through the Canadian Open Neuroscience Platform.

5.5 Results

5.5.1 Enrollment and study completion data

The naproxen- and placebo-assigned groups included 102 and 93 participants. The m-ITT analysis groups included 88 and 72 of these. The main reasons for exclusion of the remaining 35 were apparent ineligibility (discovered typically following review of Baseline cognitive testing; n = 14), early appearance of intolerable adverse effects (n = 6), or voluntary withdrawal (many reasons given; n = 12). Compliance-to-completion rates (24 months on study drug) were 62% for participants assigned to naproxen and 66% for those given placebo (P=0.579; see Figure 5.1). After the 3-month run-in, the most common cause for drug discontinuation was occurrence of new Aes (12 naproxen-assigned and 3 placebo-assigned individuals; Figure 5.1).

5.5.2 Baseline characteristics

There were no important differences between naproxen and placebo-treated groups with respect to age, sex or education. All participants were Caucasian, and there were no substantial imbalances across groups in *APOE* ε4 status, parental age of AD onset or MoCA score. As

noted below, however, we observed an unexpected difference by treatment assignment in Baseline APS values in the m-ITT pool (Table 5.2).

5.5.3 Concomitant medications

Of the 102 naproxen-assigned persons in the ITT pool, 85 (83%) initiated use of concomitant medicines, regularly or for short intervals, over the interval of the trial. The comparable figure for those assigned to placebo was 66/93 (71%; P=0.04; Appendix 2 Table 3.) The apparent imbalance was attributable principally to initiation of lipid-lowering drugs during the trial but, notably, a similar imbalance was not evident in the m-ITT analysis pool. Baseline or anytime-use of such drugs was also similar across the treatment arms in the m-ITT group and, accordingly, *post-hoc* statistical adjustment for their use brought no appreciable change in the primary outcome results.

Table 5.2: Baseline characteristics of INTREPAD participants

Characteristics	Total	Naproxen	Placebo	*p* For difference
No. of participants	195	102	93	NS
Age, y, mean ± SD	63.3 ± 5.6	64.0 ± 5.9	62.6 ± 5.0	NS
Male sex, n (%)	51 (26)	25 (25)	26 (28)	NS
Education, y, mean ± SD	15.2 ± 3.4	15.0 ± 3.2	15.5 ± 3.6	NS
Parental age at AD onset, y, mean ± SD	73.3 ± 7.7	72.8 ± 7.6	73.8 ± 7.8	NS
MoCA score (out of 30), mean ± SD	28.0 ± 1.6	28.0 ± 1.5	28.0 ± 1.6	NS
***APOE* ε4 carriers, n (%)**	73 (37)	37 (36)	36 (39)	NS
Baseline data (mITT)	n = 160	n = 88	n = 72	
APS	−0.09 ± 0.86	0.021 ± 0.8	−0.214 ± 0.9	0.06

Abbreviations: AD = Alzheimer disease; APS = Alzheimer Progression Score; mITT = modified intent-to-treat; MoCA = Montreal Cognitive Assessment.
No observable difference between the 2 groups, except for APS at baseline.

5.5.4 Safety outcomes

Table 5.3 shows the frequency of AEs occurring in ≥10% of either treatment arm. Gastrointestinal AEs prompted study drug discontinuation in nine naproxen-assigned and three placebo-assigned participants. Constipation, dyspnea, hypertension and petechiae were also substantially more common in persons receiving naproxen. However, as expected, this group reported pain less frequently. Overall, reports of any AE were more common in naproxen-assigned persons (98% *vs* 89%; P=0.015). The principal contributors to this excess were vascular disorders, which were more common in the naproxen-assigned group (P=0.023).

Table 5.3: Adverse Events (AEs)

	Naproxen (n = 102)	Placebo (n = 93)	*p* Value
AEs			
Constipation	19 (19)	6 (6)	0.011
Dyspnea	23 (23)	10 (11)	0.028
Heartburn	31 (30)	17 (18)	0.05
Peripheral edema	25 (25)	14 (15)	0.1
Hypertension	19 (19)	8 (9)	0.04
Petechiae	12 (12)	2 (2)	0.009
Pain	20 (20)	28 (30)	0.09
Serious AEs	8 (8)	2 (2)	0.07
Vascular/cardiac events	4[a]	1	
Cancers	1	1	
Musculoskeletal/ injuries	3	—	

AE table represents n (%) of participants. If an individual had the same adverse experiences multiple times during the trial, he or she was counted only once.
[a] Two SAEs in this category were judged to have a clear or possible relationship with study drug. For detailed description of SAEs, see e-Results (doi.org/10.5061/dryad.r58d342).

Of 10 participants reporting serious adverse events (SAEs) over two years (Appendix 2 Table 4), eight had been assigned to naproxen. Three SAEs required study drug discontinuation, while two prompted treatment interruption. A further three participants were unable or unwilling (some upon physician's advice) to continue participation. No SUSAR (suspected unexpected serious adverse reaction) occurred during the trial.

Among blood safety measures, both hemoglobin and hematocrit decreased in persons assigned to naproxen, but not in placebo-treated individuals. Between-group comparison of these measures showed strong differences at all time points except at 24 months (Appendix 2 Figure 2). No differences were found in the two treatment groups with respect to other safety measurements (weight, pulse rate, systolic and diastolic blood pressure).

5.5.5 Primary efficacy outcome

Analyses of the primary outcome are summarized in Figure 5.3. Among the combined treatment groups, the APS showed a clear increase over the two-year trial period ($\beta = 0.101$ standard units / year; S.E. = 0.014; 95% CI 0.074 – 0.130 ;$P < 0.001$). However, this change did not differ meaningfully between naproxen- and placebo-assigned participants after 3, 12, or 24 months of treatment. A longitudinal linear random effects model for APS showed a slight

increase, if anything, in rate of change among naproxen-assigned persons, but this was well within chance expectation (time-by-treatment interaction β = +0.019 APS units / year for naproxen-vs.-placebo; S.E. = 0.03; 95% CI –0.037 - +0.074; P = 0.51). The APS ratio for rate of change comparing naproxen- vs. placebo-assigned m-ITT participants was 1.16 (bootstrapped 95% CI 0.64 – 1.96).

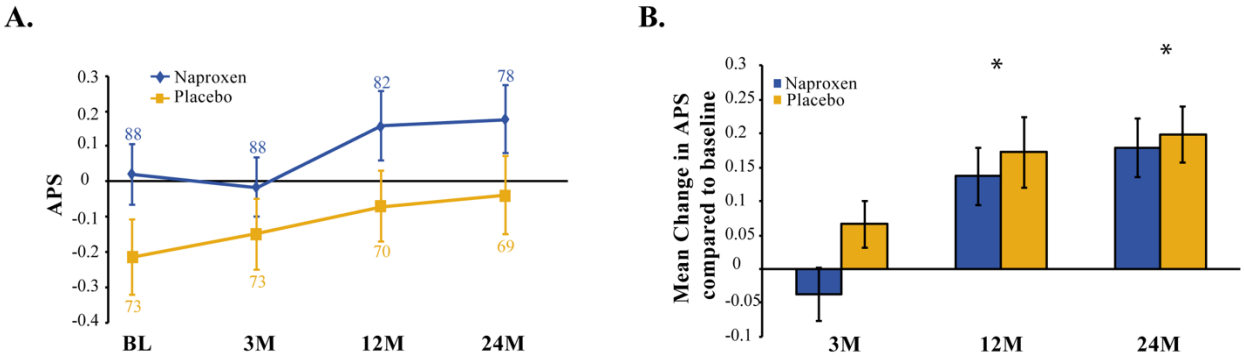

Figure 5.3: Treatment effects on APS.
Results for primary and exploratory secondary outcomes are represented. (A) There was no meaningful difference in APS rate of change between treatment groups. (B) Mean change from Baseline (± standard error of the mean) in APS did not differ between the two treatment groups at any time during the treatment interval. However, APS for both groups increased after 12 and 24 months (* P < 0.05). Data are represented as point estimates (group means) with error bars (standard error of the mean). APS: Alzheimer Progression Score

5.5.6 Secondary efficacy analyses

We used similar linear random effects models in the m-ITT sample to evaluate treatment-related differences in rate of change of RBANS total index score, UPSIT score, and CSF AD biomarkers ($A\beta_{1-42}$, t-*tau*, P-*tau*, t-*tau*/ $A\beta_{1-42}$ and P-*tau*/ $A\beta_{1-42}$). None of these comparisons suggested any benefit of naproxen treatment. Indeed, UPSIT results suggested a trend toward harm (β = -0.320, S.E. = 0.196; 95% CI –0.704 - +0.064; P = 0.102; Fig. 5.2B). Over the treatment interval, four INTREPAD enrollees (three naproxen-assigned and one placebo-assigned) developed Mild Cognitive Impairment[22] or other suggestive cognitive deficiency sufficient to prompt discontinuation of study drug.

5.5.7 Exploratory efficacy analyses.

Addition of covariates for *APOE* ε4 carrier status, age at Baseline, years of education and sex, as well as their interaction with time, did not materially affect the above findings.

As noted above, we observed that the naproxen-assigned group had higher Baseline APS scores than placebo-assigned participants. This finding prompted us to carry out a *post hoc* addition of a term for Baseline APS in the model used for the primary endpoint analysis. Addition of this term brought no consequential change from the primary endpoint results. We also analyzed whether higher Baseline APS was associated with an increased rate of change in the outcome, regardless of treatment assignment. No such association was found. After partitioning participants into tertiles of $A\beta_{1-42}$ concentration and testing for a triple interaction with time and treatment assignment, we also observed no effect of naproxen treatment on rate of change in APS among the different CSF $A\beta_{1-42}$ tertiles.

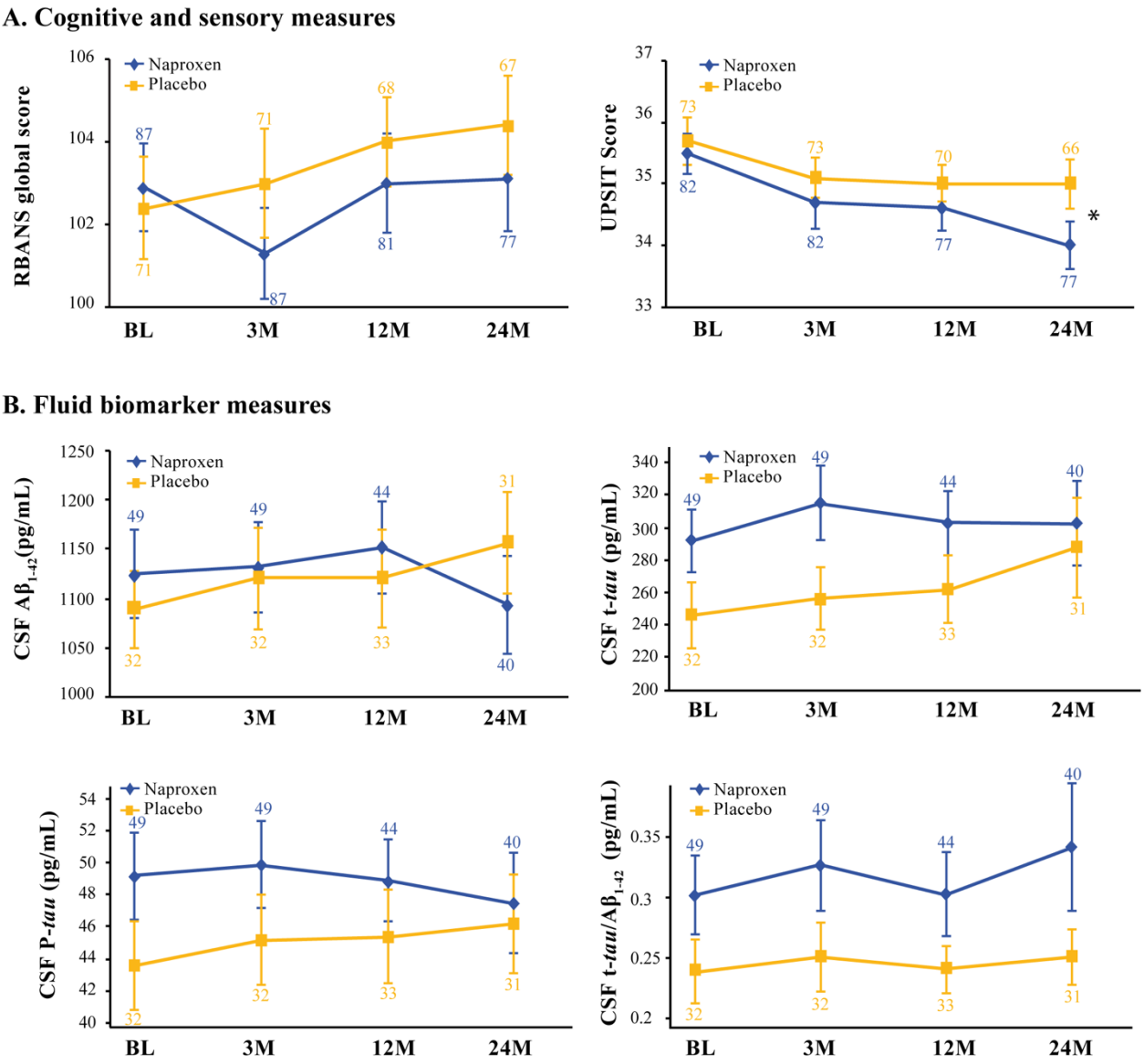

Figure 5.4: Treatment effects on neuro-sensory and CSF biomarker measures.
Linear mixed effect models did not indicate any difference between naproxen- and placebo-assigned groups in rate of change of (A) cognitive or neurosensory or (B) biological markers of AD. UPSIT scores decreased over the two-year trial period for the whole group (* $P < 0.05$). Data are represented as point estimates (group means) with error bars (standard error of the mean). RBANS: Repeatable Battery for the Assessment of Neuropsychological Status; UPSIT: University of Pennsylvania Smell Identification Test; CSF: Cerebrospinal Fluid.

5.6 Discussion

To assess the potential of the common NSAID naproxen sodium for prevention of AD, we tested this agent in INTREPAD, a two-year randomized placebo-controlled trial among cognitively healthy persons at increased risk of dementia. We observed a clear increase over

time in the trial's primary efficacy outcome (the Alzheimer Progression Score composite representing several imaging metrics, a neuro-sensory modality, three cognitive measures and, when available, CSF biomarkers). Assignment to naproxen produced an unambiguous increase in adverse events but no meaningful alteration in the rate of change for this primary efficacy outcome. Overall, our results suggest two points for discussion: 1) evaluation of a design intended to increase efficiency of AD prevention trials; and 2) the trial's safety and efficacy results, and their ethical implications.

We employed several design features intended to improve efficiency for INTREPAD and, by implication, other AD prevention trials. First, we attempted to enrich participants' proportion of persons vulnerable to AD by requiring that enrollees have an affected parent or multiple siblings.[23] Enrichment by requirement of a first-degree relative with AD is not novel, but the INTREPAD family history criteria were stronger. Especially in today's environment of greater longevity and increased awareness of AD-affected status (thus, ready identification), this more restrictive method is increasingly practical. It would seem easier to implement than a requirement of amyloid pathology demonstrable by PET[24] or CSF analysis, or even homozygosity at the *APOE* ε4 risk allele.[25] How this "enhanced family history" method of enrichment compares with the latter, more costly or invasive methods is unknown. However, we have recently shown that proximity to an index relative's age at symptom onset is related to increased AD biomarker load.[26] This same metric was associated in a separate cohort with brain changes predictive of time to symptom onset.[27] Given the method's reduced costs and subject burden, a cost/benefit comparison with other methods probably deserves consideration.

Second, we selected the composite APS as the trial's primary outcome. Relative to any single cognitive or biomarker indicator, composite outcomes of this sort should logically offer greater inference, especially in relatively early-stage pre-symptomatic AD. We chose the APS in preference to several similar composite indicators that had been less extensively validated.[28,29] While additional efforts to validate the APS are warranted, its has shown demonstrable utility for evaluation of pre-symptomatic AD progress. Specifically, in BIOCARD a 1 standard unit increase in APS predicted a 5-fold greater hazard of diagnostic progression over time.[3] In the parallel PREVENT-AD cohort, we performed several simulations to compare the statistical power of the APS to that of its constituent markers. The APS provided more information than did any single endpoint, including all cognitive measures, and it also offered improved performance over a simple summing of z-scores of its individual components (data not shown).

We therefore suggest that the APS or similar multi-modal composites show promise for prevention trials like INTREPAD.

Nonetheless, *post hoc* analyses suggested that our methods resulted in a trial with substantially less statistical power than had been originally projected. Our original power estimates were based in part on expectation that considerable information would be contributed by CSF biomarker data, available from more than half of the participants. That turned out not to be the case and, accordingly, the trial outcome's confidence interval was sufficiently broad to suggest a notable possibility of Type II error. Now having data on rate of change in the trial's outcomes, we can estimate that 2,250 person-years of observation (e.g., a sample of 1,125 followed over two years, or 563 followed for four years) would have been required for 80% power to detect a 30% reduction in the rate of APS change. Although much higher than originally estimated, this number still represents a considerable improvement over requirements of conventional prevention trial designs. For example, the ongoing A4 trial will follow ~1150 persons over ~4.6 years (5,290 person-years) for its primary cognitive outcome.[24,30] Similarly, the TOMMORROW trial had originally estimated a requirement of 5800 persons over ~4 years (29,200 person-years).[31] By comparison, even ignoring costs of PET scans in the former or serial detailed psychometric assessments in the latter, and assuming costs proportional only to person-years of observation, the INTREPAD design may achieve cost savings of 57% – 90% over traditional methods.

The INTREPAD safety results affirm prior data suggesting that, even in relatively low dosage among "younger" elderly persons, naproxen and other NSAIDS provoke harm in several health outcomes.[32-34] Our findings that these risks can be held within bounds by careful monitoring does not obviate the ethical concern that NSAID treatments are potentially harmful and should be given for AD prevention *only if they produce substantial reduction of AD risk.* The INTREPAD results provide no evidence for such a reduction. This result is especially salient, inasmuch as the trial sample was relatively young for this sort of work, and chosen for a favorable risk/benefit balance in relation to NSAID treatment. Specifically, participants were on average ~10 years younger than their affected parent or first-affected sibling, and were meticulously screened for incipient cognitive disorder.[35] These attributes appear to weaken arguments that earlier NSAID trials failed to show benefit because their aging samples were too old or too near the "cusp" of symptom onset.[6,36,37]

The "null" efficacy outcomes of INTREPAD are reinforced by several observations. The confidence interval around the trial's efficacy rate ratio suggests 95% certainty that the "true"

treatment-related reduction of AD risk in this trial (or, presumably, in similar samples) does not exceed 36%. This conclusion is buttressed by a consistent absence of apparent benefit on *any* of the trial's secondary or exploratory outcomes. These results recall observations in ADAPT[8] and prior NSAID treatment and prevention trials with null or negative results. While we cannot exclude possible benefits of NSAIDs in middle-life, we can now suggest that such benefits would be nearly impossible to demonstrate in randomized prevention trials.

As regards our further objective of testing or demonstrating methods for improving efficiency in AD prevention trials, we offer several observations. Our "enrichment" strategy requiring parental or multiple-sibling family history of AD might have been improved by a further requirement specifying participants' age in relation to their index relatives' onset(s). While evidently more informative than any single biomarker, or a composite of only a few such markers, our outcome could today quite probably be improved by incorporation of newer salient markers of preclinical disease. These might include threshold values for the traditional CSF biomarkers $A\beta_{1\text{-}42}$, t-*tau* or p-*tau*, or possibly neurofilament light chain as a *pre-condition* to enrollment. Power estimation and sample size requirement for such work should be clearer now than when we began this work. In this last sense INTREPAD may be viewed as providing an approximate benchmark for work with samples having similar baseline characteristics.

In all events, this work has left us with extreme pessimism regarding any possible role of NSAIDs in AD prevention. Instead, our results may suggest re-consideration of inflammatory diseases (or a pro-inflammatory diathesis) as a possible explanation for the reduced AD incidence among NSAID users in observational studies.[8]

Acknowledgment

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Chapter 6 – No apparent effect of naproxen on CSF markers of innate immune activation.

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6.1 Preamble

Chapter 6 is an exploratory analysis investigating the effects of naproxen in the INTREPAD trial. It has long been assumed that the benefits of NSAIDs stemmed from their anti-inflammatory properties.[1,2] However, pre-clinical evidence suggests a limited ability of these drugs to enter the central nervous system.[3,4] Here, we investigated whether an NSAID like naproxen entered the CNS and had any effect on typical markers of inflammation.

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6.2 Abstract

We studied 78 participants having a parental or multiple-sibling history of Alzheimer's disease (AD) in a two-year randomized placebo-controlled trial of naproxen 220 mg b.i.d for mitigation of early AD pathogenesis. Naproxen was detected in cerebrospinal fluid at concentrations ~100 times lower than in plasma but produced negligible change in immune markers. The repeated lack of benefit in AD prevention trials using naproxen and related drugs may reflect limited CNS permeability, lack of expected drug effects, or both. These findings suggest reconsideration of implications from results of AD prevention trials using anti-inflammatory drugs.

6.3 Introduction

Alzheimer's disease (AD) amyloid-β and *tau* deposits are accompanied by microglial activation and signs of inflammation. Early clinical observations,[1] and subsequent pharmaco-epidemiological data,[2] suggested that non-steroidal anti-inflammatory drugs (NSAIDs) prevent subsequent development of AD symptoms. Clinical trials failed to confirm this effect, however,[3-5] and we know of no human studies regarding NSAID effects on CNS immune activity. Among participants in a recently completed two-year trial of oral naproxen-sodium 220mg b.i.d. for mitigation of pre-symptomatic AD biomarker progression,[6] we therefore explored CNS permeability of naproxen and the corresponding change in immune markers.

6.4 Methods

6.4.1 Participants

INTREPAD, a recently completed two-year placebo-controlled trial of naproxen 220 mg BID for AD prevention,[6] enrolled 101 cognitively unimpaired (CU) serial CSF donors with a parental or multiple-sibling history of "sporadic" AD.[7] These were 55 or more years of age (most aged 60+). Two or more lumbar punctures were available from 78, the first at baseline in all but two instances (Table 6.1). Each participant and study partner provided written informed consent. All procedures were approved by the McGill University Faculty of Medicine Institutional Review Board. All research complied with ethical principles of the Declaration of Helsinki.

6.4.2 CSF measurements

CSF collection and storage as well as *APOE* genotyping were performed as described.[8] We measured concentrations of the "classic" AD biomarkers $A\beta_{1\text{-}42}$, total-*tau* (t-*tau*) and P_{181}-*tau* (P-*tau*) using the Innotest ELISA kit (Fujirebio, Ghent, Belgium). CSF apolipoprotein-E (apoE) levels were assessed using the Milliplex APOMAG-62k multiplex kit, and 29 immune proteins were assayed using the Milliplex HCYTMAG60PMX29BK xMap kit (EMD-Millipore, Billerica, MA, USA). We excluded marker analyses with coefficient of variation > 15% or missing data >20%, leaving 13 protein species for analysis. We also used mass spectrometry to assay naproxen concentrations in plasma and CSF of 57 and 30 participants, respectively, using methods described elsewhere.[9] Data collected at the trial's 3- and 12-month evaluations were discarded for two participants who had previously discontinued treatment.

6.4.3 Statistical analyses

Group comparisons of summary statistics used t-tests and Fisher's exact test when appropriate. Mann-Whitney-U tests compared baseline levels of CSF protein markers along with plasma and CSF Naproxen concentrations.

Table 6.1: Sample characteristics.

	Baseline				3 months				12 months				24 months			
	All	Placebo	Nap.	*P*	All	Placebo	Nap.	*P*	All	Placebo	Nap.	*P*	All	Placebo	Nap.	*P*
N	76[1]	33	43		73	29	44		72	32	30		66	30	36	
Age (years)	62.70 (5.53)	61.90 (5.35)	63.31 (5.66)	0.27	63.01 (5.35)	61.84 (4.59)	63.80 (5.71)	0.11	63.62 (4.85)	62.88 (5.13)	64.21 (4.59)	0.26	63.86 (4.54)	62.96 (4.36)	64.61 (4.61)	0.14
Sex (M:F)	24:52	9:24	15:28	0.62	22:51	7:22	15:29	0.44	22:50	7:25	15:25	0.20	19:47	6:24	13:23	0.18
%APOE	38.2	42.4	34.9	0.63	38.4	30.2	34.1	0.46	37.5	43.8	32.5	0.34	37.9	43.3	33.3	0.45
CSF A$\beta_{1\text{-}42}$ (pg/mL)	1136.04 (286.80)	1160 (239.67)	1117.28 (319.83)	0.50	1124.69 (296.92)	1146.78 (283.63)	1110.13 (307.71)	0.60	1125.54 (298.57)	1131.67 (281.43)	1120.64 (315.08)	0.88	1109.03 (298.15)	1173.33 (279.99)	1055.44 (305.99)	0.11
CSF t-*tau* (pg/mL)	274.56 (131.01)	258.32 (118.94)	287.03 (139.65)	0.33	282.60 (141.31)	240.38 (79.79)	310.43 (165.22)	**0.02**	283.84 (132.39)	257.19 (120.92)	305.16 (138.70)	0.12	295.48 (170.78)	286.72 (177.33)	302.78 (167.30)	0.71
CSF P-*tau* (pg/mL)	47.68 (18.23)	45.57 (16.81)	49.30 (19.29)	0.37	47.19 (17.40)	43.53 (13.73)	49.61 (19.22)	0.12	47.57 (17.15)	45.20 (16.97)	49.47 (17.27)	0.30	46.72 (18.79)	45.46 (17.20)	47.76 (20.21)	0.62

[1]Two participants did not receive a lumbar puncture until the 3-months visit.

6.4.3.1 Naproxen and CSF immune markers

We tested data for normality, applied Boxcox transformation when necessary and calculated Z-scores. Paired t-tests and linear models adjusted for age and sex, as appropriate, compared within- and between-treatment group marker levels at each time point. We then tested for association of naproxen concentration and protein markers levels. Mass spectrometry assays for CSF naproxen concentration were obtained for 30 (18 naproxen-assigned, 12 placebo-assigned) participants. We then performed a linear mixed-effects analysis to test whether CSF immune marker levels changed over the trial period, adjusting for age, sex, *APOE* ε4 carrier status, and compliance as well as CSF t-*tau* and $A\beta_{1-42}$ concentrations. When there was a statistically significant change over time, we repeated the linear mixed-effects analysis, now adding an interaction term for treatment-by-time to test for a difference in slope of change between treatment groups. Naproxen-treated participants had concurrent measurement of CSF markers and naproxen at 3, 12, and 24-months of follow-up (16, 16 and 4 participants). We used general linear regression models, adjusted for *APOE* carrier status and age, to investigate the association of naproxen concentrations with 3- and 12-month protein marker levels. We repeated this analysis pooling all available post-baseline data while also considering participant sex, CSF t-*tau* and $A\beta_{1-42}$, because immune marker levels are associated with AD biomarkers.[8] All analyses used two-sided $\alpha = 0.05$ in Matlab software (Mathworks inc.; Natick, Massachusetts).

6.4.4 Data availability

All de-identified data and related documentation from this trial are available upon request to qualified researchers without limit of time, subject to a standard Data Sharing Agreement.

6.5 Results

6.5.1 Summary statistics

Among the 78 participants analyzed, 4 (1 placebo, 3 naproxen), 8 (3 placebo, 5 naproxen) and 66 (30 placebo and 36 naproxen) participants completed 3-,12- and 24-month visits on treatment. At baseline, naproxen- and placebo-assigned groups were indistinguishable in sex ratios, proportion of *APOE* ε4 allele carriage, or CSF P-*tau*, t-*tau* and $A\beta_{1-42}$ levels. The

naproxen-assigned group was somewhat older (P = 0.27). Baseline immune marker concentrations were comparable between groups except for IL-6 levels, which trended higher in participants assigned to naproxen (P = 0.08).

6.5.2 Naproxen enters the brain of treated individuals.

No participants had measurable naproxen levels in either plasma or CSF at baseline (before randomization). At follow-up, only naproxen-assigned participants had measurable drug in either plasma or CSF (Figure 6.1). Follow-up assays revealed two naproxen-assigned individuals who had measurable drug in plasma but not in CSF. Otherwise, CSF concentrations were typically ~100-fold lower than in plasma. Pooling all available post-baseline data revealed little if any association between CSF and plasma concentrations of naproxen (R^2= 0.02, P = 0.37), and a marginal association of CSF naproxen with age (R^2= 0.08, P = 0.08). Variability in apparent CNS permeability to naproxen (*i.e.*, CSF naproxen concentration) was not appreciably associated with compliance.

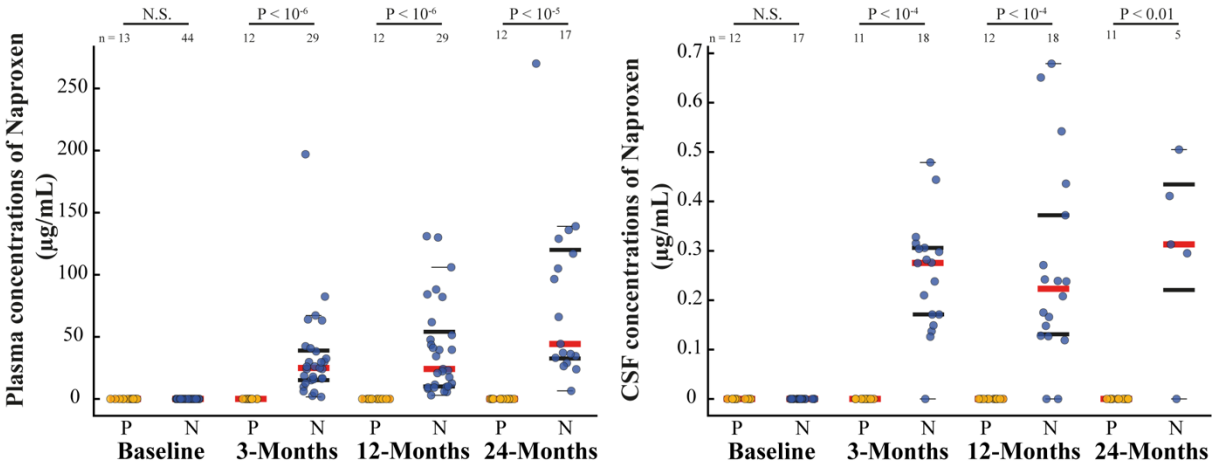

Figure 6.1: Plasma and CSF concentrations of naproxen in the trial cohort.
Plasma (left) and CSF (right) concentrations of Naproxen were measured in placebo (yellow) and naproxen (blue) assigned participants using LC-MS/MS. The placebo group showed no measurable levels of the drug at any time point. Naproxen-assigned participants had readily detectable naproxen in plasma, but ~100-fold lower concentrations in CSF at each follow-up (note the difference in y-axis scales). Red lines depict medians, bold lines represent the 25th and 75th percentile and thin black lines represent the minimum and maximum value not considered to be outliers (first and third quartile ± 1.5 times the interquartile range)

6.5.3 Naproxen does not affect concentrations of CSF immune markers.

IL-1RA, IFN-α2 and apo-E levels increased from baseline over the trial period in both treatment groups (Figure 6.2). However, these changes were comparable across the treatment groups at all time-points, suggesting that they might uniformly reflect aging of the population[10,11] and not an effect of naproxen. IL-6 levels declined significantly at 3, 12 and 24-months in naproxen-assigned participants only, but fell readily within the distribution of placebo participants. A linear mixed effects analysis indicated that apoE concentration increased substantially over the trial interval, independent of age, sex, *APOE ε4* carrier status, compliance, and CSF t-*tau* or $A\beta_{1-42}$ ($\beta = 0.13$ units/month, S.E.= 0.06, $p = 0.03$). This two-year slope of apoE concentrations appeared steeper in naproxen-assigned participants vs. placebo (time-by-treatment interaction $\beta = +0.02$ units/month, S.E.= 0.01, $p = 0.09$). Interestingly, 3- and 12-month CSF naproxen concentrations appeared to be associated with CSF apoE protein ($\beta = 4.10$, S.E.= 2.28, $p = 0.10$ and $\beta = 3.13$, S.E.= 1.24, $p = 0.03$), with adjustment for *APOE ε4* carrier status and age. Upon pooling all post-baseline data in the naproxen-assigned group, the association between drug and apoE concentration was stronger and apparently independent of age, sex, *APOE ε4* carrier status, CSF t-*tau* and $A\beta_{1-42}$ ($\beta = 3.10$, S.E.= 0.86, $p = 0.001$). Interpretation of this last observation is difficult, however, because no detectable difference appeared in mean apo-E concentrations at all time points across the two treatment arms. This finding may relate to a relative (not statistically significant) difference in baseline apoE levels between the two groups. Further adjustment for this variable indicated a difference in slope between the two groups (time-by-treatment interaction $\beta = +0.02$ units/month, S.E.= 0.01, $p = 0.04$). All results remained similar when restricting our analyses to the 66 participants who completed 24 months on study drug.

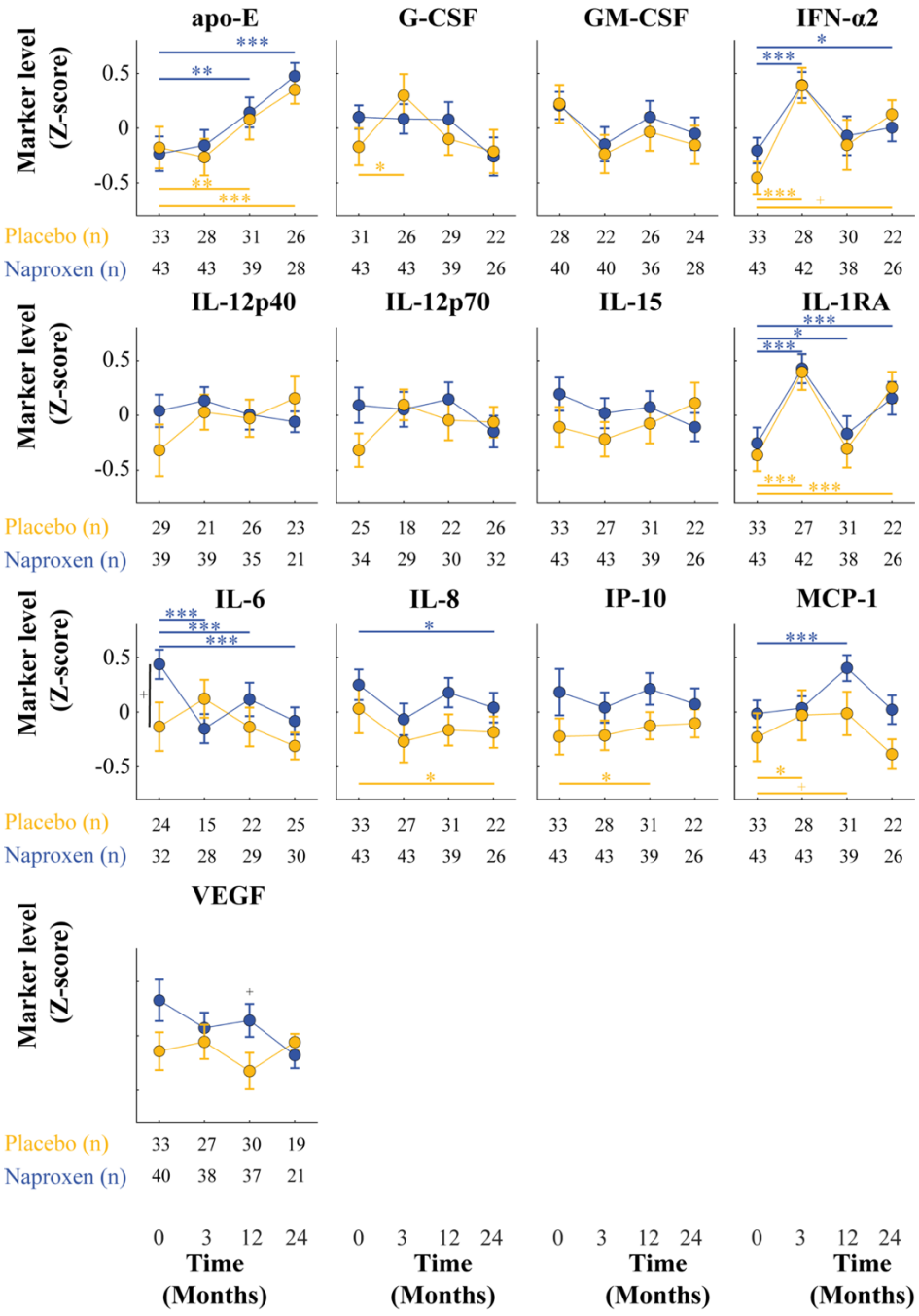

Figure 6.2: Trajectory of CSF immune markers by treatment group.
Trajectory of CSF immune markers by treatment group. Longitudinal CSF levels of immune markers in the placebo (yellow) and naproxen (blue) groups are represented. Point estimates represent group means and error bars standard error of the mean. IL-6 levels tended to be higher at baseline in the naproxen-assigned group compared to placebo ($P \leq 0.1$). IL-1RA, IFN-α2 and apoE levels increased from baseline in both treatment groups over the trial period. IL-6 levels decreased significantly at 3, 12 and 24-months compared to baseline in naproxen-assigned participants only. At all post baseline time-points, immune marker levels were comparable between both treatment arms. + $P < 0.1$; * $P<0.05$; **$P<0.01$;***$P<0.005$.

6.6 Discussion

In a sample of healthy elderly at increased risk for AD dementia we examined whether 220mg of oral naproxen b.i.d entered the CSF and thereby affected immune marker concentrations. While drug levels were measurable in plasma, CSF concentrations were ~100-fold lower. Naproxen treatment did not meaningfully alter CSF concentrations of several immune markers. CSF apolipoprotein E concentrations increased during the trial period, and the results suggested its association with CSF naproxen concentrations.

Early epidemiologic observations of reduced AD risk with NSAID treatment led to widespread speculation that these drugs produced a beneficial suppression of inflammatory responses to accruing pathology. However, this interpretation has never been verified. While the inhibition of cyclooxygenase activity (the proximate target of NSAID activity) can lead to reduced inflammation, NSAIDs also have other effects. For instance, some NSAIDs may reduce accrual of Aβ pathology itself,[12] while others may promote neuronal survival.[13] However, few human studies have examined whether NSAIDs cross the blood brain barrier. Our results suggest CSF permeability for naproxen is minimal, much like other conventional NSAIDs.[14] While it is uncertain whether CSF levels of naproxen fully reflect brain levels, the proportion of drug in CSF compared to blood is comparable to what is measurable in brain tissue in animal models.[15,16] Thus, brain levels of naproxen may never reach levels needed to observe the neuroprotective effects described *in vitro*.[17] Drug levels were, nonetheless, sufficiently measurable in CSF to suggest no meaningful effects on immune marker activity. One important limitation is that some immune markers may be actively transported across the BBB and possibly hide the apparent effects of naproxen on immune markers. However, while IL-6 may be actively transported across the BBB,[18] our IL-6 results appear to suggest nothing more than regression toward the mean among treated individuals. More importantly, the contribution of contamination – if any – to CSF concentration of immune markers is unknown, and one would expect that a decrease in global brain immune reactivity would be reflected at the CSF level, as it is for amyloid and *tau*. By contrast, CSF naproxen concentrations *were* associated with increasing CSF apoE levels. One of us (J.P.) had previously shown that NSAIDs may increase astrocytic production of apoE,[19] and that this apparent effect was obtained at NSAID concentrations below those typically required for COX inhibition.

We conclude that it is unlikely that a central "anti-inflammatory" effect would be responsible for any purported benefit of NSAIDs in protecting against development of AD. Our findings suggest instead that NSAID benefits may stem from increased apoE concentration in proportion to drug levels. However, this drug-related increase was not strong enough to obviate increasing apoE levels over time, possibly as a consequence of ageing. Before being regarded as conclusive, these results require replication, possibly in a larger group of individuals. We cannot, however, exclude that NSAIDs still bear some influence on CNS immune pathways not measured here, or that peripheral immune system activation might in some way modulate risk of AD pathology.[20]

Author Contributions

P.F.M and J.B contributed to data interpretation as well as drafting and revising of the manuscript. P.F.M analyzed the data. A.L., P.R.N and J.P. had a major role in data acquisition (measures of CSF proteins, lumbar punctures and laboratory methods, respectively). J.P and J.B contributed to study conceptualization and design. J.B. supervised the study.

Potential Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 7 – Discussion

This thesis explores the relationship of markers of immune function with biomarker evidence of progressive AD pathology at its earliest stages (Chapter 3) and with symptomatic expression and progression across the disease spectrum (Chapter 4). It also further expends upon previous work that had suggested the utility of common NSAIDs for the prevention of AD dementia (Chapter 5), and highlights the notion that it is unlikely that these drugs bear any anti-inflammatory effects in the CNS (Chapter 6). The implications of these results are discussed below.

7.1 Contributions

7.1.1 Immune activation – An integral part of AD pathogenesis

In Chapter 3, we showed that CSF immune marker changes are tightly linked to biomarker evidence of AD pathology.[1] Immune activation seemed to be more tightly linked to increasing evidence of *tau* pathology rather than accruing Aβ pathology, a finding which was in line with previous reports in early 'sporadic' and autosomal dominant AD.[2-4] More importantly, our results suggested that a decrease in CSF immune marker levels accompanied evidence of accruing AD pathology, a result recently replicated across the disease spectrum for CSF sTREM2, a recognized marker of microglial function.[5] These findings align with the notion that microglial activation in the vicinity of AD lesions is a hallmark of the disease.[6,7] However, the cross-sectional nature of the analyses does not allow any interpretation of causal associations between immune activation and subsequent change in AD biomarker levels. *In vitro* and *in vivo* pre-clinical studies suggest that Aβ is sufficient to initiate microglial activation[8,9] which in turn may lead to neuronal damage and *tau* hyperphosphorylation.[10] In general these observations prompted thee integration of the inflammatory response as a downstream effect of the amyloid cascade.[11] However, the identification of genetic variants associated with both AD and the immune response alternatively suggested that immune system is not a bystander but rather an actor in the pathogenic process.[12-14] More importantly, mutations in TREM-2 and CD33 result in impaired microglial ability to migrate to, engage with and phagocytose Aβ plaques.[15,16] Because late-onset AD is now regarded as being an issue

primarily of *clearance* rather than production of Aβ,[17] microglial inability to engage correctly with Aβ may be an initiating event of the pathogenetic process.

The results in Chapter 3 align well with these experimental findings, suggesting that an attenuated immune response might facilitate Aβ deposition. Another appealing hypothesis, however, could be that decreased CSF immune marker levels in relation to evidently accruing Aβ pathology relate to microglial 'burn out'.[18] In fact, accruing evidence suggests that microglia in the AD brain present with a non-functional or 'dystrophic' phenotype possibly resulting from exposure to long-lasting insult.[19,20] We do not discard that the trajectories presented in Figure 3.1 capture some microglial dystrophy. However, we judge unlikely that it solely represents this phenomenon because immune activity seems to 'awaken' with the increasing evidence of *tau* biomarkers.

7.1.1 Immune contribution to resilience

After having first identified a 'signature' of CSF proteins related to AD biomarker status in non-demented individuals, the work of Chapter 4 investigated whether these markers related to AD symptoms. Among the 23 proteins initially identified, we further narrowed down our investigations to a set of 10 proteins with varied biological functions that strongly predicted cognitive performance, clinical diagnosis and cognitive change over the subsequent four years. Interestingly, among proteins that predicted improved cognitive outcomes were some known to be involved in microglial activation (*e.g*, CgA, MCSF-1), microglial chemotaxis (*e.g*, VEGF) or closely related to immune pathways involved in AD (*e.g*, apoE). Most importantly, the ability of these proteins to predict cognitive and clinical outcomes was independent and complementary of AD biomarker levels. These findings therefore suggest that they may aggravate or improve symptoms independently of AD pathology.

The concepts of reserve, resilience and resistance have gained increasing recognition and importance in the AD field, such that they are now a central area of research.[21,22] These concepts were initially related to the brain's ability to circumvent pathological aggregates by using alternate pathways.[23] They have also been linked to various lifestyle or neuropsychiatric characteristics. However, the biological underpinnings of reserve, resilience and resistance have only recently been investigated.[24,25] The identification of pathways promoting resilience

and resistance hold significant potential because they may provide targets for novel pharmacological interventions for AD treatment or prevention. Despite limited research, however, the immune system has already been implicated in resilience to AD pathology. In fact, in a *post mortem* study, Perez-Nievas *et al*.[26] described individuals who had high levels of Aβ and *tau* pathologies but some of whom were 'mismatches' with no cognitive impairment. Compared to patients, mismatches had preserved neuronal density, attenuated burden of thioflavin-S positive plaques and oligomeric Aβ deposits, decreased accumulation of hyperphosphorylated *tau* at the synapse and reduced glial activation accompanying Aβ and *tau* deposits. In follow-up investigations, this group further characterized the cytokine profiles associated with resilience to AD pathology.[27] Interestingly, they observed that the response associated with resilience was characterized by higher levels of typically 'anti-inflammatory' cytokines (*e.*g, IL-4, IL1RA, IL10 and IL13) but also some important pro-inflammatory mediators (*e.g*, IL1β, IFNγ, Eotaxin). This further supports the idea that the immune response may be a modulator of developing of AD pathology and associated symptoms.

At this point, however, investigations of associations of AD biomarkers or symptoms with immune markers have been limited to single time point analyses. Our work in Chapter 4 and a PET study measuring microglial activation in AD suggest that increased immune function may portend slower subsequent cognitive decline.[28] However, the specific type of immune activation may be important to establish the benefits or harm of the response. For instance, microglia and astrocytes are highly dynamic cells which can present with diverse activation states that yield an immune response along a spectrum.[29,30] Thus, much as the *post mortem* results suggest, immune activation profiles representing *both* pro- and anti-inflammatory effects may portend better outcomes. At present, it is unclear whether TSPO ligands are specific to a given microglial response. As for our study in ADNI participants, as discussed in Chapter 4, it is notable that the set of markers identified did not include more common immune indicators possibly owing to technical (assay) difficulties encountered by ADNI investigators. We suggest that this group of markers may be only the 'tip of the iceberg' representing a larger network of underlying responses. Future investigations of this topic should leverage newer technology to assay longitudinally a more comprehensive set of markers representing immune activation simultaneously with specific AD biomarkers.

7.1.3 Targeting neuro-inflammation for AD prevention and treatment

In Chapters 5 and 6 we discussed the use of NSAIDs for AD prevention. While the epidemiological data initially appeared very strong in support of the hypothesis that NSAIDs prevent AD, all trials in healthy and demented patients have yielded null or negative results.[31-34] The results of INTREPAD in particular did not suggest any benefits of the drug on primary or secondary endpoints. While some have suggested that the trial results could have been different had we tested an NSAID with more amyloid lowering properties, or in a different population,[35] we doubt this inasmuch as an earlier meta-analysis that included specification of individual NSAID agents showed no meaningful difference between the apparent "effects" of γ-secretase modulating NSAIDs vs. others that lack this attribute.[36] Specifically, naproxen appeared to be marginally more "effective" as an AD preventive than ibuprofen (a "γ-secretase modulator"). Furthermore, the results presented in Chapter 6 seem to challenge the notion that any NSAID is likely to confer protection against AD incidence. Instead, our results appear to corroborate animal work suggesting that CNS permeability to NSAIDs is limited.[37,38] Furthermore, naproxen – when present – showed no apparent apparent effect on immune markers measurable in the CSF. As discussed in Chapter 6, we cannot exclude that naproxen affects other immune markers that we did not measure here, or has measurable effects only on cyclooxygenase, its primary target.[39] However, if naproxen were to limit the immune response at the CNS level, one could expect a broader response that should have been measurable here. Instead, it is possible that NSAIDs have a broader protective effect on AD risk by modulating systemic immune responses. Some research suggests that peripheral insults and associated immune responses throughout life may 'prime' CNS responses in late life.[40] Such microglial priming could result in a maladaptive, overactive response that might induce CNS damage.[41] But it is possible, at least, that the tendency toward some aspects of such immune activity could enhance the brain's ability to resist AD pathogenesis or symptoms. Persons with such "enhanced immune" tendencies might also have suffered in earlier life from a variety of diseases characterized by immune activation (rheumatoid arthritis being perhaps the prime example), and these would typically warrant or require treatment with NSAIDs. If later use of naproxen were in fact to suppress such a response in brain, then we suggest that prevention trials required to test this idea would require large samples followed over several decades. Such trials are simply not feasible given their cost, and risks of NSAID use that would appear to considerably outweigh the drugs' prospects for preventive effects. Finally, epidemiological

studies that investigated the "benefits" of NSAIDs on AD risk in some instances controlled the presence or absence of arthritis as a principal indication for their use. It may be notable, therefore, that statistical models that attempted to control for these indications still showed a statistically significant apparent reduction in AD risk, while the term for 'arthritis' as a confounder remained significant even when controlling for NSAID use.[42] In sum, we cannot exclude that an inflammatory diathesis may provide protection against AD risk.

As indicated in Chapter 6, we measured only minimal CNS permeability to naproxen and no change in immune marker levels. Thus, INTREPAD may have tested the 'NSAID hypothesis' rather than the 'anti-inflammatory drug' hypothesis. Immune-targeting therapies for AD remain relevant, but those currently in development have more precise targets. Anti-inflammatory drugs reducing inflammasome activation,[43] complement pathways[44,45] or even the IL-12/23 pathway[46] may be more promising as they target immune mechanisms more generally recognized as being maladaptive in disease. By contrast, other avenues meant to increase the immune response such as drugs upregulating TREM-2 or CD-33 activity[47] and, more controversially, immune checkpoint inhibitors[48,49] are also being investigated for protection against AD.

Importantly, while our findings from INTREPAD appear to be 'the nail in the coffin' of the NSAID story, they do not exclude immune targeting therapies as avenues for AD. In fact, drug development in this field may benefit from the type of studies proposed in Chapter 4 and also earlier in this discussion. Identifying pathways that influence pathological and symptomatic development – as well as the timing at which they do so – will be key.

7.2 Future directions

This work shows that diverse immune markers relate to degree of AD pathology, sometimes even more strongly than to clinical diagnosis. Combining these disease-associated markers with typical AD biomarkers leads to improved prediction of cognitive performance, clinical status and rate of symptom progression. These markers may therefore be representative of ongoing immune mechanisms that contribute to the development of AD pathology and associated symptoms. A better comprehension of these mechanisms may advance our understanding of disease processes and drive drug development. As discussed in detail in

Chapter 4 however, the study of immune mechanisms in humans is limited by contrasting results from past studies and the lack of technology able to reliably measure markers of inflammation in CSF.[50-52] Additionally, increased inflammatory signaling is also an integral component of aging.[53] Thus, it is sometimes hard to tease apart what component of immune changes is a part of the 'normal' aging process and which portends to the disease process. To further our understanding, we need to investigate longitudinal inflammatory changes in aging populations and distinguish alterations associated with aging from those associated with AD. In particular, measures of inflammatory markers should leverage high sensitivity assay technologies to measure a complete set of immune markers.

Accordingly, in preliminary work thus far unpublished, we assayed baseline CSF samples from 78 participants in the PREVENT-AD cohort,[54] for 12 immune markers using a combination of Luminex (EMD-Millipore; Appendix 1 Methods) and Single Molecular Array (SiMoA; Quanterix Billerica, MA, USA) technologies. Using a similar approach to that advocated in Chapter 4, we identified immune marker signatures associated with biological age (Figure 7.1A), P-*tau*/A$\beta_{1\text{-}42}$ ratio (Figure 7.1B) and RBANS performance (a global cognitive test, Figure 7.1C). While these preliminary data are limited by the size of the studied sample, we note that similar markers can increase with aging and evident AD pathology (*e.g*, IL-15 and IFNα2). As expected however, immune markers only explain a fraction of the variance that defines aging (19%) and AD biomarkers (12%). By contrast, they explained a similar portion (~30%) of the variance in cognitive performance as was observed in ADNI (Figure 7.1C). Interestingly, in this work we see more clearly that markers associated with immune activation *and* inhibition predicted both better and worse outcomes. Although preliminary, this observation aligns with literature suggesting that immune aspects of AD are unlikely to be unidirectional and that, while some mechanisms require inhibition (*e.g*, IL-10 signaling),[55,56] others may need up-regulation. Future work from our and other groups characterizing longitudinal change in immune markers, AD biomarkers and cognition in 'healthy aging' and pre-symptomatic AD should help identify which pathways should be targeted and when.

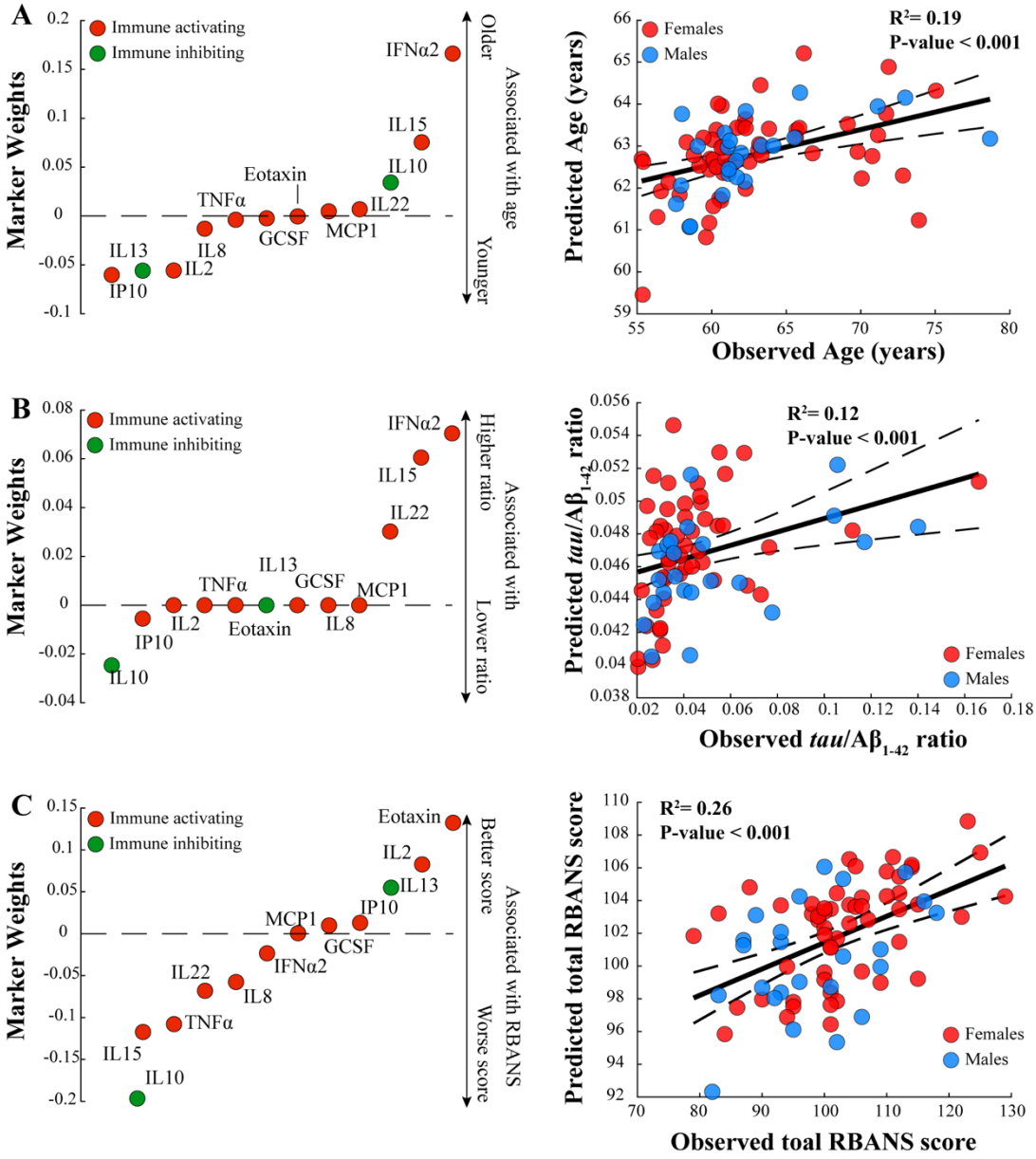

Figure 7.1: Immune associations with chronological brain aging, AD biomarkers and cognition in persons at high risk of having pre-symptomatic AD.
Shown are results of preliminary analysis of associations between immune activity, participant age, AD biomarkers and cognitive performance in the PREVENT-AD cohort of cognitively unimpaired older adults at high risk for AD dementia. ***(A)***, left, shows LASSO-assigned marker weights for the association with biological age and, right, association of model-predicted and observed age. ***(B)*** shows similar results for the association of immune markers with P-*tau*/Aβ ratio. Aging and AD pathological accumulation are both associated with increases in IL-15 and IFNα2. Results of analyses investigating the associations with cognitive performance as measured by RBANS Total Score ***(C)*** suggest that the IL-15 response may result in a worsening of cognitive performances. As suggested by prior studies, increased IL-10 signaling may also incur worse outcomes while increased signaling by Eotaxin and IL-2 may portend better outcomes.

7.3 Conclusions

In summary, this thesis explores the associations of immune markers with evidence of progressing AD pathology and symptoms as well as the potential of typical NSAIDs for AD prevention. While NSAIDs do not seem to curb the progression of a composite indicator of asymptomatic AD, biomarker studies suggest that immune and AD-related changes are closely intertwined. Immune activation may have an important role in the development of AD pathology and symptoms, thereby making it a potential target as the field moves away from Aβ-lowering therapies. The results presented in this thesis suggest the importance of characterizing *which* immune pathways are important and *when* they are involved. They also provide an experimental roadmap to investigate these processes in humans and provide preliminary data suggesting the feasibility of such studies.

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Appendix 1 – Supplementary material to Chapter 3

Supplementary Methods

CSF Apolipoprotein-E and Milliplex inflammatory marker assays

CSF apolipoprotein-E (apoE) levels were assessed using the Milliplex APOMAG-62k human apolipoprotein cardiovascular disease multiplex assay (EMD Millipore, Billerica, MA). The multiplex assay was performed in 96-well plates. The plate was wetted with 150 μL wash buffer for 10 min and left to decant. 25 μL standards or samples, 25 μL beads, and 25 μL assay buffer were added and incubated overnight at 4°C. The beads were washed three times, after which 50 μL biotinylated detection antibody cocktail was added and incubated at room temperature (RT) for 1 hour. 50 μL Streptavidin- Phycoerythrin were added and further incubated at RT for 30 min. Lastly, we washed the beads three times, added 150 μL sheath fluid and obtained readings on Luminex® instrumentation.

We used the same protocol using Milliplex HCYTMAG60PMX29BK xMap kit to assay CSF levels of 29 immune/inflammatory markers including EGF, Eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β and VEGF.

Supplementary Tables

Table A1.1: : Association of PREVENT-AD CSF immune/inflammatory markers with pathological stages.

Marker‡	Stage 1 vs 0	Stage 2 vs 0	SNAP vs 0	Stage 2 vs 1
Granulocyte Colony-Stimulating Factor	β= 0.37 P= 0.26	β= 0.62 P= 0.13	β= 1.09 **P= 0.003*****	β= 0.25 P= 0.61
Granulocyte-Macrophage Colony-Stimulating Factor	β= -0.45 P= 0.22	β= -0.03 P= 0.94	β= 0.47 P= 0.18	β= 0.43 P= 0.41
Interferon α2	β= -0.34 P= 0.31	β= 0.18 P= 0.66	β= 0.60 P= 0.09^{+}	β= 0.52 P= 0.28
Interferon Gamma-Induced Protein 10	β= -0.24 P= 0.42	β= 0.28 P= 0.45	β= 1.23 **P= 3.59E-4*****	β= 0.52 P= 0.23
Monocyte Chemoattractant Protein 1	β= -0.40 P= 0.20	β= 0.51 P= 0.19	β= 0.42 P=0.23	β= 0.91 **P=0.05***
Macrophage Inflammatory Protein 1 beta	β= -0.07 P= 0.85	β= -0.17 P= 0.75	β= -0.08 P= 0.88	β= -0.10 P= 0.86
Interleukin 12p40	β= -0.83 **P= 0.02***	β= -0.11 P= 0.81	β= 0.20 P= 0.56	β= 0.72 P= 0.17
Interleukin 12p70	β= -0.84 **P=0.03***	β= -0.53 P= 0.19	β= 0.85 **P= 0.01****	β= 0.31 P= 0.55
Interleukin 15	β= -0.60 **P= 0.04***	β= 0.38 P= 0.31	β= 1.20 **P= 0.001*****	β= 0.97 **P= 0.02***
Interleukin 6	β= 0.43 P= 0.40	β= 0.25 P= 0.59	β= 0.59 P= 0.13	β= -0.18 P= 0.78
Interleukin 8	β= -0.66 **P= 0.03***	β= 0.28 P= 0.46	β= 0.94 **P= 0.004*****	β= 0.94 **P= 0.04***
Interleukin 16	β= -0.05 P= 0.89	β= 0.47 P= 0.32	β= 1.25 **P= 0.004*****	β= 0.52 P= 0.32
Interleukin 10	β= 0.10	β= 0.24	β= 0.11	β= 0.14

	P= 0.85	P= 0.64	P= 0.80	P= 0.84
Interleukin 1 Receptor Antagonist	β= 0.16 P= 0.64	β= -0.19 P= 0.65	β= -0.39 P= 0.28	β= -0.35 P=0.48
C-Reactive Protein	β= -0.24 P= 0.54	β= -0.51 P= 0.34	β= 0.47 P= 0.32	β= -0.27 P=0.65
Serum Amyloid A	β= -0.36; P= 0.30	β= -0.44 P= 0.36	β= 0.34 P=0.43	β= -0.08 P= 0.89
Intercellular Adhesion Molecule 1	β= -0.57 P= 0.10^{+}	β= 0.98 **P= 0.05***	β= 1.12 **P=0.01****	β= 1.55 **P=0.01****
Vascular Cell Adhesion Molecule 1	β= -0.75 P= **0.02***	β= 1.29 **P= 4.85E-3*****	β= 1.27 **P= 0.001*****	β= 2.04 **P=9.42E-5*****
Vascular Endothelial Growth Factor	β= -0.26 P= 0.46	β= -0.04 P= 0.94	β= 0.18 P= 0.63	β= 0.22 P= 0.70
Apolipoprotein E	β= -0.23 P=0.45	β= 0.12 P= 0.74	β= 1.26 **P= 2.26E-4*****	β= 0.36 P= 0.41

We used linear regression models to assess the relationship between CSF marker levels and pathological stages as a categorical variable. All models were adjusted for age, gender, and APOE ε4 carrier status. Represented βs are for stage-wise comparisons of marker levels. P-values are shown uncorrected for multiple comparisons. Markers for which the names are bolded were significantly different in Stage 1 or Stage 2 vs Stage 0 (+P ≤ 0.1; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.005), although only one of these (VCAM-1) survived adjustment for multiple comparisons (not shown). ‡Data were standardized as z-scores.

Table A1.2: Association of PREVENT-AD CSF inflammatory biomarkers and AD biomarkers.

Marker‡	$A\beta_{1-42}$		Transformed t-*tau*		Transformed P_{181}-*tau*		Transformed t-*tau*/$A\beta_{1-42}$	
	Uncorrected	**FDR adjusted**	**Uncorrected**	**FDR adjusted**	**Uncorrected**	**FDR adjusted**	**Uncorrected**	**FDR adjusted**
ApoE	β=101.33 P = 1.14E-4***	**P =2.28E-3*****	β=0.22 P =1.65E-7***	**P =1.10E-6*****	β=0.20 P =4.32E-8***	**P =4.32E-7*****	β=0.52 P =5.97E-4***	**P =2.98E-3*****
GCSF	β=6.99 P = 0.80	P =0.80	β=0.09 P =0.05*	P =0.09^{+}	β=0.06 P =0.12	P = 0.19	β=0.28 P =0.08^{+}	P = 0.16
GMCSF	β=33.99 P = 0.23	P =0.33	β=0.06 P =0.23	P =0.33	β=0.06 P=0.14	P = 0.19	β=0.11 P =0.49	P = 0.58
IFNα2	β=48.29 P = 0.08^{+}	P =0.15	β=0.15 P =7.04E-4***	**P =2.01E-3*****	β=0.13 P =6.10E-4***	**P =2.03E-3*****	β=0.41 P =0.01**	**P = 0.02***
IL10	β=8.60 P = 0.78	P = 0.80	β=0.06 P =0.26	P =0.33	β=0.07 P=0.1^{+}	P = 0.17	β=0.21 P =0.23	P = 0.38
IL12P40	β=61.51 P = 0.05*	P = 0.14	β=0.09 P =0.06^{+}	P =0.12	β=0.07 P=0.13	P = 0.19	β=0.16 P =0.34	P = 0.48
IL12P70	β=84.59 P = 0.01**	**P =0.03***	β=0.05 P =0.25	P =0.33	β=0.08 P =0.04*	P = 0.08^{+}	β=-3.99E-3 P =0.98	P = 0.98
IL15	β=98.04 P = 2.29E-4***	**P =2.29E-3*****	β=0.23 P =1.90E-8***	**P =3.79E-7*****	β=0.21 P =7.28E-9***	**P = 1.46E-7*****	β=0.54 P =3.93E-4***	**P =2.62E-3*****

IL1RA	β=-36.03 P = 0.18	P =0.28	β=-0.06 P =0.18	P =0.27	β=-0.05 P =0.20	P = 0.25	β=-0.12 P =0.42	P = 0.56
IL6	β=34.22 P = 0.26	P =0.35	β=0.07 P =0.12	P =0.20	β=0.07 P =0.08^{+}	P = 0.14	β=0.21 P =0.18	P =0.33
IL8	β=75.14 P = 5.91E-3**	**P =0.03***	β=0.17 P =8.48E-5***	**P =2.83E-4*****	β=0.13 P =1.45E-3***	**P = 1.45E-3*****	β=0.42 P =0.01*	**P = 0.02***
IP10	β=50.94 P = 0.07^{+}	P =0.15	β=0.12 P =0.01*	**P =0.02***	β=0.10 P =0.02*	**P = 0.04***	β=0.31 P =0.05*	P = 0.12
MCP1	β=28.28 P = 0.31	P =0.39	β=0.15 P =1.5E-3***	**P =2.62E-3*****	β=0.12 P =2.12E-3***	**P = 0.01****	β=0.44 P =0.01**	**P = 0.02***
VEGF	β=15.07 P = 0.58	P =0.68	β=0.01 P =0.76	P =0.84	β=0.01 P =0.82	P = 0.91	β=-0.01 P =0.97	P = 0.98
MIP1β	β=12.68 P = 0.69	P =0.77	β=0.01 P =0.80	P =0.84	β=-0.01 P =0.89	P = 0.94	β=0.02 P =0.90	P = 0.98
IL16	β=62.84 P = 0.04*	P =0.14	β=0.21 P =9.96E-6***	**P =4.98E-5*****	β=0.16 P =4.00E-4***	**P = 1.60E-3*****	β=0.65 P =1.87E-4***	**P =1.87E-3*****
CRP	β=65.20 P = 0.03*	P =0.13	β=2.19E-3 P =0. 97	P =0.97	β=2.56E-3 P =0.96	P = 0.96	β=-0.19 P =0.30	P =0.46
SAA	β=43.53 P = 0.17	P =0.28	β=0.06 P =0.30	P =0.36	β=0.05 P =0.34	P = 0.40	β=0.15 P =0.44	P =0.56

sICAM1	β=57.25 P = 0.06^{+}	P =0.15	β=0.21 P =1.40E-5***	**P =5.60E-5*****	β=0.17 P =2.11E-4***	**P = 1.05E-3*****	β=0.57 P =1.24E-3***	**P = 4.97E-3*****
sVCAM1	β=54.02 P = 0.07^{+}	P =0.15	β=0.24 P =1.44E-7***	**P =1.10E-6*****	β=0.21 P =1.50E-6***	**P = 9.98E-6*****	β=0.68 P =5.36E-5***	**P =1.07E-3*****

We assessed the relationship of apoE protein and 19 CSF immune marker levels with typical CSF AD biomarkers using multivariate linear regression models. All models used age, gender, and APOE ε4 carrier status as covariates. P-values were adjusted using the false discovery rate method. ‡ Data were standardized as z-scores. In all, with adjustment for multiple comparisons, 9 of 19 immune markers and apoE showed a significant relationship with one or more specified AD biomarkers.

Table A1.3: Association of CSF markers and pathological Stages in the ADNI-1 cohort.

Marker‡	Stage 1 vs 0	Stage 2 vs 0	SNAP vs 0	Stage 2 vs 1
AXL Receptor Tyrosine Kinase	β = -0.46 **P_{FDR}= 0.02**	β = 0.43 **P_{FDR} = 0.04**	β = 1.78 P_{FDR}= 1.15E-6	β = 0.88 **P_{FDR}= 2.81E-8**
CD40 antigen	β = -0.19 P_{FDR}= 0.32	β = 0.45 **P_{FDR}= 0.04**	β = 1.22 P_{FDR}= 4.33E-4	β = 0.64 **P_{FDR}= 4.80E-5**
Interleukin-3	β = -0.45 **P_{FDR}= 0.03**	β = -0.12 P_{FDR}= 0.56	β = 0.88 P_{FDR}= 0.01	β = 0.33 **P_{FDR}= 0.04**
Macrophage Colony-Stimulating Factor 1	β = -0.16 P_{FDR}= 0.40	β = 0.59 **P_{FDR}= 0.01**	β = 1.13 P_{FDR}= 1.13E-3	β = 0.75 **P_{FDR}= 3.31E-6**
Heparin-Binding EGF-Like Growth Factor	β = -0.42 **P_{FDR}= 0.03**	β = 0.35 P_{FDR}= 0.11	β = 1.19 P_{FDR}= 6.31E-4	β = 0.77 **P_{FDR}= 1.87E-6**
Hepatocyte Growth Factor	β = 0.12 P_{FDR}= 0.56	β = 0.91 **P_{FDR}=1.59E-5**	β = 1.11 P_{FDR}= 1.22E-3	β = 0.80 **P_{FDR}= 7.18E-7**
Transforming Growth Factor alpha	β = -0.49 **P_{FDR}= 0.02**	β = 0.35 P_{FDR}= 0.12	β = 0.58 P_{FDR}= 0.1	β = 0.84 **P_{FDR}= 6.72E-7**
Vascular Endothelial Growth Factor	β = -0.64 **P_{FDR}= 0.002**	β = 0.25 P_{FDR}= 0.21	β = 1.21 P_{FDR}= 3.37E-4	β = 0.89 **P_{FDR}= 1.18E-8**
hFatty Acid-Binding Protein	β = -0.25 P_{FDR}= 0.16	β = 0.75 **P_{FDR}= 1.25E-4**	β = 0.75 P_{FDR}= 0.02	β = 1.01 **P_{FDR}= 1.98E-10**
Lectin-Like Oxidized LDL Receptor 1	β = -0.36 **P_{FDR}= 0.05**	β = 0.43 **P_{FDR}= 0.04**	β = 1.45 P_{FDR}= 4.11E-5	β = 0.79 **P_{FDR}= 4.39E-7**
Angiotensin-Converting Enzyme	β = -0.44 **P_{FDR}= 0.03**	β = 0.37 P_{FDR}= 0.10	β = 1.11 P_{FDR}= 1.24E-3	β = 0.80 **P_{FDR}= 7.18E-7**
Tissue Factor	β = -0.39 **P_{FDR}= 0.03**	β = 0.67 **P_{FDR}= 3.82E-4**	β = 1.49 P_{FDR}= 1.81E-5	β = 1.06 **P_{FDR}= 1.24E-11**

Chromogranin-A	β = -0.54 **P_{FDR}= 0.01**	β = 0.51 **PFDR= 0.01**	β = 1.20 P_{FDR}= 4.33E-4	β = 1.05 **P_{FDR}= 1.15E-10**
Fibroblast Growth Factor 4	β = 0.45 **P_{FDR}= 0.03**	β = 0.06 **P_{FDR}= 0.75**	β = -0.58 P_{FDR}= 0.09	β = -0.38 **P_{FDR}= 0.02**
Cystatin-C	β = 0.40 **P_{FDR}= 0.03**	β = -0.68 **P_{FDR}= 3.63E-4**	β = -1.39 P_{FDR}= 4.11E-5	β = -1.07 **P_{FDR}= 1.05E-11**
Matrix Metalloproteinase-3	β = -0.40 **P_{FDR}= 0.03**	β = 0.40 **P_{FDR}= 0.06**	β = 1.26 P_{FDR}= 3.37E-4	β = 0.81 **P_{FDR}= 5.19E-7**
Osteopontin	β = 0.00 P_{FDR}= 0.99	β = 0.84 **P_{FDR}= 8.72E-5**	β = 0.50 P_{FDR}= 0.14	β = 0.84 **P_{FDR}= 4.33E-7**
Tissue Inhibitor of Metalloproteinases1	β = -0.52 **P_{FDR}= 0.01**	β = -0.21 P_{FDR}= 0.31	β = 0.71 P_{FDR}= 0.03	β = 0.32 **P_{FDR}= 0.04**
Tumor Necrosis Factor Receptor2	β = -0.38 **P_{FDR}= 0.03**	β = 0.54; **P_{FDR}= 0.01**	β = 1.31 P_{FDR}= 1.13E-4	β = 0.92 **P_{FDR}= 2.41E-9**
Vascular Cell Adhesion Molecule-1	β = -0.37 **P_{FDR}= 0.04**	β = 0.18 P_{FDR}= 0.33	β = 1.20 P_{FDR}= 3.37E-4	β = 0.55 **P_{FDR}= 2.45E-4**
Apolipoprotein-E	β = -0.35 P_{FDR}= 0.06	β = 0.61 **P_{FDR}= 0.003**	β = 1.17 P_{FDR}= 6.31E-4	β = 0.96 **P_{FDR}= 2.85E-9**
Clusterin (apolipoprotein-J)	β = -0.51 **P_{FDR}= 0.01**	β = 0.19 P_{FDR}= 0.32	β = 1.35 P_{FDR}= 1.83E-4	β = 0.70 **P_{FDR}= 8.82E-6**
Trefoil Factor 3	β = -0.53 **P_{FDR}= 0.01**	β = 0.11 P_{FDR}= 0.56	β = 0.83 P_{FDR}= 9.46E-3	β = 0.63 **P_{FDR}= 2.34E-5**

We used linear regression models to assess the relationship between CSF marker levels and pathological stages as a categorical variable. 237 (90 Healthy Controls, 147 with MCI) subjects were included in this analysis. All models were adjusted for age, gender, APOE ε4 carrier status and clinical diagnostic group at baseline. Represented β are for stage-wise comparisons of marker levels. P-values are shown after adjusting for multiple comparisons using the False Discovery rate procedure. ‡ Transformed data

Table A1.4: Association of ADNI-1 CSF markers with the t-tau/ $A\beta_{1-42}$.

Marker‡	$A\beta_{1-42}$		Transformed t-*tau*		Transformed P_{181}-*tau*		Transformed t-*tau*/$A\beta_{1-42}$	
	Uncorrected	**FDR adjusted**	**Uncorrected**	**FDR adjusted**	**Uncorrected**	**FDR adjusted**	**Uncorrected**	**FDR adjusted**
AXL Receptor Tyrosine Kinase	β= 5.43 P= 0.07	P= 0.19	β= 0.20 P= 1.62E-14	P= 3.73E-14	β= 0.11 P= 8.67E-5	P= 1.17E-4	β= 0.20 P= 8.47E-7	P= 1.62E-6
CD 40 antigen	β= 4.02 P= 0.21	P= 0.34	β= 0.21 P= 4.50E-14	P= 9.41E-14	β= 0.15 P= 2.91E-7	P= 6.69E-7	β= 0.21 P= 3.54E-7	P= 8.14E-7
Interleukin-3	β= 8.87 P= 4.09E-3	P= 0.05	β= 0.11 P= 1.39E-4	P= 1.52E-4	β= 0.06 P= 0.04	P= 0.04	β= 0.07 P= 0.10	P= 0.11
Macrophage Colony-Stimulating Factor 1	β= 2.07 P= 0.51	P= 0.59	β= 0.18 P= 6.37E-11	P= 9.77E-11	β= 0.14 P= 1.02E-6	P= 2.13E-6	β= 0.19 P= 5.82E-6	P= 9.56E-6
Matrix Metalloproteinase-3	β= 2.98 P= 0.34	P= 0.46	β= 0.16 P= 4.70E-9	P= 6.01E-9	β= 0.12 P= 8.36E-6	P= 1.28E-5	β= 0.16 P= 1.08E-4	P= 1.46E-4
Osteopontin	β= -6.67 P= 0.03	P= 0.11	β= 0.20 P= 5.32E-14	P= 1.02E-13	β= 0.05 P= 0.05	P= 0.06	β= 0.27 P= 2.30E-11	P= 1.32E-10
Tissue Inhibitor of Metalloproteinases 1	β= 8.81 P= 0.01	P= 0.05	β= 0.03 P= 0.30	P= 0.30	β= 0.02 P= 0.57	P= 0.57	β= -0.02 P= 0.70	P= 0.70
Tumor Necrosis Factor Receptor 2	β= 1.48 P= 0.65	P= 0.68	β= 0.22 P= 1.49E-14	P= 3.73E-14	β= 0.15 P= 8.77E-8	P= 3.36E-7	β= 0.23 P= 2.31E-8	P= 6.65E-8

Vascular Cell Adhesion Molecule-1	β= 5.42 P= 0.10	P= 0.22	β= 0.14 P= 3.07E-6	P= 3.54E-6	β= 0.10 P= 1.34E-3	P= 1.62E-3	β= 0.13 P= 3.62E-3	P= 4.38E-3
Fibroblast Growth Factor 4	β= -6.20 P= 0.05	P= 0.16	β= -0.08 P= 6.40E-3	P= 6.69E-3	β= -0.04 P= 0.14	P= 0.14	β= -0.05 P= 0.26	P= 0.27
Heparin-Binding EGF-Like Growth Factor	β= 5.28 P= 0.09	P= 0.20	β= 0.21 P= 1.25E-14	P= 3.60E-14	β= 0.14 P= 2.07E-7	P= 6.19E-7	β= 0.20 P= 6.17E-7	P= 1.29E-6
Hepatocyte Growth Factor	β= -7.01 P= 0.02	P= 0.11	β= 0.19 P= 2.28E-12	P= 4.04E-12	β= 0.17 P= 3.72E-10	P= 1.71E-9	β= 0.26 P= 1.12E-10	P= 4.28E-10
Transforming Growth Factor alpha	β= 2.54 P= 0.40	P= 0.52	β= 0.18 P= 1.20E-10	P= 1.72E-10	β= 0.12 P= 5.55E-6	P= 9.12E-6	β= 0.18 P= 9.52E-6	P= 1.37E-5
Vascular Endothelial Growth Factor	β= 8.58 P= 0.01	P= 0.05	β= 0.22 P= 3.35E-15	P= 1.10E-14	β= 0.14 P= 1.57E-6	P= 2.77E-6	β= 0.19 P= 4.94E-6	P= 8.74E-6
Apolipoprotein E	β= 1.52 P= 0.62	P= 0.68	β= 0.22 P= 3.70E-16	P= 1.67E-15	β= 0.14 P= 2.15E-7	P= 6.19E-7	β= 0.24 P= 4.00E-9	P= 1.31E-8
Clusterin	β= 6.74 P= 0.03	P= 0.11	β= 0.17 P= 1.58E-9	P= 2.14E-9	β= 0.11 P= 1.23E-4	P= 1.57E-4	β= 0.15 P= 3.04E-4	P= 3.89E-4
hFatty Acid-Binding Protein	β= -3.73 P= 0.24	P= 0.37	β= 0.26 P= 6.73E-22	P= 5.16E-21	β= 0.22 P= 7.28E-16	P= 1.67E-14	β= 0.31 P= 1.39E-14	P= 3.20E-13

Lectin-Like Oxidized LDL Receptor 1	β= 4.95 P= 0.12	P= 0.23	β= 0.23 P= 4.35E-16	P= 1.67E-15	β= 0.15 P= 2.80E-7	P= 6.69E-7	β= 0.22 P= 1.19E-7	P= 3.03E-7
Angiotensin-Converting Enzyme	β= 4.37 P= 0.16	P= 0.28	β= 0.19 P= 7.33E-12	P= 1.20E-11	β= 0.13 P= 1.26E-6	P= 2.41E-6	β= 0.18 P= 9.31E-6	P= 1.37E-5
Tissue Factor	β= 0.99 P= 0.75	P= 0.75	β= 0.28 P= 3.11E-26	P= 7.16E-25	β= 0.19 P= 1.97E-12	P= 2.27E-11	β= 0.30 P= 3.81E-14	P= 4.38E-13
Chromogranin-A	β= 2.93 P= 0.34	P= 0.46	β= 0.24 P= 1.77E-20	P= 1.02E-19	β= 0.17 P= 1.31E-10	P= 7.54E-10	β= 0.25 P= 1.11E-10	P= 4.28E-10
Cystatin-C	β= -2.51 P= 0.43	P= 0.52	β= -0.27 P= 8.65E-24	P= 9.95E-23	β= -0.18 P= 2.79E-11	P= 2.14E-10	β= -0.28 P= 3.26E-12	P= 2.50E-11
Trefoil Factor 3	β= 7.10 P= 0.03	P= 0.11	β= 0.15 P= 1.77E-6	P= 2.14E-6	β= 0.13 P= 1.33E-5	P= 1.91E-5	β= 0.12 P= 7.49E-3	P= 8.62E-3

We assessed relation of CSF marker levels with the typical composite AD biomarker t-tau/Aβ_{1-42} using multivariate linear regression models. The analysis included 237 individuals (90 Healthy Controls, 147 with MCI). Models were adjusted for age, gender, APOEε4 carrier status and clinical diagnostic group. P-values were adjusted for multiple comparisons using the False Discovery Rate Procedure ($^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.005$).
‡Transformed data

Appendix 2 – Supplementary material to Chapter 5

Supplementary Methods

Participants – additional information

If a specialist's or other expert's diagnosis was not available, a family history of AD dementia was ascertained using a series of questions used for the Cache County Study. These were intended to establish 1) whether the parent or siblings had troubles with memory or concentration that 2) were sufficiently severe to cause disability or loss of function, and that 3) had insidious onset or gradual progression and were not an obvious consequence of a stroke or other sudden insult. Other aspects of eligibility were determined from medical questionnaires, surgical medical and history, pharmacological profile, lifestyle habits, physical and neurological examinations, blood and urine analyses as well as electrocardiogram. Beyond this, a short MRI session was included to provide structural measurements and to rule out claustrophobia in the scanner or structural brain disease. Detailed inclusion and exclusion criteria for INTREPAD trial are presented in Table 1. A complete list of demographics and clinical data gathered at the eligibility visit and throughout the trial are presented in Table 2.

Cognitive evaluation

Repeatable Battery for the Assessment of Neuropsychological Status (RBANS)

This ~30-minute battery was administered by trained research assistants and corrected by a single expert neuropsychologist for uniformity. In the trial, all participants were given the "A" version at Baseline after which the B, C or D versions were given in random order. The RBANS French versions are known not to be fully equivalent. The author of the RBANS (Dr. Christopher Randolph) recommended systematic correction of +4 for the semantic fluency section of version B. By comparing performance at the 3M visit with BL scores (assuming no treatment effects and comparable abilities at the two timepoints), we determined that additional corrections were needed to control version differences. Being uncertain but suspecting part of the difficulty traced to non-equivalence of English and French tests, we developed adjustment factors that brought the several versions into approximate equivalence. This procedure is described in [1].

We avoided correcting for age in scoring the RBANS. Whereas clinical testing may call for scoring criteria that vary by age (to compare individual performance to "normative" data), we scored all participants using norms for individuals aged 60—69 years. This method revealed actual decline in performance with age, whether or not this decline was related to disease.

Episodic memory task in fMRI set-up

During the episodic memory task fMRI acquisitions, visual stimuli were generated by a PC laptop computer and projected using an LCD projector onto a screen visible to participants *via* a mirror mounted within the standard head coil. Plastic optical corrective glasses were provided for participants who required correction for visual acuity. The six-minute encoding task presented 48 unique visual stimuli on the left or right side of a central fixation cross. After an eleven-minute break (during which structural images were acquired), 96 retrieval stimuli, including the original images, were displayed in random sequence, but at the centre of the screen. Participants used a fibre optic four-button response box to indicate whether they had observed the images initially and, if so, whether the former presentation had been on the right or left half of the screen. Responses were recorded by the E-Prime program (Psychology Software Tools Inc., Pittsburgh, PA, USA).

Neuroimaging – additional information

Magnetic Resonance Imaging (MRI) set-up

MRI sessions included 3 functional acquisitions as well as 4 structural modalities (Figure A2.2). For the functional MRI scans, heart rate and respiration are known to alter the functional Blood Oxygenated Level Dependant (BOLD) signal. To enable correction of the fMRI data, heart rate and a logic pulse marking were recorded during the scans at a sampling rate of 400Hz using the BIOPAC MP150 system (BIOPAC Systems, Inc., Goleta, CA). Chest wall motion was monitored using a respiratory belt transducer (TSD201) connected to a Respiration Amplifier module (RSP100C) in the BIOPAC system. EKG traces were recorded using the BIOPAC ECG100C amplifier.

Supplementary Tables

Table A2.1: INTREPAD Inclusion and Exclusion Criteria

Inclusion criteria:
- At least six years of formal education - Sufficient fluency in spoken and written English and/or French to participate in study visits and in psychometric testing - Family history of first degree relative(s) with Alzheimer-like dementia, as established by medical history or the brief questionnaire described above - Age 60 or older, or age 55 or older if current age was within 15 years of affected relative's estimated age at onset of AD dementia. - A collateral respondent available to provide information on the cognitive and health status of the participant, and to assist with monitoring of study interventions, if needed - Informed consent for a series of MRI / fMRI scans, to undergo a series of lumbar punctures for collection of CSF, or both. - Ability and intention to participate in regular study visits, in the opinion of a study physician - Willingness to limit use of several of different over-the-counter or prescription medicines as dictated by the protocol and described in the text - Provision of informed consent for initial evaluation and testing.
Exclusion criteria:
- Known or identified cognitive disorder diagnosed previously by a knowledgeable physician, psychologist, nurse-clinician, or other health care provider, or by PREVENT-AD staff - Past or present use of commercially available acetyl-cholinesterase inhibitors including tacrine, donepezil, rivastigmine, or galantamine - Past or present use of memantine or other approved prescription cognitive enhancement agent - Current use of vitamin E at greater than 600 i.u. / day - Any history of peptic ulcer disease complicated by perforation, hemorrhage, or obstruction - Any history of other endoscopically confirmed peptic ulcer disease - Clinically significant hypertension, anemia, liver disease, or kidney disease, in opinion of a study physician (participants with treated hypertension who were normotensive as a result of intervention were eligible.) - Concurrent regular use of systemic or inhalation corticosteroids or other anti-inflammatory or immunosuppressive agent - Concurrent use of warfarin, ticlopidine, clopidrogel, or similar anti-coagulant - Regular use of aspirin in any dose - History of hypersensitivity or anaphylactoid response to sulfonamide antibiotics (e.g., sulfamethoxazole) or to aspirin or other NSAIDs (e.g., ibuprofen, diclofenac, naproxen, celecoxib). - Any inflammatory or chronic pain condition that necessitated regular use of opiates (e.g., oxycodone, hydrocodone, tramadol, meperidine, hydromorphone), NSAIDs, or aspirin - Current plasma creatinine > 132 mmol/l (1.5 mg/dl) - Current alcohol, barbiturate or benzodiazepine abuse or dependence (in opinion of study physician) - Any other medical condition that, in the opinion of the study physician, made it inadvisable for the participant to be assigned to regular dosage of NSAIDs. - Enrolment in any trial or experimental protocol that, in the opinion of a study physician, would be likely to interfere with INTREPAD - Any other condition that, in the opinion of a study physician, made it medically inappropriate to enroll in INTREPAD

CSF: cerebrospinal fluid MRI: magnetic resonance imaging fMRI OTC: over the counter NSAID: non-steroidal anti-inflammatory drug

Table A2.2: Recorded Demographics and Clinical Characteristics

Demographics	Date of birth Gender Mother tongue Marital status Education level Professional status Language Ethnicity Residence Living situation Handedness
Clinical data	Family history of Alzheimer-like dementia Pharmacological profile Blood hematology, biochemistry, coagulation, endocrinology Urine biochemistry Electrocardiogram Blood pressure / pulse Height / Weight / Body mass index Cardiovascular risk score (CAIDE score) Medical and surgical history Physical activity level Physical and neurological examination Alcohol consumption Smoking habits Sleeping habits Medical symptoms Adverse Events / Serious Adverse Events Compliance with study drug
Genetics	Genes polymorphisms (ApoE, BchE, BDNF, HMGR, TLR4) Genome-Wide Association Study (variants not recorded in main database)

Table A2.3: Concomitant medications initiated during treatment phase of the trial; by treatment group.

Concomitant medication (as needed or regular use)	Naproxen	Placebo	P value
NSAID / pain relievers /aspirin containing medication	44 (43%)	39 (42%)	NS
Supplements / Vitamins / probiotics	22 (22%)	23 (25%)	NS
Corticosteroids (inhalator or cream)	15 (15%)	12 (13%)	NS
Antibiotics	13 (13%)	17 (18%)	NS
Histamine H2 receptor antagonist / other GI medication	12 (12%)	14 (15%)	NS
Corticosteroid (systemic)	11 (11%)	7 (8%)	NS
Cardiovascular / antihypertensive medication	10 (10%)	5 (5%)	NS
Antivertigo /antiepileptic / sedative hypnotic CNS stimulant	7 (7%)	8 (9%)	NS
Allergy medications	6 (6%)	9 (10%)	NS
Lipid lowering agent / statins	1 (1%)	8 (9%)	0.011
Oestrogen / hormone replacement	4 (4%)	6 (6%)	NS

Data are presented only for medications started by >5% of participants in either group.
Data include changes to new medication within the same category.

GI: gastrointestinal; NSAID: non-steroidal anti-inflammatory drug;

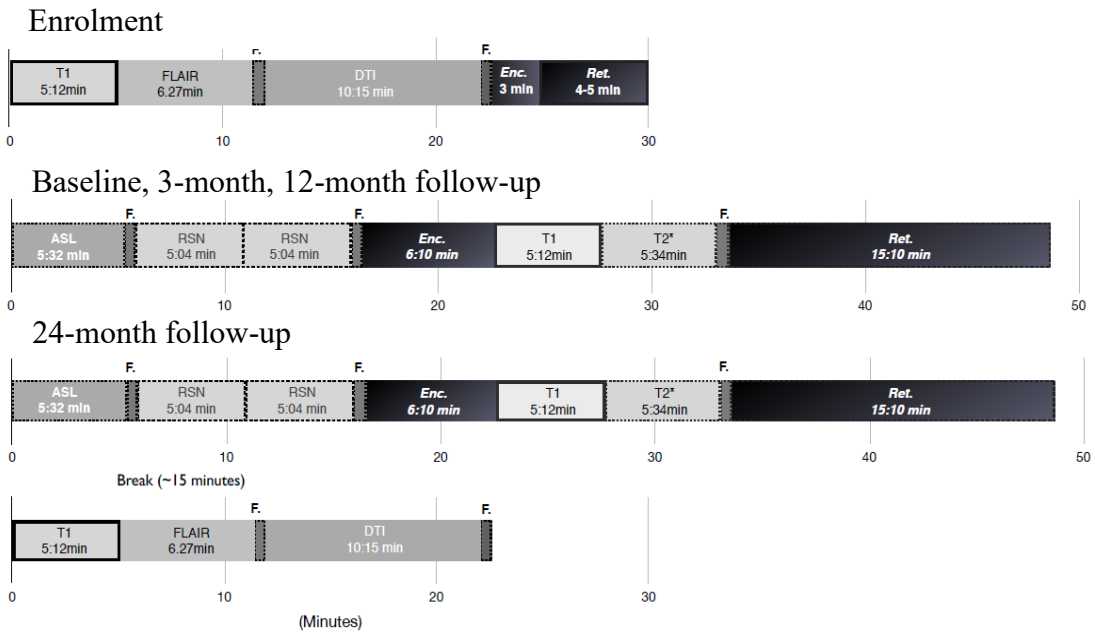
Table A2.4: Description of SAEs

SAE	Assessed Relationship	Action taken	Assigned group
Melena and low hemoglobin	Related	Study drug discontinuation	Naproxen
Subarachnoid hemorrhage	Possibly related	Ceased participation	Naproxen
Unplanned hip replacement	Likely unrelated	Study drug interruption	Naproxen
Cerebral hematoma following a fall	Not related	Ceased participation	Naproxen
Temporal arteritis	Not related	Ceased participation	Naproxen
Pelvic factures	Not related	Study Drug interruption	Naproxen
Fall	Not related	None	Naproxen
Breast cancer (carcinoma)	Not related	Study drug discontinuation	Naproxen
Myocardial infarction	Likely unrelated	Study drug discontinuation	Placebo
Anal cancer (death)*	Not related	None	Placebo

*was off study drug at the time of SAE

Supplementary Figures

A) MRI acquisitions



B) MRI parameters

Modality	TR (ms)	TE (ms)	TI (ms)	∝	In-plane resolution (mm)	Slice thickness (mm)	Matrix Size	Number of volumes
Structural modalities								
MPRAGE	2300	30	-	9°	1 x 1	1	256 x 256	-
FLAIR	5000	388	1800	-	1 x 1	1	256 x 256	-
GRE T2*	650	20	-	20°	0.8 x 0.8	2	256 x 256	-
DWI	9300	92	-	-	2 x 2	2	96 x 96	65
Functional modalities								
SE-pCASL	4000	10	-	90°	4 x 4	7	64 x 64	80
rs-fMRI	2000	30	-	90°	4 x 4	4	64 x 64	150
Task fMRI	2000	30	-	90°	4 x 4	4	64 x 64	183/453

Figure A2.1: MRI acquisitions and parameters
A) MRI acquisitions: DWI = 64-direction DWI; Enc. = Encoding task fMRI; F. = Fieldmaps; Ret. = Retrieval task fMRI; RSN = Resting state fMRI; T1 = MPRAGE (ADNI protocol); T2* = GRE T2* weighted. **B) MRI parameters** TR = Repetition Time; TE = Echo Time; TI = Inversion Time; ∝ = Flip Angle; FLAIR = FLuid Attenuated Inversion Recovery; GRE = GRadient Echo; Quantitative T2* = 12-Echo T2* (2.84, 6.2, 9.56, 12.92, 16.28, 19.64, 23, 26.36, 29.72, 33.08, 36.44 and 39.8 ms); DWI = Diffusion Weighted Imaging; SE-pCASL = Single-Echo Pseudo-Continuous Arterial Spin Labeling; rs-fMRI = Resting State Functional

MRI; DE-pCASL = Dual-Echo Pseudo-Continuous Arterial Spin Labeling (for the Quantitative O2 acquisition).

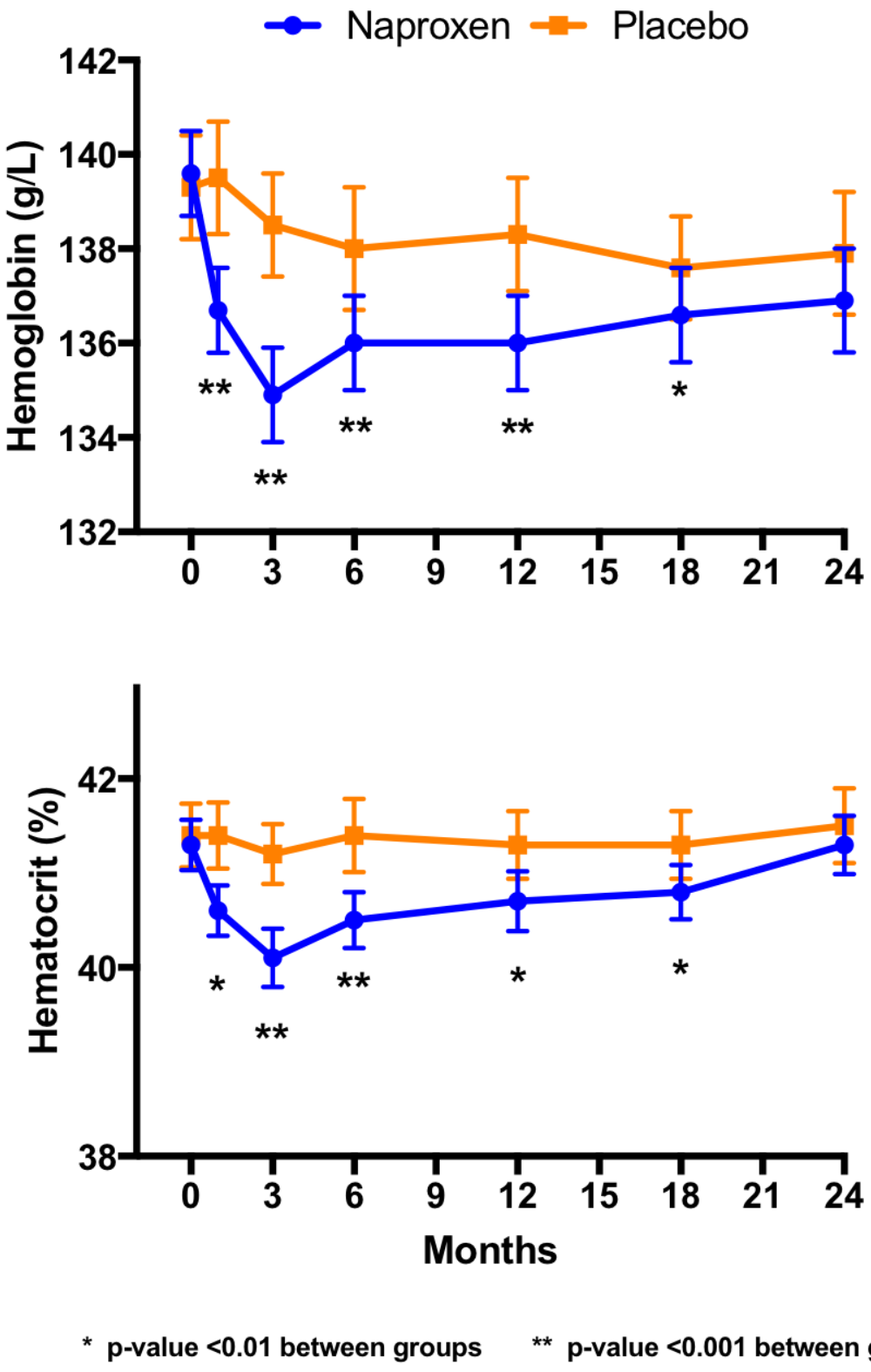


Figure A2.2: Mean hemoglobin (g/L) and hematocrit values (%) over time by treatment.

Supplementary References

1. Lafaille-Magnan ME, Leoutsakos JM, Fontaine D, et al. Crosstalk Between English and French Forms of the RBANS in an Alzheimer's Disease Prevention Trial. Alzheimer's & Dementia 2018.