Evaluation of Molecular Biomarkers of Amyloidosis in the

Alzheimer's Disease Spectrum.

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Abstract

Presently, the major difficulty in finding a therapeutic strategy to cure Alzheimer's disease (AD) comes from the lack of accessible and precise biomarkers. AD is a multifactorial disease in which two main pathologies interplay to lead to neurodegeneration: amyloid and neurofibrillary tangle (NFT). A large body of evidence suggests that amyloid accumulation starts decades before clinical symptoms arise, followed by NFT pathology which is more closely related to cognitive decline. Although the current diagnostic criteria of AD pathophysiology require the presence of both pathologies in the brain, in the clinical framework, this diagnostic determination is challenging. Neuropathological and imaging studies have shown that about 30% of cognitively unimpaired subjects have amyloid pathology. Conversely, a similar proportion of individuals clinically diagnosed with AD do not present AD pathologies. Thus, there is a critical need of precise biomarkers to indicate pathological changes. In the context of drug trials, biomarkers would be helpful in the selection of the correct target population for the study and to precisely monitor the drug effect. Additionally, early biomarkers provide the advantage of identifying subjects at initial stages of the disease, which offers the optimal window for therapeutic interventions specially in the context of AD. Blood and genetic biomarkers are preferred as imaging and cerebrospinal fluid (CSF) biomarkers are costly or of difficult access. Hence, in this thesis we aimed to identify blood and genetic biomarkers of amyloid pathology assuming that they would also be early markers of AD. In Chapters 2 and 3 we sought to find new genetic variants associated with amyloid pathology using PET imaging data. In Chapters 4 and 5 we combined imaging, genetics and CSF data to explore a potential blood biomarker. In summary, we first identified genetic variants that, independently or in combination, were associated with brain amyloid

load. Despite that the effect size of these genetic markers were modest to allow their individual use as biomarkers, these results shed light on pathways of amyloid clearance that deserve further investigation. Secondly, we showed that plasma Neurofilament light (NfL) protein is able to detect amyloid-related neurodegeneration even at early pathological stages. We also provided evidences that APOE-e4 carriage status, together with plasma NfL information, could be used for identification of cognitively unimpaired subjects with amyloidosis.

Resumé

Actuellement, la difficulté majeure à trouver une stratégie thérapeutique pour guérir la maladie d'Alzheimer (MA) provient du manque de biomarqueurs précis et accessibles. La MA est une maladie multifactorielle dans laquelle deux pathologies principales interagissent pour mener à la neurodégénérescence: l'amyloïde et l'enchevêtrement neurofibrillaire (EN). Un grand nombre d'études suggère que l'accumulation d'amyloïde commence plusieurs décennies avant l'apparition des symptômes cliniques, suivie de la pathologie de la TNF, qui est plus étroitement liée au déclin cognitif. Bien que les critères diagnostiques actuels de la physiopathologie de la MA exigent la présence des deux pathologies dans le cerveau, dans le cadre clinique cette détermination diagnostique est difficile. Les études neuropathologiques et d'imagerie ont démontré qu'environ 30% des individus cognitivement normaux sur le plan cognitif étaient atteints d'une pathologie amyloïde. À l'inverse, une proportion similaire d'individus diagnostiqués cliniquement comme atteints de MA ne présentent pas de pathologies de la MA. Il est donc essentiel de disposer de biomarqueurs précis afin de dévoiler les changements pathologiques. Dans le contexte des essais cliniques de médicaments, les biomarqueurs seraient utiles pour sélectionner la population appropriée pour l'étude et pour surveiller précisément l'effet des médicaments. De plus, les biomarqueurs précoces présentent l'avantage d'identifier les individus aux premiers stades de la maladie, ce qui constitue la fenêtre optimale pour des interventions thérapeutiques, en particulier dans le contexte de la MA. Les biomarqueurs sanguins et génétiques sont avantageux puisque l'imagerie et les biomarqueurs du liquide céphalo-rachidien (LCR) sont coûteux ou difficiles d'accès. Ainsi, dans cette thèse, nous avons cherché à identifier des biomarqueurs sanguins et génétiques de la pathologie de l'amyloïde en présumant qu'ils seraient également des marqueurs précoces de la MA. Dans les chapitres 2 et 3, nous avons cherché à trouver de nouvelles variantes génétiques associées à la pathologie de l'amyloïde en utilisant des données d'imagerie TEP. Dans les chapitres 4 et 5, nous avons combiné des données d'imagerie, de génétique et de LCR pour explorer un biomarqueur sanguin potentiel. En résumé, nous avons d'abord identifié les variantes génétiques qui, indépendamment ou en combinaison, étaient associées à la quantité cérébrale d'amyloïde. Bien que la valeur de l'effet de ces marqueurs génétiques soit modeste pour permettre leur utilisation individuelle en tant que biomarqueurs, ces résultats permettent de mieux comprendre les voies de l'élimination de l'amyloïde qui méritent d'être d'avantage étudiées. Deuxièmement, nous avons montré que la protéine plasmatique du neurofilament (NfL) est capable de détecter une neurodégénérescence liée à l'amyloïde, même à un stade précoce de la pathologie. Nous avons également fourni des preuves qu'être porteur de l'allèle e4 d'APOE, en combinaison avec les résultats de la NfL plasmatique, pourrait être utilisés pour l'identification de sujets ayant une accumulation d'amyloïde sans symptômes cognitifs.

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about the Alzheimer's disease. It is our aim and our hope to cure this devastating disease shortly.

Preface

This thesis contains six chapters. It starts with an introductory review of the current literature, in Chapter 1, providing the most relevant information to a better understanding of the subsequent chapters. Chapters 2 and 3 present two studies, based on a priori hypothesis, that searched for genetic biomarkers of Alzheimer's disease (AD) -related amyloidosis. Chapters 4 and 5 show how plasma Neurofilament light chain (NfL) protein is associated with amyloid PET and other imaging biomarkers of AD as well as how the most relevant genetic factor for AD affects NfL associations. The Chapters 2 and 3 were published at *Neurology: Genetics* and *Journal of Neuroinflammation*, respectively. Chapter 4 is in press and will be published in the *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring* journal. Chapter 5 is under review in the *Lancet: Neurology*. Chapter 6 then presents a summary and discussion of all the findings from previous chapters and ends with concluding remarks.

This thesis is based on the following original papers:

Chapter 2. Benedet AL, Yu L, Labbe A, Mathotaarachchi S, Pascoal TA, Shin M, Kang MS, Gauthier S, Rouleau GA, Poirier J, Bennett DA, Rosa-Neto P, Alzheimer's Disease Neuroimaging Initiative. **CYP2C19 variant mitigates Alzheimer disease pathophysiology** in vivo and postmortem. *Neurology Genetics*. 2018 Feb 1;4(1):e216.

Chapter 3. Benedet AL, Labbe A, Lemay P, Zimmer ER, Pascoal TA, Leuzy A, Mathotaarachchi S, Mohades S, Shin M, Dionne-Laporte A, Beaudry T, Picard C, Gauthier S, Poirier J, Rouleau G, Rosa-Neto P; Alzheimer's Disease Neuroimaging Initiative. **Epistasis analysis links immune cascades and cerebral amyloidosis.** *Journal of neuroinflammation*. 2015;12:227.

Chapter 4. Benedet AL, Leuzy A, Pascoal TA, Ashton NJ, Mathotaarachchi S, Savard M, Therriault J, Kang MS, Chamoun M, Schöll M, Gauthier S, Labbe A, Zetterberg H, Rosa-Neto P and Blennow K., Alzheimer's Disease Neuroimaging Initiative. **Stage-specific associations between Plasma NfL and biomarkers of AD pathophysiology.** Submitted.

Chapter 5. Benedet AL, Ashton NJ, Pascoal TA, Leuzy A, Mathotaarachchi S, Kang MS, Therriault J, Savard M, Chamoun M, Schöll M, Zimmer ER, Gauthier S, Labbe A, Zetterberg H, Blennow K and Rosa-Neto P, Alzheimer's Disease Neuroimaging Initiative. Plasma neurofilament light associates with Alzheimer's disease metabolic decline in amyloid positive individuals. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring.* In Press.

Contribution of authors

Chapter 2. ALB study concept, design, analysis and interpretation of data, compose figures

and manuscript draft. LY, analysis, interpretation of data and manuscript draft. AL, statistical analysis supervision and critical review of manuscript for intellectual content. SM, TAP, MS and MSK image data processing and manuscript draft. study concept, design and manuscript draft. SG, GAR and JP critical review of manuscript for intellectual content. DAB manuscript draft and critical review of manuscript for intellectual content. PRN study concept, design, study supervision and critical review of manuscript for intellectual content.

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Chapter 5. ALB, NJA, TAP, AL, KB and PRN participated in the design of this study. KB and PRN supervised the study. AB and SM carried out the statistical analysis. NJA carried out the biochemical analyses. ALa provided support in the statistical analysis. ALB, SM and MS

performed the imaging processing and quality control. SM, MS, JT and MSK provided support in the imaging analysis. ALB and NJA wrote the paper. AL, MC, MSc, SG and HZ contributed with revision of the paper. All authors read and approved the final version of the manuscript.

Contribution to original knowledge

The original contributions of this thesis include:

Chapter 2. We showed the association between a SNP in the *CYP2C19* gene and AD-related amyloidosis. This was the first report presenting a closer link between the important cytochrome P450 enzyme family and amyloid- β metabolism.

Chapter 3. Adding to the initial findings on genetic factors affecting amyloid- β metabolism, we employed epistasis analysis to look for a combination of immune-related genetic markers that together could be related to the amyloid- β load. We found that the joint effect of two genetic markers in the *C9* and *IL6r* genes can potentially modulate amyloid levels.

Chapter 4. We show for the first time that plasma NfL reflects amyloid-related neurodegeneration in cognitively unimpaired subjects. We also presented the association of plasma NfL with tau PET, gray and white matter atrophy. In addition, we found that in cognitively unimpaired subjects, NfL was associated with gray matter loss only in *APOE-e4* carriers.

Chapter 5. Last, we investigated how plasma NfL is associated with brain glucose metabolism in amyloid positive and negative subjects. We found that plasma NfL is associated with reduced brain metabolism mostly in amyloid positive groups. These findings corroborate the previous study by showing that amyloid-related neurodegeneration can be detected in cognitively unimpaired subjects by plasma NfL.

List of Abbreviations

Aβ: Beta-amyloid

AD: Alzheimer's disease

ADNI: Alzheimer's disease Neuroimaging Initiative

ApoE: Apolipoprotein E

APOE: Apolipoprotein E gene

APP: Amyloid precursor protein

BBB: Brain-blood barrier

BDNF: Brain derived neurotrophic factor

CCL2: Chemokine ligand 2

CD14: Cluster of differentiation 14

CDR: Clinical dementia rating

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

CI: Cognitively impaired

CNS: Central nervous system

CSF: Cerebrospinal fluid

CU: Cognitively unimpaired

CYP: Cytochrome P450

DNA: Deoxyribonucleic acid

DTI: Diffusion tensor imaging

EDTA: Ethylenediaminetetraacetic acid

[¹⁸F]FDG: [¹⁸F]2-fluoro-2deoxyglucose

FDR: False Discovery Rate

FWHM: Full width half maximum

GAP43: growth-associated protein-43

GM: Gray matter

GWAS: Genome wide association studies

HC: healthy control

IWG: International Working Group

LD: Linkage disequilibrium

LTD: Long term depression

LTP: Long term potentiation

MA: Minor allele

MAC: membrane attack complex

MAF: Minor allele frequency

MAO-A: Monoamine oxidase A

MAO-B: Monoamine oxidase B

MCI: Mild Cognitive Impairment

MMSE: Mini Mental State Examination

MNI: Montreal Neurological Institute

MRI: Magnetic Resonance Imaging

NADPH: Nicotinamide adenine dinucleotide phosphate

NfL: Neurofilament light chain

NFT: Neurofibrillary tangle

NIA-AA: National Institute on Aging and the Alzheimer's Association

NIA-REAGAN: National Institute on Aging and Reagan Institute Working Group on

Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease

NINCDS-ADRDA: National Institute of Neurological and Communicative Disorders and

Stroke-Alzheimer's Disease and Related Disorders Association

NMDA: N-methyl-D-aspartate

PiB: Pittsburgh Compound-B

PET: Positron Emission Tomography

PHF: Paired helical filament

POR: NADPH-cytochrome P450-reductase

RFT: Random field theory

ROI: Region of interest

SNAP: Suspected non-AD pathology

SNAP25: Synaptosome Associated Protein 25

SNP: Single nucleotide polymorphism

SUVR: Standard Uptake Value Ratio

SV2A: Synaptic vesicle glycoprotein 2A

ThT: Thioflavin-T

TREM2: Triggering receptor expressed on myeloid cells 2

TRIAD: Translational biomarker in aging and dementia

VBM: Voxel-based morphometry

WGS: Whole genome sequencing

WM: White matter

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Chapter 1 : General Introduction

Advances in public health, medicine, science and technology considerably expanded life expectancy in the past few decades. A consequence of the growing aging population around the globe is the high incidence of dementia. It is estimated that 65.7 million people will be living with dementia in 2030¹, causing an increase of 85% in its costs in the Health Care system. Of all dementias, Alzheimer's disease (AD) is the most prevalent, accounting for 50-75% of the cases ².

1.1 Alzheimer's disease: the original report

In 1906 Alois Alzheimer first described a case of a 51-year old patient, known as August D., with progressive loss of memory, sleep disorders, aggressiveness and increasing confusion state ^{3, 4}. Over the course of the disease her speech became unintelligible and on her last year she was completely apathetic. After five years of the initial clinical assessment, August D. dies and her brain was autopsied. Using staining techniques, Alzheimer was able to detect and describe the presence of abnormal agglomerates of proteins in the brain, which later came to be known as amyloid plaques and neurofibrillary tangles. In 1910, this case report was published in a book written by Emil Kraepelin, who first proposed the name "Alzheimer's disease" for this condition.

1.2 The Alzheimer's disease dementia

Since its discovery until present day, the definition of Alzheimer's disease has changed several times (Figure 1-1). In 1984, the National Institute of Neurological and Communicative

Disorders and Stroke–Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)⁵, similarly to what was later presented by the DSM-III-R⁶, termed "Probable AD" a progressive condition clinically characterized by amnestic presentations; together with evidenced decline of at least one other cognitive domain which significantly affects one's independency and autonomy. The definitive diagnosis of AD would only come after autopsy confirmation.

Years later, several neuropathological studies have linked the formation of amyloid plaques, neurofibrillary tangles (NFT) and neurodegeneration ⁷, ⁸⁻¹⁰ to the clinical diagnosis of Alzheimer's dementia. However, these studies have also shown that AD hallmarks could be detected in cognitively normal subjects ^{7, 11, 12}, suggesting that pathological changes start prior to clinical dementia symptoms.

With the development of PET imaging and biofluid biomarkers, it has been possible to detect *in vivo* pathobiological changes that were previously only evaluated *postmortem*. These new biomarkers have changed clinical practice and how clinical trials are designed. Several working groups, such as the International Working Group (IWG) ^{13, 14}, the DSM-5 ¹⁵ and the National Institute on Aging and the Alzheimer's Association (NIA-AA) ^{16, 17, 18} have been trying to conciliate clinical symptomatology and neuropathological diagnosis with biomarker profiles. In combination, these groups have proposed the separation of the clinical diagnosis from the classification of the underlying pathophysiological process ¹⁷, ¹⁸. AD dementia is then characterized by an amnestic presentation, while AD pathology is characterized by the presence of amyloid and NFT pathology, quantified by its biomarkers. AD biomarkers together with non-amnestic cognitive impairment was then classified as "atypical AD" ¹³.



Figure 1-1 Evolution of AD diagnostic criteria

A timeline of the major modifications in the diagnosis of Alzheimer's disease. Adapted from Lee et al.¹⁹

"Mixed AD" would be used for cases in which there are other pathobiological processes in addition to AD pathology. Mild cognitive impairment (MCI) with the presence of AD biomarkers was classified as "prodromal AD" ¹⁴. Similarly, the presence of AD biomarkers but no clinical manifestations were labeled preclinical AD. More recently, the NIA-AA ¹⁶ proposed a research framework to diagnose the AD pathophysiology only based on the AT(N) biomarkers system ¹⁸. It suggests that AD biomarkers are grouped into three categories: "A" for amyloid pathology, and it comprises CSF or PET amyloid biomarkers; "T" for tangle pathology, being indexed by CSF phosphorylated tau and tau PET; and "N" for neurodegeneration, being this measured with CSF total tau, [¹⁸F]fluorodeoxyglucose PET

hypometabolism, or atrophy on structural MRI in regions characteristic of AD. In summary, individuals are classified as being in the AD continuum if are positive for "A" and as "suspected non-amyloid pathology" (SNAP) if "A" negative.

1.3 Mild Cognitive Impairment

The term mild cognitive impairment (MCI) refers to a syndrome characterized by cognitive decline, not expected for the age and educational level of the patient, that is not sufficient to lead to the diagnosis of dementia ^{20, 21}. MCI subjects are capable of performing daily life activities independently, but cognitive decline can be already detected and the rate of progression to AD is 10%-15% per year ²⁰. MCI can have diverse etiology, but when linked to underlying AD pathophysiology it is conceptualized as "MCI due to AD" and it is presumed to represent a prodrome of "probable AD" ²¹.

1.4 Preclinical Alzheimer's disease

Several neuropathological studies have shown that a proportion of cognitively normal older individuals present significant AD pathophysiology, suggesting that the pathology starts years prior to the clinical symptoms ^{7,22,23}. With the advancement of imaging techniques and biofluid biomarkers, it was possible to show *in vivo* evidence that corroborated the initial neuropathological studies. The framework of the "AD continuum" then assumes that asymptomatic subjects with biomarker evidence of AD pathophysiology are the "preclinical subjects" at risk of developing AD ^{22,24}. Over time, these individuals are very likely to present

some cognitive decline and meet criteria for MCI due to AD, and later on progress to AD dementia.

In 2011, the NIA-AA workgroup proposed a 3-stage criteria to better describe the preclinical AD based on biomarkers and thus optimize the selection of target populations for research and clinical studies ²⁴. In this framework, the stage 1 comprises asymptomatic individuals with evident amyloidosis; in the stage 2 subjects are expected to have amyloidosis and neurodegeneration; and, in stage 3, the amyloidosis and neuronal injury are accompanied by subtle cognitive or behavioral decline. A year later, two other groups of subjects were defined: the stage 0, that includes individuals with no cognitive impairment and no evidence of AD pathophysiology (1) and the "Suspected Non-AD Pathophysiology" (SNAP) (2), in which subjects present normal amyloid levels but abnormal biomarkers of neuronal injury (2)²⁵.

1.5 Neuropathological diagnosis criteria

Currently, despite the progress made in using biomarkers to detect pathological profiles, the neuropathological assessment identifying the presence of both amyloid and NFT pathology, together with the clinical information, remain the *gold standard* diagnosis of "definite AD" ²⁶. The initial proposition of a consensus for pathological diagnosis of AD was published in 1985, and it suggested specific tissue quantifications of amyloid plaques and neurofibrillary tangles per microscopic field of view, depending on the patient's age ²⁷.

In 1991, the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) published a protocol for semiquantitative evaluation of neuritic plaques using silver-staining methods, such as the modified Bielschowsky ²⁸. In the same year, Braak & Braak published a study on the stageing of AD-related changes, in which they proposed how amyloid and NFT pathologies progress topographically in the brain. Amyloid stages were then named A, B and C, while NFT stages became known as Braak stages I-VI⁷.

The National Institute on Aging and Reagan Institute Working Group on Diagnostic Criteria for the Neuropathological Assessment of Alzheimer's Disease (NIA-REAGAN) published, in 1997, a consensus recommendation that united both CERAD and Braak stages for NFT, in order to classify the dementia cases into pathological profiles according to the likelihood (low, intermediate and high) of being due to AD ²⁹.

In 2002, Thal and collaborators created the amyloid- β (A β) phase scoring system, where they used immunohistochemistry to detect all A β plaques, regardless of their morphology ¹¹. This system has 5 stages that reflect the spatial distribution of presence or absence of plaques across several neocortical limbic and subcortical brain regions. The Thal A β score, together with the Braak stages and the CERAD score were then combined in the ABC classification, proposed by the NIA-AA working group in 2012, which has been used to determine the likelihood of having AD pathology ¹⁷.

1.6 Pathological phenotypes associated with Alzheimer's disease

1.6.1 The amyloid pathology

Although the debate on AD pathology remains, it is a consensus that the core hallmarks of the AD pathophysiology are brain amyloid plaques and neurofibrillary tangles (NFT). Amyloid plaques consist of aggregates of $A\beta$, which is a small peptide generated from the amyloidogenic

processing pathway of the amyloid precursor protein (APP). The APP is present in several cell types and, in the brain, it has been related to the regulation of synaptic structure and function ³⁰. Its prevalent cleavage follows the non-amyloidogenic pathway, from which soluble peptides are generated ³¹. However, when APP is cleaved by β -secretase and γ -secretase, in the amyloidogenic pathway, A β fragments are formed. The A β peptides vary in length, but the predominant forms are composed by 38 (A β ₁₋₃₈), 40 (A β ₁₋₄₀) or 42 (A β ₁₋₄₂) residues, being the latter the most prone to aggregate ³². A β peptides can also constitute dimers, oligomers, protofibrils and fibrils ²⁶. These insoluble fibrils then associate to form plaques, which are also stockpiles of a variety of components, such as carbohydrates, lipids, metal ions and lipids ³³. It is proposed that, in sporadic cases of AD, the brain's clearance system fails at washing out A β fragments, which further propitiates plaque formation.

The brain aggregation of amyloid plaques seems to progress in a specific pattern. Braak et al has proposed 3 stages of amyloid plaque progression: the "stage A" shows amyloid load in the basal isocortical areas whereas in "stage B" all isocortical association areas are affected. In "stage C" plaques can be found at all isocortical areas and the number of plaques is also increased ⁷. Thal et al suggested a 5-phase progression model in which "phase 1" diffuse amyloid deposits are found in frontal, parietal, temporal or occipital neocortex ¹¹. In "phase 2" plaques are detected in the entorhinal and hippocampal cortices (phase 2) and in the striatum and diencephalon nuclei in "phase 3". Certain brainstem nuclei are affected in "phase 4" and, lastly in "phase 5" the cerebellum and the remaining brainstem nuclei are impaired.

1.6.2 The tau pathology

Tau is a microtubule-associated protein that has an important role at maintaining microtubule assembly and stabilization ³⁴⁻³⁶. This protein is mostly found intracellularly, in the neuronal axon. In humans, tau is coded by the *MAPT* gene, which can generate different tau isoforms by alternative splicing (for review see ³⁷). The carboxy-terminal region of the protein can present basically two isoforms, 3R and 4R, which contain correspondently 3 or 4 repeat domains. In AD, both isoforms undergo abnormal phosphorylation, leading to the formation of structures called paired helical filaments (PHF) ³⁸.

NFT are deposits of PHF and other conformations of abnormally hyperphosphorylated tau that accumulate inside the neurons ³⁹. When hyperphosphorylated, this microtubule-associated protein loses its function, disrupting microtubule stabilization and axonal transport, ultimately leading to synaptic impairment and neuronal damage ^{40, 41}. Despite the debated role of tau in AD pathology, several lines of evidence suggest that amyloid- β (A β) is the trigger of tau's "bad behaviour", based in the idea that amyloid accumulation can be detected early whilst NFT are detected in later stages ⁷.

Similar to what was described for amyloid pathology, tau pathology also presents a spreading pattern in the AD brain. According to Braak & Braak ⁷, NFT starts in trans-entorhinal regions (stages I/II), followed by limbic (stage III/IV) and neocortical regions (stages V/VI).

1.6.2 Neurodegeneration

Considered a progressive atrophy and loss of neuronal function ¹², the neurodegeneration is an unspecific process associated with many pathologies ^{42, 43}. Several pathways can lead to neurodegeneration, such as inflammation, imbalance of neurotransmitters, epigenetics, oxidative stress and several other intracellular mechanisms that lead to cell death ⁴⁴.

Neurodegeneration has been always associated with AD and it is believed to occur as a consequence of the toxic effects of amyloid and NFT pathology (for review see ⁴⁵). As expected, neurodegeneration has been highly correlated with cognitive decline in AD ⁴⁶⁻⁴⁸. The neuronal function in AD can be impaired, not only through the actions of the immune system, as mentioned in the next section, but also by several other mechanisms in which A β and hyperphosphorylated tau can affect synaptic modulation and plasticity and cause neuronal damage.

Experiments *in vivo* and *in vitro* have demonstrated that, in normal conditions, $A\beta$ is produced in the axonal terminal as result of neuronal activity, and is then released in the synaptic cleft to modulate neuronal excitability (for details see ⁴⁹). Studies suggest that low to intermediate levels of $A\beta$ may act in the presynaptic end, through the activation of α 7-nicotinic acetylcholine receptor, facilitating glutamatergic release ^{50, 51}. In this case, a positive feedback loop would maintain presynaptic influx of Ca²⁺ and increase exocytosis of $A\beta$ ⁴⁹. However, higher levels of oligomeric $A\beta$ are able to reduce synaptic plasticity postsynaptically. $A\beta$ affects N-methyl-D-aspartate (NMDA) receptor function and promotes the internalization AMPA receptors, leading to reduced long-term potentiation (LTP) and enhanced long-term depression (LTD) (for review see ³⁴). Although the role of $A\beta$ in glutamate receptors remains unclear, increases in intracellular Ca²⁺ are suspected to mediate this effect, which can also lead to neurotoxic events. In addition to $A\beta$, hyperphosphorylated tau was found to contribute to synaptic failure by impairing synaptic anchoring and trafficking of glutamate receptors ⁵². In addition, as consequence of tau pathology, axonal transportation becomes compromised, resulting in generating organelle depletion, causing oxidative stress and reducing ATP production in synapses ⁵³. Summing up to these events, mitochondrial support of energy to axonal transportation ⁵⁴, that is critical for the normal function of neurons ⁵⁵, becomes insufficient due to the actions of A β in inducing disruption of organelle structure and metabolism (for review see ⁵⁶). Beyond the synaptic failure, another downstream effect of mitochondrial dysfunction and impaired axonal trafficking is the blockage of retrograde transport of neurotrophins, such as the brain derived neurotrophic factor (BDNF) ⁵⁶. BDNF, whose activity is altered in AD, is known for stimulating cell survival and synaptic plasticity, mediating learning and memory ⁵⁷.

Besides the mentioned possibilities of involvement of $A\beta$ and tau in neuronal injury in AD, numerous molecules are reported to link these proteins to convergent cascades that possibly lead to oxidative stress and cell death. However, it is still challenging to identify the precise pathways that are affected in AD. Researchers in the field, most of the times, have to work based on theoretical assumptions, studying animal models or *in vitro* cells, due to technical difficulties of studying human brain *in vivo*.

1.6.3 Neuroinflammation

A factor known to be involved in the pathogenesis of AD is the immune response. Firsts reports of the participation of the immune response in the disease date from the 70's and 80's, when immunoglobulins and complement factors were detected, surrounding amyloid deposits ^{58, 59}. Since then, there is emerging knowledge on components of the innate immunity associated

with the pathology, as well as increasing discussion concerning the beneficial and detrimental effects of the immune response in the pathogenesis of AD.

Another conflicting topic is regarding the start point of the neuroinflammatory process observed in AD brains, which raises the question whether inflammation is a cause or a consequence of the brain amyloid load. Despite the initial assumption to occur in late stages of the disease, inflammatory changes in the CSF can be detected in MCI patients, revealing the possibility of involvement of the immune system in very early stages of AD ⁶⁰. Supporting this argument, genes encoding myeloid cell surface antigen CD33, complement receptor 1 (CR1), and triggering receptor expressed on myeloid cells 2 (TREM2), were all associated with an increased risk of developing AD.

Besides the genetic factors linking the innate immune response to the disease, several studies have confirmed the presence of key innate components in the pathological process of AD ⁶¹. Despite astrocytes and perivascular myeloid cells being part of the central nervous system (CNS), resident cells that react to antigens, known as microglia, have been the most associated with AD (Figure 1-2). Initial histopathological studies have shown activated microglial cells surrounding amyloid plaques ^{62, 63}. Later on, *in vitro* and *in vivo* experiments showed that A β oligomers and fibrils can activate microglial response by binding to its receptors, including CD14, CD36, RAGE and TLR4, which in turn, can stimulate the release of cytokines that are upregulated in animal models of AD and human CNS samples (for reviews, see ^{64, 65}. Besides the function of recruiting peripheral macrophages, microglia present another neuroprotective role, as they are capable of digesting soluble A β , by using extracellular proteases, such as insulin-degrading enzyme and neprilysin. Surprisingly, to date, it is uncertain whether or not microglial cells *in vivo* have phagocytic function (for reviews see ^{64, 65}).

The humoral component of the immune system, mostly associated with AD, is the complement system. Complement factors are circulating proteins that, once cleaved, are activated in a cascade. Three different pathways, starting with C1q or C3, can initiate the cascade that will ultimately lead to the formation of the membrane attack complex (MAC). Eikelenboom and Stam ⁵⁹ first reported the presence of complement molecules surrounding amyloid plaques in AD brains, which was confirmed later with studies showing that plaques were immunoreactive to C1q, C3b, C4d, C5b-C9 and MAC ^{66, 67}. Additionally, Rogers et al. ⁶⁷ discovered that A β binds to C1q, leading to the initiation of the classical complement pathway. Once triggered, the cascade of events leads to amyloid plaques opsonized by complement factors, promoting their phagocytosis, causing microglial activation. However, the problem comes with the MAC formation at the end of the process. As it requires lipidic membranes to get attached, MAC inserts itself into surrounding dystrophic neurites, causing neuronal damage (for review see ⁶⁸). These events suggest that, despite the potential beneficial effect of the complements in clearing A β , a deregulation or constant activation of this system could favour neurotoxicity and upregulated inflammation ⁶⁴.

Cytokines are also key players in neuroinflammatory processes and neurodegenerative diseases. Produced in the CNS by microglial cells and astrocytes, cytokines are members of the innate immunity, known as chemical mediators. Studies with AD patient samples and AD animal models have shown increased levels of pro-inflammatory cytokines, such as IL-1 β , IL4, IL-6, IL10, IL12, IL18, IL23, TNF- α and TGF- β ⁶⁹⁻⁷⁶. A β aggregates can bind to several microglial receptors and promote the release of these cytokines ⁷⁷, which in turn, recruit more defence cells and other chemical messengers of inflammation. Additionally, cytokines can

potentiate tau hyperphosphorylation and NFT formation by increasing p38-MAP kinase activity and deregulating cdk5/p35 pathway ⁷⁸⁻⁸⁰.

Besides the aforementioned components, many other immune-related molecules have been associated with AD, not forgetting to mention the systemic components of the immune system, that possibly affects and/or induces inflammation in the CNS ⁸¹⁻⁸³. Despite the increasing knowledge about the molecules involved in the immune response due to A β exposure, the mechanisms that regulate these reactions are poorly understood. The *Janus face* of the neuroinflammation in AD brains is still very debatable. Some may argue that immune cells are efficient in clearing A β and can repair brain damage. However, strong arguments are in favour of the dangerous feed-forward loop of the inflammatory response in the brain. In the case of inflammation caused by microbial invasion, the immune response lasts until the moment the pathogen is destroyed. Different from that, in AD brains, the inflammation does not cease due to the continuous production and accumulation of A β . As a consequence, microglial cells are kept active, releasing even more enhancing factors. Prolonged activation and A β exposition can damage microglial cells, impairing its cleansing function and production of neurotrophic factors, further impacting neuronal integrity and cognitive performance (for details see ⁶⁴ and ⁸³).



Figure 1-2 Neuroinflammation in AD.

Microglial and astrocytic activation and the generation molecular mediators of inflammation. Adapted from Meraz-Rios et al.⁶⁴

1.7 In-vivo biomarkers of Alzheimer's disease

The lack of understanding of the complete scenario behind this dementia limits the definition of biomarkers that can precisely determine the early diagnosis of the disease and/or to predict its onset. To clarify, biomarker is a "substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" ⁸⁴.

Not having specific biomarkers for AD also affects the ability of the clinical trials to establish the efficiency and efficacy of drug development. The implications of this limitation go far beyond research facilities. Nowadays, there are no disease-modifying therapies available. The current medical treatments are symptomatic, and are being used to ameliorate cognition in initial phases and to control neuropsychological symptoms ²⁶.

The major challenge in diagnosing AD is the similarity that this disease has with other dementias, such as Lewy body dementia or subcortical vascular dementia, for example, where clinical presentations and biomarker patterns can closely resemble each other ⁸⁵. This reduces the diagnostic accuracy of the current biomarkers, especially in early stages of the disease.

1.8 Quantification of Alzheimer's disease phenotypes using imaging biomarkers

A very important progress in human brain research was the development of imaging techniques, which allows the visualization of brain characteristics *in vivo*, providing information about temporal and spatial evolution of disease pathology.

1.8.1 Brain structural changes

In AD, structural data from magnetic resonance imaging (MRI), T1 sequence, shows a progressive cerebral atrophy as a probable reflection of dendritic and neuronal loss. Atrophy is initiated in the medial temporal lobe, with the entorhinal cortex, hippocampus and amygdala, and spreads to temporal neocortex and the association areas (for review see ⁸⁶, in a similar fashion to what was described for NFT accumulation ⁸⁷. Studies have suggested that atrophy starts years before diagnosis, based on findings of the reduced rate of atrophy detected in AD patients (~3-5% per year) compared to what is observed in mildly affected individuals (~15-25% per year) (for details see ⁸⁶. Additionally, the rate of brain atrophy was associated with

cognitive decline, indicating a potential use of the volume measurement as a marker of disease progression ^{88, 89}.

Several methodological approaches generate structural information about the changing brain during the disease process. The analyses can be performed using the brain as a unique entity or based on regions of interest (ROI). For example, changes in hippocampal and temporal lobe volumes have been reported in early stages of AD and were associated to initial cognitive decline ⁹⁰. Other than specific regions, brain compartments, such as the white (WM) and the gray matter (GM), can also be studied separately. In addition to broader volumetric comparisons, voxel-based morphometry (VBM) can also be employed to evaluate GM and WM atrophy. Overall, progressive cortical thinning and reductions in WM and GM VBM have been linked to symptom severity, NFT pathology, disease progression and diagnostic status ⁹¹⁻⁹⁷.

Additional structural changes can be detected using other MRI sequences. T2-weighted images, for example, are used to identify white matter hyperintensities that reflect ischemic tissue damage. Diffusion tensor imaging (DTI), on the other hand, evaluates the diffusion of water molecules in the tissue, enabling the detection of losses in tract integrity ⁹⁸. There are evidences that these brain alterations play a causative role in cognitive decline and dementia, and might interact with other pathological hallmarks to lead to AD ⁹⁸⁻¹⁰¹.

1.8.2 Brain Glucose Metabolism

To measure cerebral metabolism and synaptic activity with PET, it is possible to use a fludeoxyglucose (FDG) labeled with Fluorine-18 and identify the brain regions of more or less FDG consumption. In the brain, the most energetically costing process is the maintenance of

the membrane potential of the neuron ¹⁰², which is necessary for neurotransmission. It is estimated that synaptic activity uses more than 70% of the total energy consumed by the brain, and this is maintained through glucose consumption (for review see ^{102, 103}). Based on this mechanism, researchers developed a radiolabeled glucose analog ([¹⁸F]FDG) that is able to trace glucose utilization (for review see ¹⁰⁴). In the past decades, many studies have shown a tight link between glucose consumption and neuronal activity, demonstrating that local stimulations are coupled with higher [¹⁸F]FDG signal in efferent regions and lesions have the opposite effect on [¹⁸F]FDG ^{105, 106}. In contrast, in several diseases (including dementias), metabolic reduction was observed in brain regions associated to the symptoms, probably reflecting neurodegeneration (for details see ¹⁰⁷).

Studies with AD patients have shown that the hypometabolic regions are mainly the precuneus, the posterior cingulate gyri, the medial temporal cortices, the inferior parietal lobule and the posterior lateral parts of the temporal lobe, suggesting that they compose the [¹⁸F]FDG signature of the disease ¹⁰⁸⁻¹¹⁰. It is believed that the abnormalities depicted with [¹⁸F]FDG correspond to the combined response of several biological processes, such as excitotoxicity, inflammation, cell death, neuronal injury and mitochondrial impairment, indicating brain dysfunction and neurodegeneration in AD. Additionally, hypometabolism was associated with high amyloid load in parietal regions ^{111, 112} and is believed to act as a mediator between preceding changes in CSF biomarkers and subsequent cognitive decline ¹¹³.

1.8.3 Amyloid accumulation

Brain amyloid load is an important AD hallmark that can also be detected with PET imaging. Several radioligands have been developed for that, being the first generation of tracers carbon-
11-labeled (¹¹C) and the second generation, fluorine-18-labeled (¹⁸F). The first specific tracer developed for human A β was the Pittsburgh Compound-B ([¹¹C]PiB) ¹¹⁴ being this a derivative of the histological dye Thioflavin-T (ThT), which has been extensively used to label fibrillary A β . As a typical molecule, ThT does not a have perfect specific binding for its substrate. Studies have shown an unspecific binding of ThT to non-fibrillar structures (for review see ¹¹⁵, and this was suggested to happen with PET tracers as well, based on the evidence of the non-specific binding detected in cerebral regions where amyloid plaques are absent ¹¹⁶. The major limitation of the [¹¹C]PiB compound is its very short half-life (20 minutes) that restricts its use to specialized facilities.

The second generation of tracers brought some advancements in the field with the increased half-life of the ¹⁸F (110 minutes), enabling the use of these tracers in clinical and research settings. Several ¹⁸F amyloid ligands have been developed, including ¹⁸F-florbetapir (AmyvidTM), ¹⁸F-flutemetamol (VizamylTM), 3'F-PiB or GE-067, ¹⁸F-florbetaben (NeuraceqTM), and ¹⁸F-NAV4694 ¹¹⁷. In a similar fashion to [¹¹C]PiB, ¹⁸F tracers show, with comparable efficacy, cerebral regions where amyloid accumulation is higher than normal levels ^{118, 119}. Studies have also shown a good agreement between the imaging measurement and the immunohistochemical exam, supporting the use of amyloid imaging modality for research purposes ¹²⁰⁻¹²².

A substantial contribution of amyloid imaging for AD research came from the detection of amyloidosis in healthy older adults and MCI subjects, helping studies at predicting converters to AD, and to better understand the progression of the pathology. In this regard, it was shown that, despite the correlation found between brain amyloid and CSF-A β in AD patients, these

measurements might partially reflect independent processes occurring in different stages of the disease ¹²³.

1.8.4 Tau accumulation

Developing tracers for tau has been very challenging. Different from amyloid plaques that are found extracellularly, NFT are intracellular aggregates that require the tracer to cross an extra barrier, other the blood-brain barrier, which is the cell membrane. On top of that, the several isoforms of tau, and their different conformations when aggregated, adds more difficulty when developing the tracer: should it be very specific to one ultrastructural conformation or to all of them? The issues concerning tau tracer development have been previously discussed ^{124, 125}.

To date, several radiotracers have been tested ([¹⁸F]AV-1451, [¹⁸F]THK5117, [¹⁸F]THK5317, [¹⁸F]THK5351, and [¹¹C]PBB3) and overall they have shown abnormal cortical uptake in MCI and AD subjects that are in agreement to what has been published with *post-mortem* data (for review see ^{126, 127}. However, *in vitro* and *in-vivo* studies detected that some of the signal generated by these first-generation tracers comes from non-specific biding to molecules such as monoamine oxidase A (MAO-A) and B (MAO-B) as well as α -synuclein ^{128, 129}. Fortunately, recent studies have presented a lot of progress by showing that the new second-generation tracers are more sensitive and specific to tau tangles than were their first-generation ^{130, 131}.

1.8.5 Other imaging phenotypes

As previously presented, in the AD pathophysiology several processes become impaired as a consequence of the amyloid and tau accumulation. Synaptic depletion, neuroinflammation and gene expression are examples of that. Imaging tracers targeting these processes are under investigation and hopefully they will improve our knowledge about the mechanisms underlying AD pathology. Together, these imaging techniques will, in a near future, contribute in determining pathological profiles that better suit prevention or disease-modifying therapy studies and in assisting clinical diagnosis.

1.9 Quantification of Alzheimer's disease phenotypes using fluid biomarkers

With the discovery that AD has a long prodromal phase, the use of biomarkers became essential not only to provide accurate diagnosis of AD dementia but also to identify asymptomatic subjects with AD pathophysiology *in vivo*, and thus enable clinical trials to test therapies in the appropriate population. Biofluid biomarkers have been preferred over imaging biomarkers as these are costly and not easily accessible at basic primary care units. Thus, researchers have devoted efforts in the race to find the best biomarkers, mostly using blood and CSF.

CSF biomarkers have the advantage of being closely related to the neuronal environment and thus reflect more precisely brain metabolic changes. The drawback side of using CSF is its limited utility due to the invasiveness and possible individual contra-indications to the lumbar puncture procedure, what makes it difficult to be used routinely to track biomarker progression over time. Contrarily, blood biomarkers have been warranted alternatively to CSF, as blood collection is a common procedure at any primary care facility and is inexpensive, as compared to other methods. However, biomarkers are found in much lower concentrations in the blood as compared to CSF, requiring ultrasensitive methods to detect these molecules within many others at much higher concentrations. Further, concentrations of blood biomarkers are susceptible to systemic metabolism, possibly affecting reliable comparisons to the central nervous system environment (for review see ¹³²).

1.9.1 Fluid biomarkers of amyloid pathology

The most used biomarker of amyloid pathology to date is the CSF $A\beta_{1-42}$, which has been extensively proven to be in reduced concentrations in the CSF of AD patients as consequence of its accumulation into plaques in the brain. In addition, CSF $A\beta_{1-42}$ was also found to be in reduced levels in MCI and preclinical AD ¹³³. Reinforcing this framework, CSF $A\beta_{1-42}$ showed strong correlation with imaging and neuropathological data ¹³⁴⁻¹³⁷. However, the disadvantage of using this biomarker alone is its reduced specificity to AD and the fact alterations in this biomarker do not reflect AD pathology only, but are also related to individual baseline total levels of A β peptide concentrations leading to the discovery of either false positive or negative results (for review⁸⁵). An alternative that has been frequently employed to detect more precisely the changes in the amyloid metabolism is the use of the ratio CSF $A\beta_{1-42}/A\beta_{1-40}$. $A\beta_{1-40}$ is another derivative of $A\beta$ that can also be detected in CSF. Despite that $A\beta_{1-40}$ levels have not been found to be different between cases and controls in AD ^{138, 139}, the ratio CSF $A\beta_{1-42}/A\beta_{1}$. ₄₀ has improved the reliability of the CSF $A\beta$ biomarker for diagnosis and prediction of AD pathology in MCI subjects ^{138, 140, 141}. Blood A β assays are under development and, despite conflicting results between studies, have already shown promising results (for review ¹⁹). Plasma A β fractions and their ratios have been correlated with amyloid CSF and PET and were able to predict amyloid status ¹⁴²⁻¹⁴⁴. However, studies are still needed to validate initial findings and to evaluate the diagnostic value of this biomarker.

1.9.2 Fluid biomarkers of tangle pathology

CSF phosphorylated tau, or p-tau, is thought to reflect the phosphorylated state of neuronal tau and indicates the presence of NFT pathology *in vivo*. Higher concentrations CSF p-tau are found in AD patients as well as in prodromal AD ¹³³, as compared to age matched controls. Although being the most specific biomarker of AD and associated with disease progression, CSF p-tau is not highly correlated with NFT pathology, as shown by imaging and neuropathologic studies ^{134, 145, 146}. The reason for that is still under investigation.

Blood p-tau biomarkers are under development and seem to be promising, but so far, they are not yet reliable ¹⁴⁷.

1.9.3 Fluid biomarkers of neurodegeneration

Total tau (t-tau), together with p-tau and the amyloid biomarkers, is part of the "core" biomarkers of AD. CSF t-tau levels are increased in AD dementia cases, as well as in its prodromal state, and have been liked to disease progression ¹³³. As this biomarker is also found

elevated in other conditions, such as traumatic brain injury and stroke, it is considered a proxy of the neurodegenerative state of an individual and thus, is less specific to AD pathophysiology ¹³².

Blood t-tau studies are few and still not accurate, but they are being evaluated as a screening tool due to the non-specificity to AD.

Another protein that has got attention more recently is the neurofilament light chain (NfL), which is a structural protein mostly found in the axon of large myelinated neurons. In the event of neuronal damage, NfL is released extracellularly and can reach the biofluids, such as CSF and blood ¹⁴⁸. As it is associated with neurodegeneration, this biomarker is not specific to any disease but has been found to be in higher concentrations in prodromal and AD dementia as compared to cognitively normal subjects ¹⁴⁹⁻¹⁵¹. Longitudinal studies have also suggested that this biomarker tracks the disease state and could be used as early indicator of neuronal degeneration for several diseases, including AD ^{152, 153}.

1.9.4 Other fluid biomarkers

With the known involvement of pathways other than the ones related to amyloid and tangle formation in AD, the search for alternative biomarkers have focused on molecules that would be a proxy of neuroinflammation or synaptic depletion.

Currently, CSF neurogranin is the most promising candidate biomarker. Neurogranin is a postsynaptic protein involved in long term potentiation signaling, especially in the hippocampus and basal forebrain. CSF neurogranin is elevated in AD and it predicts cognitive decline and neurodegeneration ¹⁵⁴⁻¹⁵⁶. Moreover, it was associated with AD but not with other

dementias ¹⁵⁷, indicating a high specificity for AD. Synaptic biomarkers such as Synaptic vesicle glycoprotein 2A (SV2A), Synaptosome Associated Protein 25 (SNAP25), growth-associated protein 43 (GAP-43) and others are being evaluated.

Biomarkers of neuroinflammation often target microglial activation. With this aim, studies have investigated biomarkers such as TREM2, Chitinase 3-like 1 (YKL-40), chemokine ligand 2 (CCL2) and Cluster of differentiation 14 (CD14). Out of those, TREM2 has gained more attention due to its role in the AD pathology evidenced by genetic and neuropathological data. Recent studies have shown that CSF TREM2 is increased in AD and associated with p-tau and t-tau levels ^{158, 159}.

1.10 Genetic biomarkers of Alzheimer's disease

1.10.1 Risk factors and Genetics of Alzheimer's disease

Several papers have been published in an attempt to address the environmental and the genetic causes of AD pathophysiology. In 2006, a twin study performed by Gatz ¹⁶⁰, probably the largest to date, reported that AD heritability is as high as 79%, and that the balance is due to shared and non-shared environments. Epidemiological studies indicated that age is the most important risk factor for sporadic AD; the risk doubles every 5 years after the age of 65. ¹⁶¹. Age is followed by diabetes, stroke, head injury, depression, smoking, hypertension, and obesity in decreasing order ^{2, 26}.

Despite the big impact genes can have at causing the disease, there is no known genetic variant associated to the disease that can independently be the cause of AD. In early-onset dominant

AD, approximately 70% of known genetic variations (amyloid processing genes: APP, PSEN1 and 2) are highly penetrant ^{162, 163}. However, in sporadic cases, the most impacting variation is in the APOE gene and only 50% of the affected individuals carry the risk allele ¹⁶⁴. For this reason, researchers have used many different approaches to identify other genes involved in the pathology. After doing candidate-based association analysis in case-control studies, what did not unveil any consistent finding (except for APOE), genome wide association studies (GWAS) revealed several genetic variations related to the disease. Collaborative efforts were able to collect data from thousands of patients and healthy controls (for review see ¹⁶⁵, and identify more than 10 genetic variants from 3 major processes and pathways associated with AD: (I) immune response, including CR1, CD33, MS4A, TREM2 and EPHA1; (II) cholesterol metabolism, including CLU, SORLI, APOE and ABCA7; and (III) endocytosis processes, including PICALM, BIN1, SORL1, and CD2AP (Figure 3). The variants found in GWAS studies are common in the human population and these variants have low contribution to the risk for AD. As the findings did not explain disease susceptibility, researchers also tried to run GWAS with quantitative endophenotypes of AD instead of doing case-control analysis. Characterized as internal or subclinical traits of a disease ¹⁶⁶, the endophenotypes can help detect small genetic effects in multifactorial diseases due to their quantitative nature and closer proximity to the genetic variation (as compared to the diagnosis) ¹⁶⁷. However, in AD, association studies using endophenotypes -such as measurements of fluid biomarkers- did not reveal any promising results so far. One alternative to find AD causative genes, which has recently drawn the attention of geneticists, is the search for low frequency and rare variants. The alternative hypothesizes that they may have higher effect and could explain the disease phenotype ^{165, 168}. To date, studies have reported some new rare variants in genes associated to AD, such as APP, PSEN 1 and 2, and TREM ¹⁶⁹⁻¹⁷³, but they did not solve the puzzle of the missing heritability in AD.



Figure 1-3 Genetic variants that contribute to AD.

Rare and common variants that have been associated with AD in GWAS studies. Adapted from Karch CM, Goate AM. ¹⁶⁵

1.10.2 Genetics and Imaging Endophenotypes

In the past few years imaging data have been used as endophenotypes to study the effects of known genetic risk factors and for the discovery of new ones. Several studies have shown that asymptomatic subjects who have AD first-degree relatives present AD-related biological changes, which indicates that heritability plays an important role in these features $^{174-176}$. The first genetic works revealed that the most studied genetic factor of AD, the *APOE-* ε 4 allele, is associated with increased amyloid accumulation –measured by PIB and [18 F]florbetapir PET-, increased hippocampal atrophy, decreased glucose metabolism and brain connectivity $^{177-180}$. Besides the *APOE* gene, *CR1* was also associated with brain amyloid load while *BIN1* and *PICALM* were related to entorhinal cortical thickness $^{181-183}$.

Trying to find new susceptibility genes for AD, GWAS analyses were performed with structural and functional neuroimaging. *FASTKD2* was reported to be associated with memory and hippocampal structure ¹⁸⁴, while several other genetic variants were identified in analyses using hippocampal volume and rate of atrophy ^{180, 185}. Regarding functional neuroimaging, GWAS revealed *BCHE* and *APOE* as modulators of brain amyloid load measured by [¹⁸F]florbetapir PET ¹⁸⁶. Additionally, a recent GWAS using longitudinal [¹⁸F]florbetapir PET suggested the involvement of *IL1RAP* with amyloidosis, which implicates microglial activation in the process ¹⁸⁷.

1.10.3 Genetic interactions in Alzheimer's disease

In the last decades, researchers have investigated possible genes implicated in pathologic mechanisms that promote AD, in the hope that they may bring new clues about the pathologic mechanisms that promote disease onset and progression. Furthermore, it is expected that specific treatments and prevention strategies can be developed based on the genomic profile of the subject. However, to date, there is no consensual method to predict the subjects that will develop AD ¹⁸⁸. The traditional methodological approaches have not yet unveiled any determinant genes of AD, indicating that we may not find an answer by looking for the independent effect of single common variants in the disease. Likewise, there is evidence that the information about genetic architecture of complex diseases, such as AD, is missing due to ignored epistatic effects ¹⁸⁸⁻¹⁹⁰.

Epistasis is defined by Fisher ¹⁹¹ as the interaction between two or more genes that contribute, not merely additively, to a single phenotype. In this context, epistasis analysis is an important

tool to capture the jointly effect of variants (possibly from different pathways) on endophenotypes, revealing genetic factors that cannot be detected due to the little or no effect they have alone. Despite the potential application of this method, the *a priori* definition of the biological framework that sustains the genetic hypothesis must be considered ¹⁹². This is what connects the mathematical/statistical model with the biological context, which allows insights about possible pathological mechanisms underlying the disease. The hypothesis preceding the analysis is of a great importance as it makes the findings intelligible and reduces multiple testing burden.

In AD, the largest review about epistasis was done by Combarros *et al* ¹⁹³, which has found over 100 claims of epistasis in sporadic AD. The reports examined are all based on case-control studies and, in most of them, no interaction analysis had been performed. From the 27 significant interactions found, 4 (*APOE x BACE1*; *APOE x* IL6; APOE x BCHE; APOE x ACT) were replicated at least once in independent samples (for details see ¹⁹³. Aside from those findings, more recent studies have reinforced the importance of interactive effects of genes in AD ^{194, 195}, showing interactions between GSK3 and amyloid processing genes affecting brain amyloid burden, as well as a masked effect of HMGCR over APOE regarding disease progression in MCI subjects. In spite of the strong suggestions towards the importance of epistasis in AD, and despite the genetic interactions already reported with AD phenotype, little is known about the epistatic effects on AD endophenotypes, leaving an open field with opportunity for new discoveries.

1.11 Alzheimer's disease hypotheses

1.11.1 The amyloid hypothesis

In fact, mutations in the APP gene, or in the genes coding proteins involved in APP processing, are known to cause autosomal dominant AD, which is usually early onset ¹⁹⁶⁻¹⁹⁸. Also, similar brain amyloidosis happens in cases of Down's syndrome in which the APP gene is triplicated ¹⁹⁹, reinforcing the role of amyloid in the pathology and being the basis of the most accepted theory about AD pathology: the amyloid cascade hypothesis.

Firstly proposed in 1992 by Hardy & Higgins ²⁰⁰ and updated in 2002 by Hardy & Selkoe ²⁰¹ the amyloid cascade hypothesis suggests that the increased production and accumulation of $A\beta_{1.42}$ triggers a series of events that cause both activation of immune response (astrocytic and microglial recruitment) as well as synaptic and neuritic impairment. These progressive alterations would cause neuronal oxidative injury, tangle formation, and cell death, leading to symptoms found in dementia. Despite this hypothesis being widely accepted, there remains a discussion regarding the initial events in the pathological cascade. It is undeniable that amyloidosis can lead to neuronal damage and that it is essential in the disease process; however, some studies show evidence that tau hyperphosphorylation can occur before $A\beta_{1.42}$ formation ²⁰²⁻²⁰⁴.

1.11.2 Biomarker modeling of Alzheimer's disease pathophysiological progression

Following the leading principles of the amyloid cascade hypothesis, and based on many studies from the last two decades, Jack *et al.* ^{12, 205} proposed the biomarker model of Alzheimer's disease. According to this model, AD biomarkers can be classified into two major categories: 1) the amyloid pathology, revealed by $A\beta_{1.42}$ level in the cerebrospinal fluid (CSF) and positron emission tomography (PET) amyloid imaging, 2) neurodegeneration, depicted by tau level in CSF, PET imaging of hypometabolism with [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG), and atrophy seen with structural magnetic resonance imaging (MRI) atrophy. These biomarkers were then presented in a temporal perspective, showing how they possibly evolve in the AD pathology (Figure 1-4).

The model is mainly based on early onset AD due to the homogeneity of its pathology, which is often caused by genetic mutations that lead to amyloidosis. In these cases, it is expected to detect abnormalities first in CSF A β_{1-42} , and then in amyloid PET imaging, reflecting amyloid plaque accumulation. In the sequence, CSF tau measures begin to increase and [¹⁸F]FDG PET to decreases, revealing advancing neuronal damage. At last, neuronal loss is reflected by brain atrophy in MRI, and the drastic worsening of the clinical symptoms makes the disease even more evident.



Figure 1-4 Biomarker model of Alzheimer's disease.

Temporal evolution of biomarkers showing that amyloid biomarkers are the first to become abnormal. Adapted from Clifford R., et al⁴⁵.

Different from what is described for early onset, late onset AD is sporadic and is frequently observed to coexist with other brain pathologies and/or age-related changes ²⁰⁶, which makes it difficult to determine the causative factors of the AD pathology. For this reason, Jack et al ¹² suggested two possible biomarker models for late onset AD. One follows the amyloid-first model described for early-onset AD, in which tau-related changes become abnormal only after abnormal changes are detected in amyloid biomarkers. The other model is in line with the theory presented by Price & Morris ²⁰⁴, in which neurodegeneration happens first. In this case, any biomarker of neurodegeneration, or their combination, becomes abnormal first and consequently, but independently, $A\beta_{1.42}$ biomarkers arise. Despite the initial events, tau-related effects would be potentiated by increasing $A\beta_{1.42}$, resulting in accelerated neurodegenerative process that drives the worsening of AD symptoms.

The models present plausible sequences of events and are widely accepted in the field, however, they also have limitations. Besides the possibility of explaining the disease progression in two different ways, the biomarkers used can be also altered in non-AD pathologies, which means that, if taken independently, they are not specific to AD pathology. Furthermore, although the models predict the order and the time frame that changes can be detected, it is not fully understood why and how they happen.

1.12 Rationale and Objectives

Because AD is a multifactorial disease, it is imperative to evaluate how the biomarkers relate to each other to better understand the pathophysiological processes that lead to AD, which ultimately allows definitions of new therapeutic strategies. More specifically, the use of biomarkers depicting early pathological stages brings the advantage of the identification of individuals at risk of developing the disease further on time. Considering that amyloid pathology is an early hallmark of the disease, according to the most accepted framework on disease progression, the overarching goal of this thesis is to explore how amyloid pathology is related to multimodal early *in-vivo* biomarkers –genetic, fluid and imaging. This would indicate individuals with higher probability of developing amyloid pathology or that are suffering the early pathophysiological changes associated to AD-related amyloidosis.

Towards this goal, we designed 4 studies with the specific objectives as follows. Chapters 2-3 focus on the discovery of genetic variants associated with amyloidosis, more specifically with pathways that would lead to brain amyloid accumulation and could impact its downstream effects. In **Chapter 2**, we employed a hypothesis-driven approach, using data from two independent cohorts, to investigate whether genetic variants in Cytochrome P450 genes could be associated with increased predisposition to amyloid accumulation, considering that the enzymes coded by these genes are involved with several metabolic pathways impaired in AD. Further, in **Chapter 3**, we performed epistasis analysis to verify if the interaction between variants related to immune-related cascades is associated with in vivo measures of amyloid

load, reflecting possible impairments in the amyloid clearance mechanisms. In Chapters 4-5 we combined information provided by genetic and imaging biomarkers to evaluate how the new plasma biomarker of neurodegeneration, the Neurofilament light chain protein (NfL), is associated with amyloid load and with early AD-related pathophysiological changes. More specifically, in **Chapter 4** we investigate the association between plasma NfL and amyloid and tau PET (using data from 2 cohorts). We also verify how plasma NfL reflects white and gray matter atrophy over time, considering the *APOE*-e4 carriage status. In the same line of investigation, **Chapter 5** finally evaluates how plasma NfL is associated with brain metabolic changes longitudinally, considering the amyloid load status.

Chapter 2 : CYP2C19 variant mitigates Alzheimer disease pathophysiology *in vivo* and *post-mortem*

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complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wpcontent/uploads/how to apply/ADNI Acknowledgement List.pdf

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2.1 Preface

Currently, in the AD research field, identifying early biomarkers of AD is being considered the most urgent goal. For this purpose, genetic markers would be preferable due to their unchangeable nature, allowing an early prediction of future pathophysiological changes. As previously mentioned, it is expected that a large proportion of AD is caused by genetic factors although only part of this has been already explained ¹⁶⁰. Thus, the search for genetic markers is made necessary. A possible strategy to increase statistical power in this investigation, is to use intermediate phenotypes of the disease as they are closer related to the genetic factors ¹⁶⁷. Here we used amyloid load, quantified by PET imaging, as an early endophenotype of AD. We also chose to select genes related to a poorly explored amyloid clearance pathway, hoping that we would find new genetic factors associated with amyloid.

2.2 Abstract

2.2.1 Objectives

To verify whether *CYP* polymorphisms are associated with amyloid- β (A β) pathology across the spectrum of clinical AD using *in vivo* and *postmortem* data from two independent cohorts.

2.2.2 Methods

A candidate-gene approach tested the association between 5 genes (28 SNPs) and A β load measured *in vivo* by global [¹⁸F]florbetapir PET standardized uptake value ratio (SUVR) in 338 ADNI participants. Significant results were then tested using plasma A β and cerebrospinal-fluid (CSF) A β and A β /phosphorylated-tau (A β /p-tau) ratio in the same cohort. The significant association was also generalized to *post-mortem* A β load measurement in Rush-ROS/MAP cohorts. Additionally, global cognition was used as phenotype in the analysis in both cohorts.

2.2.3 Results

Analysis of A β PET identified a variant in the *CYP2C19* gene (rs4388808;*P*=0.0006), in which carriers of the minor-allele (MA) had lower global SUVR. A voxel-wise analysis revealed that the variant is associated with lower A β load in the frontal, inferior temporal, and posterior cingulate cortices. MA carriers also had higher CSF A β (*P*=0.003) and A β /p-tau ratio (*P*=0.02), but had no association with A β plasma levels. In *post-mortem* brains, MA carriers had lower

A β load (*P*=0.03). Global cognition was higher in MA carriers, which was found to be mediated by A β .

2.2.4 Conclusions

Together, these findings point to an association between *CYP2C19* polymorphism and $A\beta$ pathology, suggesting a protective effect of the MA of rs4388808. Despite the several possibilities in which *CYP2C19* affects brain A β , the biological mechanism by which this genetic variation may act as a protective factor merits further investigation.

2.3 Introduction

In in sporadic Alzheimer's disease (AD), accumulation of amyloid- β (A β) seems to be associated with reduced A β clearance but little is known about the biochemical pathways underlying cerebral A β metabolism.

Cytochrome-P450 (CYP) is a family of enzymes known to metabolize several endogenous and exogenous substrates. Besides metabolizing drugs, brain CYPs are also involved in the modulation of blood flow, metabolism of fatty acids, cholesterol and neurotransmitters, and mobilization of intracellular calcium ¹⁻³, all pathways that have been somehow linked to AD and/or amyloid metabolism. Additionally, studies have shown that CYP proteins and its genetic variants are associated with the brain immune response ² and neurodegenerative diseases ^{2, 4, 5}.

Although previous evidence shows CYP expression in areas affected by AD such as the amygdala, frontal, and temporal cortices and the hippocampus ^{6, 7}, there are only few reports documenting a link between CYPs and AD pathophysiology. In one study, the overexpression of human APP on the Tg2576 mice model was associated with elevated CYP hepatic function, while their renal counterparts were depressed ⁸. Additionally, another study presented evidences that A β stimulates NADPH-cytochrome P450-reductase (POR), a CYP inducer, in APP transgenic mice and human AD brains, which may impact the redox status ⁹.

In line with the evidence that $A\beta$ can stimulate metabolic cascades in a similar fashion as foreign compounds ^{9, 10}, and knowing that brain CYP could potentially affect amyloid metabolism also through other mechanisms, we tested the hypothesis that variants in *CYP* genes could be associated with $A\beta$ and, consequently, have an impact in cognitive performance. We examined the association of *CYP* variants with biomarkers of $A\beta$ load *in vivo* and *postmortem*, as further specified.

2.4 Materials and methods

2.4.1 Study participants

Discovery analysis data were obtained from the AD Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). For this study cognitively normal (CN) individuals had a mini–mental state examination (MMSE) score of 24 or higher and a clinical dementia rating (CDR) of 0. The operational criteria adopted for mild cognitive impairment (MCI) were participants who had a MMSE score equal to or greater than 24, a CDR of 0.5, subjective memory concern, objective

memory loss measured by education adjusted scores on delayed recall of 1 paragraph from Wechsler Memory Scale Logical Memory II (\geq 16 years: \leq 8; 8-15 years: \leq 4; 0-7 years: \leq 2), and essentially normal activities of daily living. AD participants were individuals with MMSE score lower or equal 26, CDR higher than 0.5, who meet the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association criteria for probable AD. All individuals had absence of any other neuropsychiatric disorders. The ADNI inclusion/exclusion criteria are described in detail at www.adni-info.org.

Our report also used data from the Religious Orders Study (ROS) and Rush Memory and Aging Project (MAP), two longitudinal clinical pathologic cohort studies of aging and dementia ^{11, 12}. All participants enrolled without known dementia and agreed to annual clinical and neuropsychological assessments and brain donations after death. The clinical diagnose of dementia and AD followed the National Institute of Neurological and Communicative Disorders and Stroke–Alzheimer's Disease and Related Disorders Association recommendations. The MCI diagnose is given to participants who were rated as impaired based on MMSE and CERAD neuropsychological measures, but who were not found to have dementia by the examining neurologist. Our study includes only MCI participants with one impaired domain and no other cause of cognitive impairment. Notably, the studies are conducted by the same team of investigators and share a large common core of testing batteries, which allow for combined analyses.

The discovery sample consisted of a subset of ADNI participants who had [¹⁸F]florbetapir PET, genetic data, and CSF data available. The findings obtained with the discovery sample were then generalized to the Rush-ROS/MAP participants that died at age 90 years or younger.

2.4.2 Standard Protocol Approvals, Registrations, and Patient Consents

This secondary analysis study followed all IRBs regulations, which are detailed in the supplementary methods.

2.4.3 Phenotypes

From ADNI we obtained demographics, neurocognitive scores, cerebrospinal fluid and plasmatic data available after passing rigorous quality control. The preprocessed imaging and genetics data, underwent additionally processing and quality control before the statistical analysis in our study, as described below. From Rush, all the data used in this project to perform statistical analyses were already processed and quality controlled with very rigorous criteria.

In ADNI cohort, brain Aβ load was estimated using [¹⁸F]florbetapir PET standardized uptake value ratio (SUVR, details figure 1 and e-methods). Separately, Aβ and hyperphosphorylated tau (p-tau) levels were measured in the CSF and plasma. These data were obtained by the Biomarkers Consortium CSF, and the description of the methodology regarding the sample acquisition, processing and analysis is available at ADNI website (http://adni.loni.usc.edu/data-samples/biospecimen-data/).

In Rush-ROS/MAP cohort, post-mortem data for A β load and paired helical filaments (PHF) tau tangle density were obtained from eight brain regions using immunohistochemistry ¹³.

Cognitive function was evaluated in both ADNI and Rush-ROS/MAP. For ADNI, the global cognition scores were developed by making a z-score of the sum of the memory and the executive function composite scores. In Rush-ROS/MAP, the global cognitive scores were based on 17 cognitive performance tests and we only included participants who had undergone autopsies, thus last valid cognitive score proximate to death was used for this analysis (for further details see supplementary methods).

We used gene expression data to examine cis eQTL. For ADNI, total RNA was obtained from the peripheral blood–at the same visit when the imaging data was obtained–and, after quality control, was hybridized to Affymetrix Human Genome U219 array plate as described ¹⁴. The normalized and quality-controlled expression data ¹⁴ were used. For Rush-ROS/MAP, RNA was extracted from the gray matter of dorsolateral prefrontal cortex blocks using the miRNeasy mini kit (Qiagen, Venlo, Netherlands) and the RNase free Dnase Set (Qiagen, Venlo, Netherlands). These samples were quantified and qualified as described¹⁵, and then sequenced on the Illumina HiSeq with 101 bp paired-end reads. The normalized and quality-controlled expression data were used. In both analyses, the RNA integrity measurement was used as a covariate.

2.4.4 Genotyping, imputation and gene selection

We interrogated SNPs from the *CYP* genes of the five–probably–most important metabolizers: *CYP3A4*, *CYP2D6*, *CYP2C9*, *CYP1A2*, and *CYP2C19*^{3, 16-18}. Methods involved in DNA processing are detailed in the supplementary methods. Using PLINK ¹⁹, the SNPs within these genes were recoded based on the dominant model with respect to the MAs, and the ones in

high linkage disequilibrium (LD) ($r^2>0.8$) were removed from the analysis to avoid unnecessary testing. A total of 28 SNPs was used in the initial step of the association analysis.

For Rush-ROS/MAP cohorts, DNA was extracted, genotyped, imputed, and quality controlled according to procedures described in supplementary methods.

2.4.5 Statistical analysis

The statistical tests were performed in R statistical software ²⁰, where linear regression models were used to test the association between the genotypes and the phenotypes in both cohorts, separately. All statistical models included the covariates of age, sex and *APOE*- ε 4 carriage status, except for the models in which global cognition was the outcome measure, where *APOE*- ε 4 status was replaced by years of education. The initial analysis–using global [¹⁸F]florbetapir SUVR as phenotype–was carried out to interrogate a total of 28 SNPs (Table e-1). To correct for multiple testing, the statistical significance for SNP discovery was set at *P*<0.0017 using a Bonferroni correction for 28 tests and a type I error α =0.05. The significant SNPs were further tested for associations with other phenotypes in ADNI and Rush-ROS/MAP cohorts. To test for group differences in diagnostic status we added diagnosis as a covariate in the regression models and also tested the interaction between diagnostic status and the desired independent variable. Effect size calculations (Cohen's d) were also employed.

A voxel-based analysis was carried out using the RMINC imaging tool ²¹, where parametric images were obtained contrasting [¹⁸F]florbetapir SUVR between the genotype groups of the

SNP found in the discovery analysis. After Random Field Theory correction for multiple comparisons, the T value threshold of significance was \leq - 3.1 ($P \leq 0.001$).

Mediation analysis was employed to examine whether the SNP associations with cognition were mediated through the effect of A β . To test this hypothesis, firstly the association of CYP SNPs with global cognitive performance was evaluated using linear regression models. Then, if the association was significant, the measure for A β was added to this model to check whether the SNP associations with cognition were attenuated due to A β .

2.5 Results

Demographics and general information of the ADNI and Rush-ROS/MAP cohorts are presented in Table 1. After imaging and genetic QC, a total of 338 ADNI participants were studied, including 186 CN, 105 single- or multiple-domains amnestic MCI, and 47 AD. A total of 738 Rush-ROS/MAP participants were analyzed, including 301 CN, 179 amnestic MCI, and 258 AD. As expected, in both cohorts, CN individuals had higher MMSE scores and fewer were *APOE*-ɛ4 carriers, as compared to both MCI and AD. There was no difference in sex or years of formal education between diagnostic groups in both cohorts (details provided in Table e-2).

2.5.1 *CYP2C19* polymorphism is associated with global [¹⁸F]florbetapir SUVR in the ADNI cohort.

The discovery analysis tested the association of 28 SNPs from *CYP* genes with global [¹⁸F]florbetapir SUVR. The analysis unveiled an association of a SNP (rs4388808) in the *CYP2C19* gene (t=-3.43; *P*=0.0006) (Figure 2a). The MA frequency (MAF) of rs4388808^G in the ADNI cohort was 0.19, and the MA carriers displayed less [¹⁸F]florbetapir binding as compared with the non-carriers. Importantly, the association remained significant when adjusting the model for diagnosis. In addition, no interaction was found between the SNP and diagnostic status. The estimated effect size for rs4388808 in the whole sample was Cohen's *d*= 0.36. Interestingly, the effect size was greater for the subset of *APOE*-ε4 carriers (Cohen's *d*= 0.51) as compared to *APOE*-ε4 non-carriers (Cohen's *d*= 0.33).

2.5.2 Minor allele carriers of *CYP2C19* polymorphism have less $A\beta$ in AD-related regions in the ADNI cohort.

To identify the brain areas responsible for the global difference observed in [¹⁸F]florbetapir uptake revealed in the discovery analysis, we performed a voxel-wise analysis. The analysis showed that carriers of rs4388808^G had less [¹⁸F]florbetapir binding in the frontal, posterior cingulate, and inferior temporal cortices (Figure 3), with the voxels in these regions presenting p-values equals or smaller than 0.001.

2.5.3 Findings with brain imaging are corroborated by CSF data in the ADNI cohort.

Due to the well-known relationship between brain and CSF levels of A β , we tested the association between rs4388808 and CSF A β . Because not all participants in the ADNI sample had CSF data, we performed the analysis in a subset of 260 individuals. As expected, MA carriers presented more CSF A β than non-carriers (t=2.94; *P*=0.003) (Figure 2b). The *CYP2C19* polymorphism was associated with A β /p-tau ratio (t=2.29; *P*=0.02), and higher ratios were found in carriers of rs4388808^G (Figure 2c). No association was found using p-tau as a single outcome measure (t=-1.24; *P*=0.21).

2.5.4 Association between *CYP2C19* polymorphism and A β is exclusive to the A β brain levels in the ADNI cohort.

To examine whether the *CYP2C19* polymorphism is also associated with A β levels outside the brain, we tested the association of rs4388808 with plasmatic levels of A β in a subsample of 113 participants. We didn't find an association between the SNP and free (t=-0.60; *P*=0.54) or total (t=0.49; *P*=0.62) plasmatic A β_{1-42} levels (Figure 2d), indicating that the findings reported above are specific to CNS.

2.5.5 Results from the ADNI cohort were generalized to Rush-ROS/MAP cohort

We next generalized the findings to postmortem indices of A β load and to neurofibrillary tangles in Rush-ROS/MAP cohorts. From the eight brain regions where A β load was measured, we selected six regions (angular gyrus, anterior cingulate, entorhinal cortex and mesial and inferior temporal cortices) and calculated an average per subject. These six regions were chosen to match with the brain areas where we detected differences between genotype groups in the voxel-wise analysis. When testing the association between the average of A β load and the *CYP2C19* polymorphism, we found that rs4388808^G (MAF=0.15) carriers had a lower A β load than non-carriers (t=-2.15; *P*=0.03) (Figure 2e), consistent with findings from the ADNI cohort. The difference between rs4388808^G carriers and non-carriers in PHF tau tangle density did not reach statistical significance (t=-1.65; *P*=0.09).

2.5.6 Minor allele carriers of *CYP2C19* polymorphism had better cognitive performance than non-carriers in both cohorts.

To examine whether the *CYP2C19* polymorphism impacts the cognitive performance, we used composite measures of global cognition as the outcome in linear regression models. In ADNI cohort, there was a clear tendency to an association with *CYP2C19* polymorphism (t= 1.92; P=0.05) (Figure 2f), while in Rush-ROS/MAP cohorts, the association was such that rs4388808^G carriers had a higher cognition than non-carriers (t=2.08; P=0.03) (Figure 2g).

2.5.7 The effect of *CYP2C19* polymorphism on cognition is mediated by A β .

A mediation analysis was employed to determine whether the *CYP2C19* polymorphism was directly or indirectly associated with cognitive performance. We retested the association between the *CYP2C19* polymorphism and cognition, but now adding the measures of

 $[^{18}F]$ florbetapir or A β load in the model. Neither of the previous genotype-phenotype associations remained significant, indicating that the observed effect of the genotype was mediated by A β .

2.5.8 Association between rs4388808 and gene expression of *CYP2C19* suggests that the SNP is functional.

To check if the polymorphism has an effect in the gene expression, we tested the association between rs4388808 and *CYP2C19* RNA levels. In ADNI cohort, with 304 participants for this analysis, we found a cis eQTL such that minor-allele carriers displayed higher RNA levels in the blood (t=2.38; *P*=0.01) (Figure 2h). In Rush-ROS/MAP cohorts, very low levels of *CYP2C19* expression were detected in postmortem brains, and the data did not give consistent results (data not shown) as found in the blood expression data from ADNI.

2.6 Discussion

In the present study, we found an association between *CYP2C19* polymorphism and A β burden across the spectrum of AD, in which carriers of rs4388808^G presented less A β load and downstream cognitive impairment. This association was initially detected using [¹⁸F]florbetapir SUVR and posteriorly confirmed with CSF A β in ADNI cohort. No association was found between the polymorphism and diagnostic groups, suggesting that the SNP is associated with amyloid load rather than with the disease status. Effect size analysis suggests that the benefic effects of the polymorphism is larger in *APOE*- ε 4 carriers, as compared to noncarriers or the whole sample, in which the effect is similar to what has been described for a *BCHE* gene polymorphism ²². Subsequently, this association was generalized to *post-mortem* data from Rush-ROS/MAP cohort. Finally, both cohorts showed a protective association between the MA of *CYP2C19* and cognition. Importantly, subsequent mediation analysis suggested that the effects of *CYP2C19* on cognition were mediated by A β .

Overall, these results support the notion that the MA of *CYP2C19* (rs4388808) is a protective variant against A β and downstream cognitive impairment. Interestingly, voxel-wise analysis in *in vivo* participants revealed that the MA was associated with reduced A β burden in AD-related regions in the frontal, inferior temporal, and posterior cingulate cortices ²³. Additionally, the *CYP2C19* SNP was associated with the CSF A β /p-tau ratio but not with plasmatic A β . Beyond the fact that the A β /p-tau ratio is postulated to better represent neuritic plaques than the single CSF A β information ²⁴, these results provide further evidence that the polymorphism affects brain accumulation of neuritic plaques in a tissue- and disease-specific manner ²⁴. In both cohorts, the referred polymorphism was not associated with PHF tau or single CSF p-tau, agreeing with the framework that A β and tau have independent upstream triggers ²⁵. In line with overall significant results, the *CYP2C19* polymorphism also presented a protective effect in global cognition, being supported by previous studies that linked A β pathology to cognitive changes ¹³. Indeed, the mediation analysis supported that the association between the genetic factor and cognitive abnormalities is likely due to the upstream accumulation of A β .

Levels of the CYP2C19 protein are associated with the rs4388808 polymorphism ²⁶, which is an intronic variant of the *CYP2C19* gene. Indeed, we found here a similar association showing that minor-allele carriers expressed higher levels of *CYP2C19* when compared to non-carriers, suggesting that the SNP affects the expression of its gene. To corroborate this finding, we retrieved the SNPs previously excluded due to high LD and verified that none was associated with *CYP2C19* expression in the blood (data not shown), reinforcing the idea that rs4388808 is functional. This association could not be detected in *post-mortem* data probably due to the low *CYP* expression or due to its limited detection by the method used ⁷.

Despite the SNP not being widely studied to provide information about association with other phenotypes, other polymorphisms in the gene have been correlated with metabolic variability, atherosclerosis, and behavioral traits ²⁷⁻²⁹. *CYP2C19* expression has been detected in several brain regions ^{6, 7}, however its function in the central nervous system has not been fully elucidated. Some reports have suggested that, besides its role at metabolizing exogenous substrates, *CYP2C19* also participates in other important biological cascades, such as the metabolism of serotonin and sexual hormones ¹ and the metabolism of the arachidonic acid, where it functions as an epoxygenase ³⁰.

The biochemical mechanism underlying the association of *CYP2C19* with A β remains speculative. A study has shown that A β is able to act as a foreign body and trigger POR, activating CYPs to initiate catabolic reactions ⁹. In turn, CYP2C19 is involved in the catalysis of estradiol ³¹. Since several studies have reported the beneficial effects of estrogens on AD– demonstrating that these steroids are able to act as anti-inflammatory compounds ³² and even inhibit A β production ³³–, one could think that MA carriers of rs4388808 have a reduced expression of this *CYP* in the brain or that they have an increased structural incompatibility between CYP2C19 and estradiol. Consequently, minor-allele carriers could present a reduction in the catabolism of estrogen and a reduction in A β load. Alternatively, A β load would be affected by the levels of epoxyeicosatrienoic acids (EETs) produced via the metabolism of arachidonic acid (AA) by CYP epoxygenases. In the brain, studies have shown that EETs have beneficial effects at regulating blood flow, cortical angiogenesis, and at promoting anti-

inflammatory reactions (for review see ²). By contrast, when AA is metabolized via other cascades, the protective effect of EETs is lost, and there is a possibility of induction of A β production and/or accumulation ³⁴. Additionally, it was demonstrated that A β is able to reduce epoxygenase activity and consequently decrease EET production³⁵, probably leading to more susceptibility to neuronal damage (for review see ³⁶). In this scenario, it would be possible that the minor-allele of rs4388808 increases the expression of *CYP2C19*, leading to an "A β -resistant" epoxygenase metabolism of AA, with a maintained production of EETs and a more protective phenotype against amyloidosis.

It is also plausible to think, however, that the association described here between *CYP2C19* polymorphism and $A\beta$ is indirect, being mediated by the effect of a drug. CYP2C19 is known to metabolize several drugs ³⁷, including medications to treat depression which has been mentioned as a risk factor for Alzheimer's disease (for review see ³⁸). Citalopram, for example, is an antidepressant metabolized by CYP2C19 that has been shown to decrease $A\beta$ production ³⁹. Thus, one may think that the polymorphism rs4388808 is then associated with an improved action of Citalopram, leading to decreased brain $A\beta$ burden, rather than associated with $A\beta$ metabolism itself. Because it is difficult to track all the medications the study participants have taken before been enrolled, it is unfeasible at the moment to check if this association holds true.

The interpretation of the results should take into consideration a few limitations. The use of the two cohorts does not allow us to detect and measure A β pathology with the same methodology, inserting in the analysis some variability specific to the method used. However, despite being measured with different techniques, the imaging radiotracer and the antibody used in the brain tissue are both expected to bind to the fibrillar form of A β ¹³. Similarly, the cognitive composite scores from the two cohorts have some differences that should be considered. There are also

some intrinsic characteristics of the samples that have to be taken into account: (1) the inclusion criteria of each study; (2) the average age difference between the studies; (3) and the disease stage in which A β measures were performed–at the end or during the disease process. Sample size restrictions impose some degree of caution when interpreting findings, including, for example, the lack of association between genotype and plasmatic levels of A β , or cognition, in ADNI cohort. Additionally, both cohorts are mostly composed of non-Latino Caucasians, limiting the extrapolation of the present findings to other population groups. Longitudinal analysis together with functional genomics and biochemistry experiments, not performed here, would also be necessary to determine the effect of the SNP in the protein function as well as to support any theoretical framework proposed in the discussion section.

Results obtained from the two independent cohorts provide compelling evidences linking *CYP2C19* polymorphism and A β pathology, suggesting that the MA of rs4388808 confers protective effects against A β accumulation in the brain and its downstream cognitive consequences. These results could have implication for anti-amyloid clinical trials designs as preclinical rs4388808^G would present a protective factor against amyloid load. Therefore, the biological mechanism by which the genetic variation would alter A β build up and clearance merits further investigation.

2.7 Tables and Figures

	ADNI			Rush-ROS/MAP		
	CN	MCI	AD	CN	MCI	AD
No. Participants	186	105	47	301	179	258
Males (%)	91 (48.9)	64 (61)	26 (55.3)	182 (60.5)	101 (56.4)	154 (59.7)
Age ¹ (SD)	75.70 (6.63)	74.29 (8.12)	75.67 (7.37)	83.7 (5.19)*	85.26 (4.35)	85.95 (3.81)
APOE- ɛ4 (%)	52 (28)*	57 (54.3)*	30 (63.8)*	49 (16.4)*	50 (27.9)*	112 (43.4)*
MMSE (SD)	29.09(1.14)*	27.24(1.95)*	20.34(3.66)*	28.69(1.42)*	27.91 (1.83)*	25.49 (5.17)*
Education ¹	16.41 (2.75)	16.67 (2.68)	16.13(2.47)	16.53 (3.74)	16.34 (3.47)	16.66 (3.94)
MAF ² /sample						

Table 2-1 Demographic and key characteristics of the samples.

SD standard deviation, MMSE Mini-Mental State Examination

*Statistically different from the other groups of the same sample.

¹Measured in years.

²MAF= Minor allele frequency of rs4388808.





Flowchart showing acquisition methods (purple), image processing (blue), and outcomes (green). PET= positron emission tomography, MRI= magnetic resonance imaging, GM = grey matter, WM = white matter, CSF = cerebrospinal fluid, FWHM = full width half maximum, ROI= region of interest.



Figure 2-2 Comparison between non-carriers(-) and carriers(+) of the MA of rs4388808 (*CYP2C19*).

In ADNI cohort, a difference was observed in brain A β load (a), CSF A β levels (b), and CSF A β /p-tau ratio (c) but no difference was detected in plasmatic levels of A β (d). Results were generalized to post-mortem data of Rush-ROS/MAP cohorts, where a concordant pattern was
observed in A β load (percentage area occupied by amyloid) (e). A tendency towards significance in global cognitive performance (f) was found in ADNI cohort while in Rush-ROS/MAP, it was significant (g). *CYP2C19* expression levels were also different between non-carriers (-) and carriers(+) of the MA of rs4388808 (h) in ADNI cohort. All linear models were adjusted for age, sex, and APOE-e4 carriage status, with the exception of the models using global cognition and gene expression, which were adjusted for age, sex, and years of education or RNA integrity, respectively.

Figure 2-3 T-statistical parametric maps showing differences between non-carriers(-) and carriers(+) of the MA of rs4388808 (*CYP2C19*).



T-statistical parametric maps superimposed on an average structural MRI show brain regions with lower SUVR values in MA carriers (*CYP2C19* (+)) of the polymorphism in *CYP2C19*. Statistical differences overlap with brain regions vulnerable to AD pathophysiology, such as the posterior cingulate, frontal, and temporal cortices.

2.8 Acknowledgements

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2.9 Supplementary material

Gene	Chromosome	SNPs
CYP1A2	15	rs2472304
CYP3A4	7	rs3735451, rs2246709
CYP2C9	10	rs9332108, rs2253635, rs4086116, rs17443251, rs10509679, rs4918766, rs2153628, rs9332172, rs1856908, rs9332177, rs1934967, rs9332222, rs2298037, rs9332238, rs1057911
CYP2C19	10	rs12768009, rs7916649, rs4388808, rs7068577, rs17878673, rs1853205, rs10509678, rs10786172, rs11592737, rs4917623200
CYP2D6	22	No SNP passed QC

Supplementary Table 2-1. List of single nucleotide polymorphisms (SNP) used in the analy

Supplementary Table 2-2. Main results of the ANOVA analyses and their respective post-

hoc tests (when applicable) for the variables presented in Table 1.

	ADNI						Rush-ROS/MAP					
Variable	ANOVA p-value	Groups compared	Mean difference	Lower Bound	Upper Bound	Tukey HSD Adjusted p-value	ANOVA p-value	Groups compared	Mean difference	Lower Bound	Upper Bound	Tukey HSD Adjusted p-value
Age (years)	0.23	MCI-CN AD-CN AD-MCI	NA NA NA	NA NA NA	NA NA NA	NA NA NA	9x10 ⁻⁸	MCI-CN AD-CN AD-MCI	1.47 2.17 0.69	0.46 1.26 -0.34	2.48 3.07 1.73	0.001 1x10 ⁻⁷ 0.25
Education (years)	0.50	MCI-CN AD-CN AD-MCI	NA NA NA	NA NA NA	NA NA NA	NA NA NA	0.68	MCI-CN AD-CN AD-MCI	NA NA NA	NA NA NA	NA NA NA	NA NA NA
MMSE	2x10-16	MCI-CN AD-CN AD-MCI	-1.85 -8.75 -6.89	-2.41 -9.49 -7.69	-1.29 -8.00 -6.09	5x10 ⁻⁹ 5x10 ⁻⁹ 5x10 ⁻⁹	2x10-16	MCI-CN AD-CN AD-MCI	-0.77 -3.19 -2.42	-1.52 -3.86 -3.18	-0.03 -2.53 -1.65	5x10-9 5x10-9 5x10-9

NA = not applicable.

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Chapter 3 : Epistasis analysis links immune cascades and cerebral amyloidosis.

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3.1 Preface

In the previous chapter we found an association between brain amyloidosis and a genetic variant in the *CYP2C19* gene. This association did not differ between diagnostic groups and had a modest effect size, indicating that this variant would not be a valuable biomarker for amyloidosis or AD if used isolated. We thus experimented another methodological approach based on the fact that, in biological systems, the expression of a given gene affects the expression of others. This is the so-called epistasis and there is evidence that the information about genetic architecture of complex diseases, such as AD, is missing due to ignored epistatic effects ¹⁸⁸⁻¹⁹⁰. In data analysis, epistasis can be suggested if there is a statistical interaction between the predictive factors. Thus, we tested here if amyloid load could be inferred by interactions between genes from immune-related pathways.

3.2 Abstract

3.2.1 Background

Several lines of evidence suggest the involvement of neuroinflammatory changes in Alzheimer's (AD) pathophysiology such as amyloidosis and neurodegeneration. In fact, genome wide association studies (GWAS) have shown a link between few neuroinflammatory genes and Alzheimer's disease (AD). In order to further investigate whether interactions between candidate genetic variances coding for neuroinflammatory molecules are associated with brain A β fibrillary accumulation, we conducted an epistasis analysis on a pool of genes associated with molecular mediators of inflammation.

3.2.2 Methods

 $[^{18}\text{F}]$ Florbetapir positron emission tomography (PET) imaging was employed to assess brain A β levels in 417 participants from ADNI-GO/2 and posteriorly 174 from ADNI-1. IL-1 β , IL4, IL6, IL6r, IL10, IL12, IL18, C5, and C9 genes were chosen based on previous studies conducted in AD patients. Using the $[^{18}\text{F}]$ florbetapir standardized uptake value ratio (SUVR) as a quantitative measure of fibrillary A β , epistasis analyses were performed between two sets of markers of immune-related genes using gender, diagnosis, and apolipoprotein E (APOE) as covariates. Voxel-based analyses were also conducted. The results were corrected for multiple comparison tests. Cerebrospinal fluid (CSF) A β 1-42/phosphorylated tau (p-tau) ratio concentrations were used to confirm such associations.

3.2.3 Results

Epistasis analysis unveiled two significant SNP-SNP interactions (FDR threshold 0.1) being both between *C9* gene (rs261752) and *IL6r* gene (rs4240872, rs7514452). In a combined sample, the interactions were confirmed ($p \le 10-5$) and associated with amyloid accumulation within HC and AD spectrum groups. Voxel-based analysis corroborated initial findings. CSF biomarker (A β_{1-42} /p-tau) confirmed the genetic interaction. Additionally, rs4240872 and rs7514452 SNPs are shown to be associated with CSF and plasma concentrations of IL6r protein.

3.2.4 Conclusion

Certain allele combinations involving *IL6r* and *C9* genes are associated with $A\beta$ burden in the brain. Hypothesis driven search for epistasis is a valuable strategy for investigating imaging endophenotypes in complex neurodegenerative diseases.

3.3 Background

Alzheimer's disease (AD) is the most common form of dementia worldwide and has been recently reconceptualized as a dynamic and progressive process in which pathological changes start decades prior to the onset of clinical symptoms (1, 2). According to the amyloid cascade hypothesis (3), the accumulation of brain amyloid- β (A β) sets a cascade of progressive neurodegenerative changes—including the formation of intracellular inclusion of neurofibrillary tangles (NFTs)—resulting in cognitive impairment and, ultimately, dementia. Imaging and cerebrospinal fluid (CSF) biomarkers have successfully advanced our knowledge

in terms of the evolution of AD (1). However, the most recent hypothetical model of AD biomarkers (4) has not explored the role of neuroinflammation, a phenomenon implicated in the pathogenesis of AD by several lines of evidence (5-7).

Previous studies have shown important interactions between immune responses and brain amyloidosis (8), with both *in vitro* and *in vivo* studies demonstrating altered cytokine expression in AD. In addition, neuroinflammation secondary to systemic infections, traumatic brain injuries or other neurologic conditions have been shown to increase the risk of sporadic AD (9, 10).

Currently, it is widely accepted that $A\beta$ triggers innate immunity pathways—as well as molecular mediators such as cytokines, chemokines and complement molecules—leading to neuroinflammation and disturbance in brain homeostasis. However, it is not known whether the activation of the immune system secondary to brain amyloidosis is harmful or protective in nature. In fact, it has been hypothesized that impaired immune response either fails to clear $A\beta$ from the brain or drives an overreaction against this protein, resulting in chronic inflammation and neurodegeneration.

Endophenotypes associated with variations in immune-related genes particularly related to AD neuropathological features remains elusive. Genome-Wide Association Studies (GWAS) and meta-analysis have found immunogenetic variants associated to AD, namely *CR1*, *CLU*, *TREM2*, *PICALM*, *CD33*, *MEF2C*, reasserting the role of the immune system in AD pathophysiology (11-15). However, recent investigations did not reveal a link between brain amyloidosis and immunologic genetic variants (16, 17), suggesting that some endophenotypes might be affected by gene-to-gene interactions or epistasis.

In multifactorial diseases such as AD, the power to detect isolated genetic variants can be reduced due to epistatic effects, which occurs when one locus masks or alters the effect of another (18-20). In this respect, approaches moving beyond single marker outcomes may better capture heritability links (21).

In this study we aimed to investigate the interactions between immune related genes primarily molecular mediators of inflammation—and the accumulation of A β *in vivo*, as quantitated by amyloid imaging with positron emission tomography (PET). We hypothesize that differential amyloid burden is associated with the deregulation of innate immunity response, which could be evidenced by epistasis analysis of genes that encode for immune proteins reported to be related to AD.

3.4 Results

3.4.1 Epistasis analysis indicates that interaction between *C9* and *IL6R* genes is associated with brain amyloid deposition.

After applying the quality control steps as previously described, one pair of subjects was found to be genetically related. One of the subjects was thus randomly selected and excluded from the study. No difference was seen in the Q-Q plots for SNPs as main effects, before and after adjustment for the first two principal components (data not shown). This suggests that our sample is genetically homogeneous. Epistasis analysis unveiled two significant SNP-SNP interactions after FDR correction (FDR threshold p=0.1; see Table 2 and Supplementary Table

The most significant interaction was between the SNP rs261752 of the *Complement 9* (*C9*) gene and the SNP rs7514452 annotated to the *Interleukin 6 receptor* (*IL6r*) gene (t=3.92, unadjusted $p = 1.0 \times 10^{-4}$). This interaction showed a trend level association in the ADNI-1 group (t=1.82, unadjusted p=0.06) and a very significant association in the combined dataset (t=4.42, unadjusted $p = 1.1 \times 10^{-5}$). A second interaction was noted between this *C9* SNP and another SNP in *IL6r* (rs4240872) (t= 3.73, unadjusted $p = 2.1 \times 10^{-4}$). Similar to the first interaction, this association was found to be significant only in the combined sample (t=4.15, unadjusted $p = 3.7 \times 10^{-5}$; ADNI-1, t=1.85, unadjusted p=0.06).

Group comparison between genotypes showed similar results for the two interactions reported. The *IL6r* and *C9* SNP interaction showed that, despite being not AD and having only one subject *ApoE* $\varepsilon 4$ positive, subjects homozygous for both minor alleles (CC(*C9*)*CC(*IL6r*)) have higher mean SUVR values when compared to almost all other genotype combinations (see Figure 1). In contrast, homozygosity for the minor allele of *C9* SNP and major allele of *IL6r* SNP seems to exert a protective effect when compared to the absence of both minor alleles (for Post-hoc results, see Supplementary Table 3).

In order to assess whether the interaction was specific to individuals in the AD spectrum, we stratified the individuals in HC and AD spectrum (MCI and dementia phase). Both groups presented similar results than those obtained using the entire sample (see Table 3).

3.4.2 Voxel-based analysis revealed that the epistasis is related to amyloid deposition in AD-related brain regions.

The voxel-based analysis showed that the interaction between C9 and IL6r SNPs is associated with amyloid load in the anterior and posterior cingulate, temporal and inferior parietal cortices bilaterally (see Figure 2). Additionally, voxel-wise comparisons revealed that homozygous subjects for both minor alleles when compared to either carriers of the genotype CC(C9)*TT(IL6r) or the genotype TT(C9)*TC(IL6r) have more amyloid load in the brain regions mentioned above. These differences are corrected for multiple comparisons (at 0.05 level).

3.4.3 CSF biomarkers of AD neurodegeneration replicated the results obtained using [¹⁸F]florbetapir SUVR.

A subsample of 208 subjects (85 HC, 113 MCI and 10 AD) who had baseline CSF measures was used to confirm the interaction model. The ratio $A\beta_{1-42}/p$ -tau was used as a dependent variable (HC average= 7.73, MCI average= 6.05, AD average= 2.76; difference between all groups statistically significant p= 5.3×10^{-5}). The interaction analysis was replicated in the tested pair of SNPs (rs261752*rs7514452 t=-2.82, p= 0.005) (Supplementary Table 4). Due to sample size restrictions the group comparison could not be performed (Figure 3).

3.4.4 Plasma and CSF levels of IL6R protein were associated with the genetic polymorphisms.

The *IL6r* genotypes of both SNPs were associated with plasmatic levels of IL6r protein (rs7514452 t=-2.42, unadjusted p= 0.01; rs4240872 t=-2.94, unadjusted p= 0.003), which shows individuals carriers of the minor alleles having a lower level of the protein compared individuals non-carriers. Due to the sample size it was not possible to verify if the same effect is present within diagnostic groups, therefore it was used as covariate in the analysis. Similarly, CSF levels of IL6r protein were associated with rs4240872 (t=-3.17, unadjusted p= 0.002) but not with rs7514452 (t=-1.52, unadjusted p= 0.12). Unfortunately, there is no data available to date reflecting plasmatic levels of C9 protein that would permit us to do the correspondent analysis with the SNP rs261752.

3.5 Discussion

In the present study two interactions between two immune-related genes, *C9* and *IL6r*, were found to be associated with [¹⁸F]florbetapir SUVRs. This result suggests that A β burden in the brain may be differentially affected depending on the allelic combination of the cited variants.

The SNP rs261752 is an intronic variation of the *C9* gene, with no previously reported association to any phenotypic feature or neurodegenerative endophenotype. However, it has been associated with age-related macular degeneration, a disorder highly frequent amongst AD patients (22, 23). Moreover, several studies have described increased immunoreactivity of classical complement molecules, including C9, in the vicinity of brain Aβ aggregates (24-26).

C9 protein is also a component of the MAC, which is responsible for disrupting cellular homeostasis, causing cell death following activation of the complement pathway (27). Indeed, it is well known that extracellular A β triggers the complement cascade leading to MAC formation (25, 28, 29). Since MAC requires a lipidic bilayer structure to act upon, it binds to the surrounding neurites (29, 30), leading to neurodegeneration and cell death. Further, the protein clusterin, encoded by the AD-related CLU gene, has been shown to play an important role in reducing inappropriate MAC activity tied to physical interaction with the C9 protein (31).

The two SNPs from the *IL6r* gene are more than 1800 bp apart from each other ($r^2=0.69$), and, despite not being in high linkage disequilibrium, might reflect the same signal. The SNP rs4240872 is an intronic variant of the *IL6r* gene while the variant rs7514452 is located in the 3' untranslated region (3'-UTR), an important sequence at the end of the mRNA known to affect post-translational regulation and subsequent protein expression (32). A previous study suggests a possible association between 3'-UTR markers and diabetes mellitus type 2 (33), an association of possible relevance owing to evidence showing that insulin signalling is downregulated in AD (for review, see (34). Additionally, Waltson et al (35) reported that some IL6r SNPs are associated with plasmatic levels of interleukin 6 (IL6), a cytokine that plays an important role in the regulation of neuroimmune responses, promoting both pro-inflammatory and anti-inflammatory effects (36-38). Similar results were reported here showing that CSF levels of IL6r were associated with one IL6r SNP while plasmatic levels were associated with both SNPs (rs7514452 and rs4240872) in ADNI-1 subsample, reflecting a genotype-phenotype effect. The IL6r protein is either part of ligand-binding receptor of IL6 or a soluble form (s-IL6r), which binds to IL6 to enhance its activity (39, 40). Deregulation of immune response signalling in AD is evidenced by altered protein expression in the brain (41, 42). Differences

in cerebrospinal fluid (CSF) and serum levels of both IL6 and s-IL6r are also evident when comparing AD patients to HCs (43-45).

Voxel based findings revealed by this study further corroborated global increases of amyloid load in regions typically affected by AD pathophysiology. Homozygous subjects for minor alleles of both *IL6r* and *C9* genes show higher levels of amyloid in brain areas that correspond to regions impaired in AD (46). Interestingly amyloid plaques depicted by amyloid imaging agents are typically surrounded by neuroinflammatory changes such as astrocytosis and microglial activation (for review see (47), reinforcing a link between amyloidosis and immune response. Additionally, one could claim that a reduction in the IL6r levels causes decreases in the IL6 activity, contributing to A β accumulation through different possible mechanisms.

In agreement with [¹⁸F]florbetapir findings, the interaction between *C9* and *IL6r* genes were also associated with CSF A β_{1-42} /p-tau ratio. This finding based on an independent measurement of brain amyloidosis, provides additional evidence that *C9* and *IL6r interactions* affects brain accumulation of neuritic plaques in a disease specific manner. (48). However, it is important to take into consideration reduced sample size present in the cerebrospinal fluid population.

Based on our results, it seems plausible that a combination of gene polymorphisms in complement factors and interleukins play a synergic role in determining amyloid burden in the brain. Specifically, a particular combination of genotypes that up-regulate both C9 and IL6r may exert an additive effect via neuroinflammatory processes. Besides the supposition of how these SNPs may jointly affect amyloid accumulation in the brain, no relationship between these two genes or proteins has been reported to date with respect to amyloid metabolism. However, it has been shown that the protein IL6 is able to stimulate C9 mRNA expression in post-mortem

human astrocytes and neuroblastoma cells (49, 50), showing a metabolic link between the two proteins in nervous system cells.

In order to overcome the well-known limitations of association studies, several assumptions need to be addressed. For example, although all the cited proteins are related to the immune system, their role in A β accumulation remains unclear. Presently, the function of the reported SNPs remains elusive due to the lack of relevant literature. Regarding the association found between IL6r levels and *IL6r* SNPs, linking the genotype with the phenotype, it is important to mention that: (51) protein levels were measured in average 55 months prior to [¹⁸F]florbetapir image acquisition; (2) there was no association between the use of anti-inflammatory drugs and IL6r levels in this sample; (3) beyond the effect that *IL6r* SNPs can have at the protein level, is very important to know the effect of the *C9* genotypes on C9 protein to better understand how they jointly impact the immune response.

In addition to the limitations of the study, the ADNI cohort is a sample mostly composed of non-Hispanic Caucasians, limiting the extrapolation of the present findings to other population groups. A wider range of subjects varying in terms of ethnicity, family history and disease progression should be considered for future replication of this study. Moreover, currently it is though that the A β oligomers (soluble forms) are the most synaptotoxic (for review see (52) and the most chased by the immune system, however, it is not possible to detect these forms *in vivo* using brain imaging; [¹⁸F]florbetapir is only able to bind amyloid plaques. Recently, MRI probes for targeting A β oligomers have been developed, and will likely provide further information regarding the association between A β oligomers and the immune system (53). In fact, more studies are needed to address the biological mechanisms in which gene interactions may affect the phenotype, using both amyloid plaque and A β oligomer quantifications.

It is also important to mention that statistical analyses between genetic factors do not define their biological interactions or interferences (19), necessitating more investigation. Though ADNI-1 data was used to confirm significant associations, the reduced sample size could have been a limiting factor with respect to the achievement of statistical significance. Based on the effect size of the interactions found in the first analysis with ADNI-GO/2 data (data not shown), the sample size required to reach 95% of power and a type I error of 0.05 is 497 subjects. For this reason, a less strict FDR correction was adopted in the first step of the analysis. Sample size requirements might also explain why it was not possible to fully replicate the results using data from ADNI-1, while results were replicated in the combined sample – the p-values obtained for the interactions in the combined sample would be significant at 0.05 level if corrections for multiple comparisons had to be applied.

In conclusion, using a clinically well-characterized and genetically homogenous sample, as well as a confirmatory imaging analysis, our hypothesis driven analyses identified several epistatic links between *IL6r* and *C9* genes, suggesting genetic components linking immune system and brain amyloidosis. Though further studies are required, these results suggest that these interacting genotypes may represent potential biomarkers for differential treatment of the disease.

3.6 Methods

3.6.1 Research Subjects

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial MRI, PET, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment and early Alzheimer's disease.

This report is based on data acquired from 417 participants from ADNI-GO/2 and 174 from ADNI-1, for whom both genetic and PET data were available. Demographic data is summarized in table 1.

3.6.2 PET methods

Amyloid load was estimated using [¹⁸F]florbetapir PET standardized uptake value ratio (SUVR). A detailed description of the [¹⁸F]florbetapir imaging acquisition protocol can be found online at the ADNI website. PET image processing and estimation of global SUVRs have been described previously (54). All image processing, including generation of regions of interest, is summarized in Supplementary Figure 1.

3.6.3 Gene selection

We have chosen to verify possible interactions between the main interleukins (IL) reported to be associated with AD pathology and proteins of the Membrane Attack Complex (MAC). Selected MAC key proteins include Complement 5 (C5) and Complement 9 (C9). They are respectively the first and last proteins to be activated in the MAC cascade and both are also found to be associated with amyloid plaques in AD brain (24, 29). The interleukins selected were the most frequently reported to be related to AD (29, 55-58) IL1β, IL6 (and its receptor IL6r), IL12 and IL18 have shown to be differentially expressed in AD brain when compared to controls. These pro-inflammatory cytokines display increased expression in AD brains and/or are associated with amyloid plaques (56, 59-61). They also seem to reduce AD-like phenotype when are inhibited in animal models (62, 63). IL4 and IL10 have anti-inflammatory properties and they all have been associated with AD, either by *in vitro* studies, genetic studies or biochemical analysis of plasma, CSF and/or AD brains (55, 64).

3.6.4 Genetic Analysis

The ADNI-GO/2 subjects were genotyped using the Illumina OmniQuad (Illumina, Inc., San Diego, CA) array (65) while for ADNI-1 subjects correspondent genotypes were obtained from HumanOmni2.5 BeadChip (Illumina, Inc., San Diego, CA). Quality control was performed using PLINK software (version 1.07) (66) excluding Single Nucleotide Polymorphisms (SNPs) with a genotyping efficiency <95%, a minor allele frequency of <5%, or deviation from Hardy-Weinberg equilibrium (67) <1 x10⁻⁶. Subjects were excluded if they had a call rate <95% and if genetic relatedness was detected (PI_HAT > 0.5). Population stratification was accounted for by subtracting the effect of the first two principal components using the Eigenstrat routine (68) in the Eigensoft V5.0 package (69) and posterior visualization of the Q-Q plots.

For the epistasis analysis we selected 10 genes related to molecular mediators of inflammation as mentioned above. These genes were grouped into two sets according to their protein function, being the set 1 composed by interleukins and one interleukin receptor (*IL-1* β , *IL4*, *IL6*, *IL6r*, *IL10*, *IL12*, *IL18*) and the set 2 by proteins of the MAC (*C5*, *C9*). Using the UCSC Genome Browser (http://genome.ucsc.edu) we annotated the start and end points of each selected gene and all SNPs present within this region were obtained with PLINK. SNAP Proxy Search (version 2.2) (70) was used to verify and then remove markers in high linkage disequilibrium ($r^2 > 0.8$). The remaining SNPs were grouped together within the respective set (31 SNPs in set 1 and 21 in set 2) as summarized in Supplementary Table 1. Epistasis analysis was performed using R (71).

3.6.5 Cerebrospinal Fluid concentrations of phosphorylated tau and Amyloid- β_{142}

Baseline Cerebrospinal Fluid (CSF) Amyloid- β_{1-42} (A β_{1-42}) and phosphorylated tau (p-tau) were measured using the multiplex xMAP Luminex platform (Luminex Corp, Austin, TX) and Innogenetics/Fujirebio AlzBio3 immunoassay kits. The methodology applied for aliquot collection, peptide quantification, as well as quality control and data normalization are described in previous reports (72, 73). The normalized data was used to calculate the ratio A β_{1-42} /p-tau, which is the phenotype used to confirm findings obtained with [¹⁸F]florbetapir.

3.6.6 Cerebrospinal Fluid and Plasmatic Protein Levels

The Biomarkers Consortium CSF and Plasma Proteomics Project multiplex data is available for ADNI-1 subjects for whom protein levels were measured at the baseline for both biospecimens, and 12 months follow-up visit only in the plasma. The description of the methodology regarding the sample acquisition, sample processing and analysis, as well as quality control procedures is available at ADNI website (http://adni.loni.usc.edu/methods/biomarker-analysis/proteomic-analysis and http://adni.loni.usc.edu/data-samples/biospecimen-data/).

3.6.7 Statistical Analysis

The epistasis analysis was performed with R, in which a linear model was used to test the interaction between two SNPs in a given pair. Each SNP pair was composed of one SNP from each set, resulting in 651 pairs tested. Subject's genotypes were acquired using PLINK and categorized based on minor allele counts (additive model). The quantitative trait analyzed was the [¹⁸F]florbetapir SUVR. In the model, diagnostic status (AD, Mild Cognitively Impaired (MCI) or healthy control (HC)), gender and number of Apolipoprotein E allele 4 (*ApoE c4*) were added as covariates. False Discovery Rate (FDR) was used to correct for multiple comparisons. The significant interactions (after FDR correction at 0.1 level) found with the ADNI-GO/2 sample were tested for replication using both the ADNI-I sample and the combined dataset (ADNI-1 and ADNI-GO/2. The comparison of [¹⁸F]florbetapir SUVR means between genotype groups was performed using the combined dataset. Tukey's HSD test was used in the post-hoc analysis. The significant interactions were also tested within the two groups obtained from the combined dataset: HC and AD spectrum (MCI and Alzheimer dementia patients). In these late comparisons, the model applied followed the same criteria described for the analysis with the whole sample.

Voxel-based analysis was carried out to confirm volume of interest analysis. Parametric images were obtained using methodology summarized in Supplementary Figure 1. First, the model was tested for the most significant interaction. Then, we compared the groups participating in the two most significant contrasts found in Tukey's honest significant difference (HSD) test applied in the global SUVR analysis. Voxel based statistical differences were obtained by contrasting [¹⁸F]florbetapir SUVR between genotype groups, adjusting for gender, diagnostic status, and *ApoE* ε 4 using the RMINC imaging tool. RMINC is an imaging

package that allows images files in the Medical Imaging NetCDF (MINC) to be analyzed with the powerful statistical environment R. After Random Field Theory (RFT) (74) correction for multiple comparisons, the T value threshold of significance is ≥ 3.0 (p ≤ 0.05) for the interaction model and ≥ 3.2 (p ≤ 0.05) for the group comparison.

To confirm the association found with [18F]florbetapir phenotype, the most significant interacting pair of SNPs was tested using the baseline ratio $A\beta_{1-42}/p$ -tau as the dependent variable, which was available for a subsample of 208 subjects. The model applied had diagnosis, gender and number of *ApoE* $\varepsilon 4$ as covariates.

The effect of the polymorphisms on their respective protein levels in the plasma was tested in ADNI-1 subjects who had available proteomic data (n=114 subjects). A Linear Mixed-Effects Models –adjusted for diagnosis and gender– was applied to analyse the effect of the genotype in both baseline and 12 months follow-up data. The genotypes were categorized according to the presence or absence of the minor allele. An additional linear model tested associations between the polymorphisms and the CSF concentrations of their respective proteins (n=81 subjects).

3.7 Disclosure statement

The authors declare no competing financial interests.

3.8 Tables and Figures

Table 3-1 Demographic and key characteristics of the sample at baseline.

		ADNI-GO/2			ADNI-1			COMBINED	
							DATASET		
	Cognitively Normal (CN)	Mild Cognitive Impairment (MCI)	Alzheimer's Disease (AD)	Cognitively Normal (CN)	Mild Cognitive Impairment (MCI)	Alzheimer's Disease (AD)	Cognitively Normal (CN)	Mild Cognitive Impairment (MCI)	Alzheimer's Disease (AD)
Number of subjects (%)	123 (29.6)	266 (64.9)	27 (6.5)	73 (42)	58 (33.3)	43 (24.7)	196 (33.2)	324 (54.9)	70 (11.9)
Number males (%)	62 (50.4)	146 (54.9)	17 (63.0)	36 (49.3)	38 (65.5)	25 (58.1)	98 (50.0)	184 (56.8)	42 (60.0)
Number APOE-e4 carriers	s 32 (26.0) [*]	118 (44.4)*	17 (63.0)*	19 (26)	21 (36.2)	27 (62.8)**	51 (26.0)*	139 (42.9)*	44 (62.9)*
(%)									
Mean age (SD)	74.37 (5.62)	71.45 (7.61)**	75.56 (10.67)	80.73 (4.72)	79.64 (7.42)	76.47 (6.24)**	76.74 (6.12)	72.92 (8.19)**	76.12 (8.17)
Mean years of education	16.50 (2.64)	16.00 (2.55)	16.41 (2.32)	15.85 (2.92)	15.38 (3.17)	16.21 (2.66)	16.26 (2.76)	15.89 (2.67)	16.23 (2.49)
(SD)									
Mean CDR-SOB (SD)	$0.03 (0.14)^*$	1.35 (0.86)*	4.70 (1.20)*	$0.26(0.8)^{*}$	1.73 (1.43)*	5.63 (3.28) [*]	0.12 (0.51)*	1.42 (0.99)*	5.26 (2.69)*
Mean MMSE (SD)	29.05 (1.16)*	28.23 (1.62)*	22.52 (1.88)*	29.03 (1.3)*	27.69 (1.88)*	$21.44(5.0)^*$	29.04 (1.21)*	28.13 (1.68)*	21.87 (4.13)*
Mean [¹⁸ F]florbetapir SUVR (SD)	1.24 (0.20)*	1.31 (0.23)*	1.51 (0.24)*	1.15 (0.13)*	1.25 (0.18)*	1.40 (0.18)*	1.20 (0.19)*	1.30 (0.23)*	1.44 (0.21)*

SD – Standard Deviation. The baseline considered here is the date of the first [¹⁸F]florbetapir PET acquisition. Clinical Dementia Rating Scale Sum of Boxes (CDR-SOB).

MMSE (Mini-Mental State Examination).

SUVR (Standard Uptake Value Ratio).

* Statistically different from the other groups from the same sample.

Table 3-2 SNP-SNP interaction information

ADNI-GO/2				ADNI-1 COMBINED DATASET					
Gene Interactions	SNP Interactions	Minor Allele	MAF ^a	P value	P value adjusted ^b	MAF ^a	P value	MAF ^a	P value
C9* IL6r	rs261752 * rs7514452	C C	0.44 0.18	1.0 x 10 ⁻⁴	0.06	0.43 0.19	0.06	0.44 0.20	1.1 x 10 ⁻⁵
C9* IL6r	rs261752 * rs4240872	C C	0.44 0.24	2.1 x 10 ⁻⁴	0.07	0.43 0.25	0.06	0.44 0.24	3.7 x 10 ⁻⁵

^a MAF – minor allele frequency.

^b FDR- corrected *P* value (threshold 0.1)

Table 3-3 Interactions tested within diagnostic groups in the combined dataset.

		Cognitively Normal (CN)			AD spectrum (MCI + AD)		
Gene Interactions	SNP Interactions	β value	P value	MAF ^a	β value	P value	MAF ^a
C9* IL6r	rs261752 * rs7514452	0.07	0.02	0.42 0.20	0.10	1.2 x 10 ⁻⁴	0.45 0.20
C9* IL6r	rs261752 * rs4240872	0.07	0.02	0.42 0.25	0.09	3.3 x 10 ⁻⁴	0.45 0.24

^a MAF – minor allele frequency.



Figure 3-1 Interaction between C9 and IL6r genes

a) Representation of the SNP rs261752 (*C9* gene) in chromosome 5 and SNP rs7514452 (*IL6r* gene) in chromosome 1. b) *C9* x *IL6r* (rs7514452) on amyloid deposition. The interaction between *C9* and *IL6r* genes is associated with amyloid burden. Error bars represent the standard error. Small numbers represent the subsample size for each genotype combination. *The mean SUVR for the assigned genotypes are different between each other [p = 0.05 (2-tailed)]. **The mean SUVR for this genotype is higher than all other genotypes [p values ≤ 0.05 (2-tailed)]. P-values are adjusted according to Tukey's HSD test. c) Representation of the SNP rs261752 (*C9* gene) in chromosome 5 and SNP (rs4240872) (*IL6r* gene) in chromosome 1. d) *C9* x *IL6r* (rs4240872) on amyloid deposition. The interaction between *C9* and *IL6r* genes is associated with amyloid burden. Error bars represent the subsample size for each genotypes are different between each other [p = 0.05 (2-tailed)]. **The mean SUVR for the assigned genotypes are different between each other [p = 0.04 (2-tailed)]. **The mean SUVR for the assigned genotypes are different between each other [p = 0.04 (2-tailed)]. **The mean SUVR for the genotype CC(*C9*)*CC(*IL6r*) is higher then all other assigned genotypes [p values ≤ 0.05 (2-tailed)]. P-values are adjusted according to Tukey's HSD test.



Figure 3-2 T-maps of contrasts between genotypes.

a) T-statistical parametric maps (SPM) superimposed on a average structural MRI shows

brain regions with high SUVR values in carriers of the genetic interaction between the SNPs rs261752 (*C9* gene) and rs7514452 (*IL6r* gene). Statistical differences overlap with brain regions vulnerable to AD pathophysiology. b) Statistical parametric mapping (SPM) maps superimposed on an average structural MRI shows the t-statistical contrast [CC(C9)*CC(IL6r) > CC(C9)*TT(IL6r)]. Carriers of CC(C9)*CC(IL6r) have higher [¹⁸F]florbetapir SUVR in the frontal, parietal and temporal cortices. c) SPM maps superimposed on an structural MRI shows the t-statistical contrast [CC(C9)*CC(IL6r) > TT(C9)*CC(IL6r)]. CC(C9)*CC(IL6r) carriers have high [¹⁸F]florbetapir SUVR in brain regions typically affected by amyloidosis in Alzheimer's disease. The analyses were adjusted for gender, diagnostic status and *ApoE* $\varepsilon 4$. The T value threshold of significance after RFT correction is ≥ 3.2 (p ≤ 0.05).

Figure 3-3 Interaction between C9 and IL6r genes using CSF Aβ1-42/p-tau ratio.



The interaction between *C9* and *IL6r* genes is associated with amyloid burden. Error bars represent the standard error. Small numbers represent the subsample size for each genotype combination.

3.9 Supplementary material

Set	Gene	SNP
	IL-1β	rs1071676, rs1143634, rs1143633, rs3136558
-	IL4	rs2243268, rs2227282
Set 1	IL6	rs2069832, rs2069835, rs2069837, rs2066992
-	IL6r	rs4845617, rs1386821, rs4845618, rs8192282, rs4537545, rs11265618, rs4240872, rs7514452
-	IL10	rs3024496
-	IL12	rs2243123, rs568408, rs3212227, rs11574790, rs2853694, rs2569254, rs1003199, rs2546893, rs730691
-	IL18	rs5744256, rs2043055, rs360717
	С5	rs41258306, rs7045519, rs17612, rs7026551, rs2269066, rs4837805, rs10760135, rs17611, rs7027797, rs10985126, rs17220750, rs7031128, rs1468673

Supplementary Table 3-1. List of SNPs within each set.

C9

rs7514452 rs261752 0.0001027 0.06 rs4240872 rs261752 0.0002157 0.07 rs2069837 rs17220750 0.0009282 0.20 rs2243268 rs7026551 0.0019076 0.3 rs2243268 rs7027797 0.0049702 0.57 rs4845618 rs41258306 0.0079221 0.57 rs4845618 rs17612 0.0080400 0.57	e adj ^d
rs7514452rs2617520.00010270.06rs4240872rs2617520.00021570.07rs2069837rs172207500.00092820.20rs2243268rs70265510.00190760.3rs2243268rs70277970.00497020.57rs4845618rs412583060.00792210.57rs4845618rs176120.00804000.57	
rs4240872rs2617520.00021570.07rs2069837rs172207500.00092820.20rs2243268rs70265510.00190760.3rs2243268rs70277970.00497020.57rs4845618rs412583060.00792210.57rs4845618rs176120.00804000.57	69045*
rs2069837rs172207500.00092820.20rs2243268rs70265510.00190760.3rs2243268rs70277970.00497020.5'rs4845618rs412583060.00792210.5'rs4845618rs176120.00804000.5'	02403^{*}
rs2243268rs70265510.00190760.3rs2243268rs70277970.00497020.5'rs4845618rs412583060.00792210.5'rs4845618rs176120.00804000.5'rs5744256	014374
rs2243268 rs7027797 0.0049702 0.57 rs4845618 rs41258306 0.0079221 0.57 rs4845618 rs17612 0.0080400 0.57	104708
rs4845618 rs41258306 0.0079221 0.5' rs4845618 rs17612 0.0080400 0.5'	752360
rs4845618 rs17612 0.0080400 0.5 ⁴	752360
	752360
rs5/44256 rs835218 0.0081852 0.5	752360
rs8192282 rs263275 0.0083114 0.5'	752360
rs11574790 rs261752 0.0108487 0.5	752360
rs7514452 rs696758 0.0114193 0.5 ⁴	752360
rs11265618 rs263275 0.0137167 0.5'	752360
rs3136558 rs2269066 0.0138882 0.5'	752360
rs2243268 rs10985126 0.0147935 0.5	752360
rs2227282 rs263275 0.0160563 0.5 ⁴	752360

Supplementary Table 3-2. R epistasis results (firsts 15 lines).

^a SNP1 First SNP from set 1

^b SNP2 Second SNP from set 2

^c P value P value from R epistasis analysis ^d P value adj. FDR corrected P value ^{*} Interactions that reached significance after FDR correction at 0.1 level.

Supplementary Table 3-3. Tukey's HSD test results.

Contrasted	l genotypes	Contrasted genotypes					
<i>C9*IL6r</i> (rs7514452)	<i>C9* IL6r</i> (rs7514452)	P value	<i>C9*IL6r</i> (rs4240872)	<i>C9*IL6r</i> (rs4240872)	P value		
TT*TC	TT*TT	0.17	TT*TC	TT*TT	0.13		
TT*CC	TT*TT	0.85	TT*CC	TT*TT	0.55		
TC*TT	TT*TT	0.69	TC*TT	TT*TT	0.79		
TC*TC	TT*TT	0.55	TC*TC	TT*TT	0.16		
TC*CC	TT*TT	0.96	TC*CC	TT*TT	0.61		
CC*TT	TT*TT	0.05	CC*TT	TT*TT	0.04		
CC*TC	TT*TT	0.99	CC*TC	TT*TT	0.92		
CC*CC	TT*TT	0.03	CC*CC	TT*TT	0.24		
TT*CC	TT*TC	1.00	TT*CC	TT*TC	0.99		

TC*TT	TT*TC	0.86	TC*TT	TT*TC	0.79
TC*TC	TT*TC	0.99	TC*TC	TT*TC	1.00
TC*CC	TT*TC	1.00	TC*CC	TT*TC	1.00
CC*TT	TT*TC	1.00	CC*TT	TT*TC	1.00
CC*TC	TT*TC	0.83	CC*TC	TT*TC	0.98
CC*CC	TT*TC	0.001	CC*CC	TT*TC	0.01
TC*CC	TT*CC	0.99	TC*CC	TT*CC	0.90
TC*TC	TT*CC	0.99	TC*TC	TT*CC	0.99
TC*CC	TT*CC	0.99	TC*CC	TT*CC	1.00
CC*TT	TT*CC	1.00	CC*TT	TT*CC	1.00
CC*TC	TT*CC	0.96	CC*TC	TT*CC	0.96
CC*CC	TT*CC	0.01	CC*CC	TT*CC	0.03
TC*TC	TC*TT	0.99	TC*TC	TC*TT	0.90
TC*CC	TC*TT	0.99	TC*CC	TC*TT	0.96
CC*TT	TC*TT	0.65	CC*TT	TC*TT	0.48
CC*TC	TC*TT	0.99	CC*TC	TC*TT	1.00
CC*CC	TC*TT	0.006	CC*CC	TC*TT	0.06
TC*CC	TC*TC	1.00	TC*CC	TC*TC	1.00
CC*TT	TC*TC	0.97	CC*TT	TC*TC	0.99
CC*TC	TC*TC	0.99	CC*TC	TC*TC	0.99
CC*CC	TC*TC	0.004	CC*CC	TC*TC	0.02
CC*TT	TC*CC	1.00	CC*TT	TC*CC	1.00
CC*TC	TC*CC	0.99	CC*TC	TC*CC	0.99
CC*CC	TC*CC	0.01	CC*CC	TC*CC	0.03
CC*TC	CC*TT	0.72	CC*TC	CC*TT	0.90
CC*CC	CC*TT	0.001	CC*CC	CC*TT	0.009
CC*CC	CC*TC	0.03	CC*CC	CC*TC	0.07

P values ≤ 0.05 are highlighted in bold.

Supplementary Table 3-4. Interaction tested using $A\beta_{1-42}/p$ -tau ratio as phenotype.

Gene Interactions	SNP Interactions	MAF ^a	β value	P value
C9*IL6r	rs261752 * rs7514452	0.46/0.18	-1.98	5.2 x 10 ⁻³

^a MAF – minor allele frequency.

Supplementary Figure 3-1. [¹⁸F]florbetapir SUVR analytical method.



Flowchart showing acquisition methods (purple), image processing (blue) and outcomes (green). PET= positron emission tomography, MRI= magnetic resonance imaging, GM = grey matter, WM = white matter, CSF = cerebrospinal fluid, FWHM = first width half maximum, ROI= region of interest.

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Chapter 4 : Stage-specific associations between Plasma NfL and biomarkers of AD pathophysiology

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4.1 Preface

In the previous studies we detected new genetic variants associated with amyloid clearance pathways. However, these variants showed modest effect on amyloid accumulation and cannot be used in isolation as biomarkers. Because of this limitation, we decided to use another approach to identify an early biomarker of amyloidosis. We opted for combining information of different types of biomarkers, such as genetic and blood. The blood biomarker explored here, plasma Neurofilament light protein, was associated with brain amyloid deposition in a non-targeted proteomic study that investigated both cognitively impaired and unimpaired participants ²⁰⁷. Thus, in this study we evaluated the association of plasma NfL with amyloid

PET as well as with other imaging biomarkers and considered the *APOE-e4* status as the genetic biomarker.

4.2 Abstract

4.2.1 Background

Neurofilament light (NfL) is a marker of neuroaxonal injury and has been shown to correlate with CSF levels of both amyloid- β and tau. Studies addressing associations between plasma NfL and imaging markers are few however, and have been confined to pre-defined regions of interest and a global index of white matter change. Moreover, no studies have looked at the association between plasma NfL and tau PET.

4.2.2 Methods

In this study, we examined the associations between plasma NfL concentration and both structural MRI (whole brain voxel-wise measures of gray and white matter volume) and PET based molecular imaging (amyloid- β and tau) measures in two cohorts (ADNI and TRIAD) comprising cognitively unimpaired (CU) older participants (n=382) and in participants with cognitive impairment (CI, n=776: mild cognitive impairment and dementia due to AD). Longitudinal plasma NfL was measured using an in-house SIMOA method in patients with MRI (longitudinal) and PET (cross-sectional) imaging (amyloid- β and tau). Linear regression was implemented voxel-wise in order to examine the association between plasma NfL and imaging measures.

4.2.3 Findings

As compared to CU and CI participants, the rate of change in plasma NfL was greater among AD patients. In both cohorts, plasma NfL associations were confined to amyloid- β PET in CU participants and tau PET in CI participants; associations were found in AD related brain regions. Volumetric analysis of MRI data showed that NfL concentration was associated with both gray and white matter volume loss, with gray matter atrophy among CU participants specific to *APOE*- ϵ 4 carriers.

4.2.4 Interpretation

These findings suggest that plasma NfL may prove an early marker of amyloid- β -related neuronal injury in AD, being in turn more closely related to tau-mediated neurodegeneration during the symptomatic course of the disease.

4.3 Introduction

Alzheimer's disease (AD) is characterized by the accumulation of extracellular amyloid- β (A β) plaques and intracellular tau aggregates (*I*). These pathological hallmarks are thought to result in neurodegenerative changes via direct and synergistic effects, leading, ultimately, to cognitive impairment and functional decline. Increasingly, biomarkers of these biological processes are used in both clinical practice and research settings and in the context of clinical trials and drug development (*2*). The most commonly used AD biomarkers include structural magnetic resonance imaging (MRI) based measures of atrophy, positron emission tomography (PET) based imaging of brain metabolism, A β , and tau, and cerebrospinal fluid (CSF) measures of A β , tau and neuronal injury (*3-5*). Hampering the use of these biomarkers, however, is the

high cost and limited availability of imaging-based measures and the perceived invasiveness of CSF sampling. As a result, blood-based measures are increasingly seen as a complimentary alternative for use as a simplified initial screening step in primary care (6). One such potential measure is the axonal injury marker neurofilament light chain (NfL) (7). NfL is a key structural component of the neuronal cytoskeleton (8, 9) and is abundantly expressed in large-caliber myelinated axons (8). In response to injury, NfL is released into the CSF and blood; biofluid concentrations of NfL, however, also increase in an age-dependent manner. NfL has been shown to be elevated in both plasma (10) and CSF (11) in sporadic, familial and preclinical AD, and to correlate with cognitive, biochemical, and imaging based measures in AD (12-14), as well as with the severity tau neurofibrillary tangle pathology and immunohistochemical staining of NfL in the white matter at *post-mortem* (15). Studies addressing associations between plasma NfL and imaging markers are few (12-14, 16) however, and have been confined to pre-defined regions of interest and a global index of white matter change. Moreover, no studies have looked at the association between plasma NfL and tau PET.

Herein, we examined the associations between plasma NfL concentration and both structural MRI (whole brain voxelwise measures of both gray and white matter volume) and PET based molecular imaging (A β and tau) measures in two cohorts comprising cognitively unimpaired (CU) older subjects and in subjects with cognitive impairment (CI; mild cognitive impairment (MCI) and dementia due to AD). We tested the following hypotheses: 1) plasma NfL is increased in both CU and CI subjects, and shows increases over time; 2) plasma NfL, while showing an association with grey and white matter atrophy, shows a stronger association with white matter volume; 3) that the coupling between NfL and atrophy is more pronounced among CI subjects and 4) that amyloid PET will relate more closely to plasma NfL among CU subjects with NfL then relating more strongly to tau PET among CI subjects.

4.4 Materials and Methods

4.4.1 Study participants

This study used longitudinal data obtained from ADNI (AD Neuroimaging Initiative; adni.loni.usc.edu). The ADNI based cohort (n=1148) consisted of 382 CU controls and 768 CI participants (MCI, n=420; AD dementia, n=348) with available MRI and longitudinal plasma NfL (up to 48 months) and a second group of 110 CU and 88 CI subjects (MCI, n=67; AD dementia, n=21) who underwent amyloid and tau PET (using [¹⁸F]florbetapir and ^{[18}F]flortaucipir, respectively. For this cross-sectional analysis, we used baseline tau PET and the closest matching amyloid PET. The ADNI inclusion/exclusion criteria are described in detail elsewhere (17). Briefly, controls had Mini-Mental State Examination (MMSE) scores of 24 or greater (range, 0-30, with higher scores indicating less impairment) and a Clinical Dementia Rating (CDR) score of 0 (range, 0-3, with higher scores indicating more impairment). Patients were MCI had objective memory loss based on delayed recall performance on the Wechsler Memory Scale (logical memory II; (>1 SD below the mean), MMSE scores \geq 24, a CDR score of 0.5 and preserved activities of daily living (i.e. no dementia). Patients with AD dementia had met the National Institute of Neurological Communicative Disorders and Stroke-Alzheimer Disease and Related Disorders Association criteria for probable AD (18) and had MMSE scores between 20 and 26, and CDR scores between 0.5 and 1. Regional ethical committees of all participating institutions approved the ADNI. All study participants provided written informed consent.

Cross sectional data was also obtained from TRIAD (Translational Biomarkers in Aging and Dementia), an observational, longitudinal and biomarker-based cohort, specially designed to study pathophysiological processes underlying dementia disorders. Participants are followed over time, with yearly clinical and neuropsychological assessments, as well as fluid and imaging biomarkers. The sample from TRIAD consisted of 74 CU and 42 CI subjects (MCI, n=16; AD dementia, n=26) with available plasma NfL and PET (amyloid and tau), with CU and CI groups were defined using the same criteria as in ADNI. The Research Ethics Board of the Montreal Neurological Institute as well as by the Faculty of Medicine Research Ethics Office, McGill University approved the TRIAD. All study participants provided written informed consent.

4.4.2 Cognition

Overall cognition was assessed by the Mini-Mental State Examination (MMSE) and Clinical Dementia Rating (CDR) in ADNI and TRIAD cohorts.

4.4.3 Plasma measurements

For both cohorts, plasma NfL concentrations were measured using an in-house immunoassay on the single-molecule array (Simoa) platform with a 4-fold dilution, as previously described (*13*). Measurements were performed in a single batch of reagents for each cohort.

In ADNI, one sample did not range between the limits of quantification and was excluded. The limits of quantification were 6.7 ng/L and 1620.0 ng/L. The LCS was 11.0 ng/L and the HCS was 173.9 ng/L, with the respective intra-assay coefficients of variation being 6.2% and 4.9%. The inter-assay coefficients of variation were 9.0% and 7.2% for the LCS and HCS respectively. In TRIAD, a total of eleven samples were below the lower limit of quantification (LLOQ = 6.7 ng/L) and were removed from the analysis. Data acquisition spanned five analytical runs; for the low-concentration quality control sample (12.9 ng/L), the repeatability was 5.3% and intermediate precision was 5.4%. For the high-concentration quality control sample (178 ng/L), the corresponding coefficients of variation were 3.4% and 6.2%, respectively.

4.4.4 Voxel based morphometry

Pre-processed 1.5T and 3T T1-weighted MRI scans were downloaded from the ADNI database (adni.loni.usc.edu; for pre-processing details, see (19)). Anatomical images were segmented into probabilistic Grey Matter (GM) and White Matter (WM) maps using the SPM12 segmentation tool. Each GM and WM probability map was then non-linearly registered (with modulation) to the ADNI template using DARTEL (20), and smoothed with a Gaussian kernel

of full width half maximum (FWHM) of 8 mm. All images were visually inspected to insure proper alignment to the ADNI template.

4.4.5 Brain Amyloid and Tau imaging

In the ADNI cohort, amyloid load was estimated using [¹⁸F]florbetapir PET standardized uptake value ratio (SUVR) and tau load using [¹⁸F]flortaucipir PET SUVR. Preprocessing and spatial normalization details have been described elsewhere (*21*). SUVR maps were generated using the cerebellar grey matter for [¹⁸F]florbetapir and the inferior cerebellar grey matter as reference region for [¹⁸F]flortaucipir.

In TRIAD, PET scans were acquired with a Siemens High Resolution Research Tomograph (HRRT). Participants underwent amyloid and tau PET using [¹⁸F]AZD4694 and [¹⁸F]MK6240, respectively. [¹⁸F]AZD4694 images were acquired 40–70 minutes post-injection and [¹⁸F]MK6240 90–110 minutes post-injection. PET data was reconstructed using the OSEM algorithm on a 4D volume ([¹⁸F]AZD4694, 3x600s (*22*); [¹⁸F]MK6240, 4x300s (*23*)). SUVR maps were generated using the cerebellar grey matter as reference region for [¹⁸F]AZD4694 and the inferior cerebellar grey matter for [¹⁸F]MK6240. PET images were spatially smoothed to achieve a final resolution of 8mm full-width at half maximum. T1-weighted images were acquired at 3T for co-registration purposes and were non-uniformity and field-distortion corrected using an in-house pipeline.

4.4.6 Statistical analysis

The statistical software R (version 3.4.3) (24) was used to run statistical tests for demographic comparisons, and to perform several linear mixed effect (LME) based analysis: first, to compare the progression in plasma NfL between CU and CI subjects; these models had plasma NfL as the dependent variable and included the interaction between the independent variables time (days between baseline NfL and follow-up time points) and group and were adjusted for sex, age at baseline NfL, magnetic field strength (1.5 or 3T), with random intercepts. 95% confidence intervals were determined based on the estimated fitted value across the distribution from 1000 simulations of the model (including all variations, except theta). Statistical significance was set at $P \le 0.05$, two-sided. Two additional models were implemented at the voxel level (25): first, to address the longitudinal relationship between plasma NfL and VBM (adjusting for time (continuous), age at baseline NfL, sex, scanner type (1.5 or 3T) and the time between plasma collection and MRI, with a random intercept); second, to address the longitudinal association between the interaction of plasma NfL with time in relation to VBM (adjusting for time (categorical), age at baseline NfL, sex, scanner type (1.5 or 3T) and the time between plasma collection and MRI, with a random intercept). These voxel-based analyses were performed using VBM findings for both grey and white matter. Finally, voxel-wise linear models were used to examine the association between plasma NfL and cross-sectional PET (amyloid, tau) imaging. These models were corrected for amyloid or tau (i.e. models with tau PET as the outcome were adjusted for global amyloid and vice-versa), age at plasma collection, sex and time interval between plasma and PET.

Voxelwise findings were corrected for multiple comparisons using Random Field Theory (26), which accounts for imaging resolution and for the spatial correlation between voxels.

Considering the biological reasoning, we expected to find a negative association between VBM and plasma NfL and a positive association between PET measurements and plasma NfL. Thus, we performed one-tailed hypothesis tests with a type I error α <0.05. Degrees of freedom are informed in the text.

4.5 Results

4.5.1 Demographic characteristics

Demographic and clinical information for the study population are presented in Table 1. From the ADNI cohort, 1149 subjects were included in the study (215 subjects had only baseline), including 382 CU subjects and 768 CI subjects (MCI, n=420; AD dementia, n=348). The average age of participants was 74.1 years (SD=7.5), with males representing 54% of the participants. No difference in age was found between groups (t=1.92; P=0.055), but CU participants included more males ($\chi^2=20.30$; P<0.001) while CI had more *APOE* e4 carriers ($\chi^2=70.93$; P<0.001), fewer years of education (t=3.94; P<0.001) and performed worse on the MMSE (t=5.35; P<0.001), as compared to the CU group. The initial plasma NfL concentrations were on average higher in the CI than were in the CU group (t=6.65; P<0.001; Figure 1A), adjusting for age and sex. Age was highly associated with plasma NfL (t=19.40; P<0.001), but not with sex, education or *APOE-e4* status (when adjusting for diagnosis). Plasma NfL was also different between groups over time (t=6.58, P<0.001; Figure 1C), as shown by the LME analysis with the longitudinal data. However, no difference between the slopes was found (t=1.40; P=0.16), indicating that the rate of increase in plasma NfL levels did not differ between groups. The TRIAD population comprised 116 participants, including 74 CU subjects and 42 CI subjects (MCI n=16; AD dementia, n=26). The average age of TRIAD participants was 74.1 years (SD=7.5), with males representing 38% of the cohort. There was no difference in age (t=1.44; P=0.15), sex (χ^2 =2.78; P=0.09) or *APOE* ε 4 status (χ^2 =4.48; P=0.10) between groups. CI participants, however, had on average fewer years of education (t=2.00; P=0.04), lower scores in the MMSE (t=8.56, P<0.001) and higher levels of plasma NfL (t=2.55; P=0.01; Figure 1B), as compared to CU participants. Similar to ADNI, age had a large effect on plasma NfL (t=4.23; P<0.001) but sex, education and *APOE* ε 4 status (when adjusting for diagnosis), did not.

4.5.2 Plasma NfL is associated with amyloid and tau in AD related brain areas

The voxel-wise analysis showed an association between plasma NfL and [¹⁸F]florbetapir in CU participants (Figure 2A; ADNI, $t_{(103)}$ and TRIAD, $t_{(67)}>3.21$, both P<0.05). These associations were seen primarily in the posterior cingulate/precuneus, parietal cortex, frontal and temporal cortices.

No significant associations were seen between plasma NfL and amyloid PET among CI subjects. In ADNI, [¹⁸F]flortaucipir showed some association with plasma NfL in the CI group $(t_{(79)}>3.19, P<0.05;$ Figure 2B), although these results did not survive multiple comparison correction. In the TRIAD cohort, tau load ([¹⁸F]MK6240) and plasma NfL were associated only in the CI group $(t_{(35)}>3.34, P<0.05;$ Figure 2B), showing strong associations in the frontal and temporal regions.

4.5.3 Plasma NfL is associated with grey matter atrophy in AD related brain areas in *APOE* ε 4 carriers.

The voxel-wise LME demonstrated increases in plasma NfL levels associated with reduced GM volume in CU ($t_{(1160)}$ <-3.09, *T*<0.05; Figure 3A) and CI participants ($t_{(2391)}$ <-3.09, *T*<0.05; Figure 3B,). Associations in the CU group were confined to small clusters in the frontal lobe and hippocampus. Among CI participants, more widespread associations were seen in frontal and temporal cortices, as well as in the medial temporal lobe.

Given the initial findings with GM VBM, we decided to further subdivide CU and CI groups according to *APOE* ε 4 status, considering this a proxy of longitudinal A β accumulation (27). When doing so, a clear distinction was seen between *APOE* ε 4 carriers and non-carriers among CU subjects, with findings confined to ε 4 carrier group ($t_{(1160)}$ <-3.09, *T*<0.05; Figure 2A). Among CI subjects, no clear differences were seen between *APOE* ε 4 carriers and non-carriers, with both groups showing significant associations between plasma NfL and grey matter volume, mainly in the temporal cortices ($t_{(1160)}$ <-3.09, *T*<0.05; Figure 2B). *APOE* ε 4 carriers, however, appeared to have more medial temporal atrophy.

When examining the association between plasma NfL and GM volume over time (baseline, 12-, 24-, 36- and 48-months), CU subjects only showed significant findings for the contrast baseline versus 48 months ($t_{(1160)}$ <-3.09, *T*<0.05; Figure 3C), with results seen primarily in the frontal and temporal cortices. CI subjects, by contrast, showed a progressive reduction in GM volume, predominantly in the temporal cortex ($t_{(1160)}$ <-3.09, *T*<0.05; Figure 3D).

4.5.4 Plasma NfL accounts for white matter atrophy

In addition to GM loss, increases in plasma NfL concentrations were accompanied by a reduction in WM volume. Using baseline data only, CU participants ($t_{(1165)}$ <-3.09, T<0.05; Figure 4A) showed significant associations between plasma NfL and WM volume in the hippocampus, parietal and prefrontal cortices; these findings were very focal, however. By contrast, widespread associations were seen within the CI group ($t_{(2372)}$ <-3.09, T<0.05; Figure 4A). Longitudinally,

plasma NfL was found to associate with WM volume in superior periventricular areas in the CU group ($t_{(2372)}$ <-3.09, *T*<0.05; Figure 4B). Only at month 36, however, was it possible to detect clear findings in temporal regions. In the CI group, by contrast, associations between plasma NfL and WM matter volume were seen at 12-months ($t_{(2372)}$ <-3.09, *T*<0.05; Figure 4C) and spread across the additional time points.

4.6 Discussion

The present study is, to our knowledge, the first to examine the association between plasma NfL, longitudinal atrophy (using an unbiased, whole brain method) and tau PET. The main findings of this study were that plasma NfL was associated with amyloid burden (cross sectional amyloid PET) in CU subjects, and with tau burden (cross sectional tau PET) in CI subjects. Both sets of associations, however, were observed in AD related brain areas. When looking at VBM based GM atrophy, findings in CU subjects were largely driven by *APOE* ε 4 carriers, an effect not seen within the CI group. Despite increases in plasma NfL over time, GM volume loss was only clearly seen at later time points in CU subjects using longitudinal data, while CI subjects showed a progressive decrease in GM volume across time points. High NfL was linked to focal WM atrophy in CU subjects, in contrast to more widespread findings

in CI subjects. Longitudinally, associations between plasma NfL and WM volume clearly increased over time, with associations spreading from periventricular regions in CU subjects, in contrast to the CI group which showed findings predominantly in the temporal lobe that widespread over time.

The observed relationship between the concentration of NfL in plasma and amyloid pathology markers among CU subjects was in agreement with the existing literature. NfL levels were low overall, what has previously been interpreted to suggest that the degree of neuronal injury is limited in preclinical AD (i.e. amyloid- β CU), with increases in NfL levels falling below detection limits (13). Even though neuronal injury is limited in CU, our results corroborate the framework stating that the initial neural injury in AD pathophysiology, here indexed by plasma NfL, is caused by abnormal amyloid accumulation (28). In fact, plasma NfL has been already associated with amyloid in CU. In an additional study, though plasma NfL at baseline among CU subjects did not predict amyloid accumulation over a short follow-up interval, increases in plasma NfL tracked increased signal from amyloid PET (28). In addition, the brain regions found here to be linking amyloid to neurodegeneration in CU are frequently reported as having high levels of amyloid in AD. This scenario would prove consistent with the topographical overlap we observed between areas showing significant associations between plasma NfL and amyloid PET (e.g. the temporal cortex and precuneus), and areas showing declines in GM volume. Similarly, Mielke and colleagues reported in their study that the reported NfL-amyloid association was also accompanied by declines in hippocampal volume and global cortical thickness, and speculated that the observed association with amyloid PET may reflect ADrelated neurodegeneration (28).

In contrast to the CU group, plasma NfL associations were confined to tau PET among CI subjects. While there are to date no other studies that have employed tau PET when looking at plasma NfL, previous work has shown plasma NfL to positively correlate with CSF phosphorylated tau (P-tau) over time among subjects with CI (14). Similar findings have also been reported using NfL from cross-sectional plasma and CSF samples (*12, 29*). The fact that the association was specific for tau may reflect CI subjects having largely reached a plateau in their level of amyloid deposition (*30*), with tau pathology by contrast showing continued accumulation (*31*) that is most evident in tau PET at the MCI and AD stages (*23*). Voxelwise associations between plasma NfL and tau PET differed between cohorts, however, with findings that did not survive multiple comparison correction in ADNI, in contrast to robust positive associations within frontotemporal regions in the TRIAD cohort. This observed difference may relate to differing time intervals between plasma sampling and tau PET between cohorts. Although models accounted for this difference, this, combined with [¹⁸F]flortaucipir and [¹⁸F]MK6240 differing in their sensitivity to tau aggregates (*32*), may account for the observed discrepancy.

Using VBM, plasma NfL was found to associate with both grey and white matter volume loss in CU and CI subjects. Using GM VBM data, though findings were limited when looking across all CU subjects, analysis by *APOE* subgroups (ϵ 4 carriers and non-carriers) showed significant declines in gray matter volume in temporal, posterior cingulate and orbitofrontal regions among ϵ 4 carriers. As described above, these findings overlapped spatially with areas that showed a significant association between plasma NfL and amyloid imaging and may be due to *APOE* ϵ 4 carriers showing enhanced amyloid deposition (*33, 34*). Only at 48-months, however, was the relationship between GM VBM and plasma NfL seen to progress. This suggests a temporal delay between the build-up of amyloid- β pathology and neuronal injury (1). As hypothesized, among CI subjects the coupling between NfL and GM loss was more pronounced, particularly within the temporal lobe, and showed a continuous increase across time points. Moreover, associated areas overlapped with those showed to be significantly associated between plasma NfL and tau PET. These findings are consistent with neuroimaging studies showing progressive neuronal loss across the symptomatic phase of AD (*35*) and the co-localization of tau, reduced brain metabolism and atrophy (*36-38*). Not surprisingly, no notable difference was seen in the GM associations between CI *APOE*- ε 4 carriers and non-carriers. As expected, only about half of the subjects clinically diagnosed with AD are *APOE*- ε 4 carriers but all of them undergo neurodegenerative processes.

In addition to the loss of cortical neurons, white matter injury (39) and the loss of corticosubcortical connectivity (40) are features of AD. In CU and CI subjects, the extent of WM volume loss exceeded that of GM loss. Since plasma NfL levels are understood to reflect damage to large-caliber myelinated axons (8, 15), this would suggest that WM damage may precede GM loss in AD (41). Indeed, there is evidence to support the position that WM abnormalities precede GM changes (42): changes in CSF tau measures and $A\beta_{1-42}$ have been shown to predict MR based measures of WM integrity in CU individuals at risk for AD in the absence of effects on GM (43). Moreover, several studies have shown that soluble amyloid- β is toxic to oligodendrocytes (44, 45) and elevated in the WM (46). Further, while the neocortical tau pathology seen in AD mainly affects GM, glial tangles are also seen in astrocytes and oligodendroglia (47) and phosphorylated tau in GM has been shown to be associated with demyelination and WM abnormalities in AD (48, 49). Amyloid- β and tau may thus first result in WM damage, as reflected by increases in plasma NfL, which subsequently result in GM loss but a spatiotemporal offset course. This study has several limitations. First, we did not include patients with significant vascular burden, which may have prevented us from detecting more subtle associations between WM atrophy and plasma NfL. In addition, the ADNI and TRIAD cohorts differed in the average time interval between plasma and tau PET measurements and in the tau tracer used. While this was accounted for in statistical models it may have affected the comparability of findings between CI groups. Moreover, a larger sample size combined with the inclusion of non-AD subjects and longitudinal PET data, would have allowed us to investigate the ability of plasma NfL to capture synaptic effects of both amyloid and tau as well as aspects of neurodegeneration that are non-related to either proteinopathy (*12*).

4.7 Conclusions

The increase in NfL is associated with widespread grey and white matter loss in AD-related regions, providing further evidence supporting the use of NfL as marker of progressive neuronal damage. These findings further suggest that plasma NfL may prove an early marker of amyloid-related neuronal injury in AD, being in turn more closely related to tau-mediated neurodegeneration during the symptomatic course of the disease.

4.8 Tables and Figures

	ADNI		TRIAD	
	CU	CI	CU	CI
No. subjects	382	767	74	42

Table 4-1 Demographics and key characteristics of the ADNI and TRIAD cohorts.

Age ¹ (mean, SD)	73.5 (6.9)	74.4 (7.8)	72.4 (6.2)	70.4 (8.5)
Males (n, %)	173 (45.3)*	456 (59.5)*	24 (32.4)	21 (50.0)
Education ¹ (mean, SD)	16.6 (2.5)*	15.9 (2.7)*	15.3 (4.2)*	13.7 (3.8)*
<i>APOE</i> -e4 (n, %)	106 (27.7)*	395 (51.6)*	25 (33.8)	17 (40.5)
MMSE (mean, SD)	29.0 (1.1)*	26.4 (3.4)*	28.9 (1.3)*	23.4 (5.3)*
Plasma NfL ² (mean, SD)	35.2 (16.7)*	44.0 (22.2)*	27.3 (19.2)*	34.5 (13.7)*

SD standard deviation; MMSE Mini-Mental State Examination.

¹Measured in years; ²Measured in pg/mL. *Statistically different between the groups (p<0.05).



Figure 4-1 Plasma NfL.

Differences in plasma NfL levels are seen between cognitively impaired (CI) and cognitively unimpaired (CU) subjects in both ADNI (A) and TRIAD (B) cohorts at baseline. CI participants also showed higher plasma NfL concentrations as compared to CU participants(C) longitudinally.



Figure 4-2 Plasma NfL and PET biomarkers.

T-statistical parametric maps (T-maps) superimposed on average structural MRI show brain regions where higher NfL levels are associated with higher [18F]florbetapir (ADNI) and [¹⁸F]AZD4694 (TRIAD) standard uptake value ratios (SUVR) in the CU group (A). In CI group, T-maps also show brain regions where higher NfL levels are associated with higher [¹⁸F]flortaucipir (ADNI) and [¹⁸F]MK6240 (TRIAD) in CI group. T-values that are significant after random field theory (RFT) correction for multiple comparisons are indicated in the text.



Figure 4-3 Plasma NfL and GM volume.

T-statistical parametric maps (T-maps) superimposed on average structural MRI show brain regions where higher NfL levels are associated with reduced GM volume in CU (A) and CI (B) participants, being them *APOE*-e4 carriers (+) or non-carriers (-). T-maps also show that, as compared to baseline, only at 48 months there was a reduction of GM volume associated with plasma NfL in CU (C), while in CI group differences are observed at each time point (D).

T-values that are significant after random field theory (RFT) correction for multiple comparisons are indicated in the text.



Figure 4-4 Plasma NfL and WM volume.



T-statistical parametric maps (T-maps) superimposed on average structural MRI show brain regions where higher NfL levels are associated with reduced WM volume in CU and CI participants (A). T-maps also show that, as compared to baseline, there was a reduction of WM

volume associated with plasma NfL in CU (B) and CI groups (C) at each time point (D). Tvalues that are significant after random field theory (RFT) correction for multiple comparisons are indicated in the text.

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4.10 Author Disclosures

Kaj Blennow has served as a consultant or at advisory boards for Alector, Alzheon, CogRx, Biogen, Lilly, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Venture-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper. Henrik Zetterberg has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg.

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Chapter 5 Plasma neurofilament light associates with Alzheimer's disease metabolic decline in amyloid positive individuals

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http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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5.1 Preface

In Chapter 4 we showed that amyloid PET was associated with plasma NfL only in cognitively unimpaired (CU) subjects, suggesting that NfL is able to detect amyloid-related neurodegeneration at early stages in the AD spectrum. Moreover, we demonstrated that GM atrophy, in CU, was only associated with NfL in *APOE-e4* carriers. As NfL is not related to *APOE*, we hypothesized that, in CU, the APOE-e4 genotype is indexing amyloid accumulation. Following this line of investigation, in this study we aimed to verify if NfL is associated with glucose metabolism. We hypothesize that, in amyloid positive individuals, plasma NfL will be associated with reduced glucose uptake indexing neurodegeneration.

5.2 Abstract

5.2.1 Introduction

Neurofilament light chain (NfL) is a promising blood biomarker to detect neurodegeneration in Alzheimer's disease (AD) and other brain disorders. However, there are limited reports of how longitudinal NfL relates to imaging biomarkers. We herein investigated the relationship between blood NfL and brain metabolism, indexed by [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG) in the Alzheimer's Disease Neuroimaging Initiative (ADNI).

5.2.2 Methods

Voxel-wise regression models tested the cross-sectional association between [¹⁸F]FDG and both plasma and CSF NfL in cognitively impaired (CI) and unimpaired (CU) subjects. Linear mixed models were also used to test the longitudinal association between NfL and [¹⁸F]FDG in amyloid- β positive (A β +) and negative (A β -) subjects.

5.2.3 Results

Higher concentrations of plasma and CSF NfL were associated with reduced [¹⁸F]FDG uptake in correspondent brain regions. In A β + participants, NfL associates with hypometabolism in AD-vulnerable regions. Longitudinal changes in the association [¹⁸F]FDG-NfL were confined to CI A β + individuals.

5.2.4 Conclusions

These findings indicate that plasma NfL is a proxy for neurodegeneration in AD-related regions in $A\beta$ + subjects.

5.2.5 Keywords

Neurofilament light, hypometabolism, [¹⁸F]FDG, neurodegeneration, Alzheimer's disease, biomarkers, blood, PET, longitudinal.

5.3 Introduction

Blood-based biomarkers that can predict the clinical onset of Alzheimer's disease (AD) and monitor disease progression via the identification of the underlying pathology are urgently needed. These would greatly assist therapeutic trial stratification and the clinical management of patients, particularly when disease modifying drugs become available. Blood-based biomarkers for neurodegeneration can also be used to identify downstream effects on neurodegeneration in clinical trials on drugs with disease-modifying potential [1, 2]. In AD Phase I-II trials, for example, these biomarkers may be valuable for decision-making on whether to continue Phase III drug development or not. Both molecular imaging (*e.g.*, amyloid- β (A β), tau and glucose metabolism using positron emission tomography (PET)) and cerebrospinal fluid (CSF; A β , total tau (T-tau) and phosphorylated tau at 181 (P-tau)) based biomarkers accurately identify and track AD pathophysiology [3]. Nonetheless, PET imaging is costly, and access is restricted to specialized centers and implausible to be implemented widely in a general routine assessment of cognitive complaints. CSF sampling is becoming more accepted in clinical routine, but a lumbar puncture may still be regarded as an invasive procedure. Therefore, a blood-based measure would have substantial practical advantages for both clinicians and patients.

Recent advancements in proteomic assays have demonstrated the potential use of plasma $A\beta$ to identify brain $A\beta$ -positive individuals with moderate-to-high accuracy [4-8]. The measurement of T-tau in plasma has limited diagnostic value, albeit being slightly but significantly increased in AD patients [9] but promising data is emerging on P-tau [10]. The most replicated blood biomarker for AD is neurofilament light chain (NfL). NfL is abundantly expressed in large myelinated axons and is promptly released into the CSF and blood under axonal distress and degeneration. Indeed, concentrations of NfL are higher in AD patients as compared to aged-matched controls [11]. Furthermore, in familial AD studies, NfL appears to be altered around one decade before symptom onset [12, 13]. However, NfL is a disease-unspecific marker, as elevations are observed in other neurodegenerative disorders [14, 15], inflammatory conditions [16] as well as in acute traumatic brain injury [17]. Although the diagnostic specificity of NfL is lacking, the semi-automated measurement of NfL in blood offers the possibility of multiple sample collections to monitor disease progression and potentially treatment response.

There are limited studies evaluating longitudinal measures of blood NfL in sporadic AD [18, 19]. Furthermore, associations between blood NfL, particularly longitudinal measurements, and established imaging biomarkers for AD are few [11, 19-21] with no detailed investigation of the association between blood NfL and glucose hypometabolism as measured by [¹⁸F]fluorodeoxyglucose ([¹⁸F]FDG) PET. The decreased uptake of [¹⁸F]FDG is understood to largely reflect neurodegeneration, more specifically, synaptic damage [22],

and has become an essential tool in the evaluation of suspected AD and related disorders [23, 24]. AD exhibits a characteristic pattern of glucose hypometabolism, involving the precuneus/posterior cingulate, inferior parietal lobule as well as posterolateral and medial aspects of the temporal lobe, including the hippocampus and entorhinal cortex [25, 26]. This metabolic signature has been shown to predict the progression from mild cognitive impairment (MCI) to AD dementia [27].

In this study, we have investigated the cross-sectional and longitudinal associations of plasma NfL with glucose metabolism as measured by [¹⁸F]FDG PET in cognitively impaired (CI) and unimpaired (CU) participants enrolled in the Alzheimer's Disease Neuroimaging Initiative (ADNI). We hypothesized that higher levels of plasma NfL would be associated with greater brain hypometabolism, mainly in those A β positive, reflecting the underlying physiological and neurodegenerative processes.

5.4 Materials and methods

5.4.1 Study participants

This study uses data obtained from the ADNI database (adni.loni.usc.edu), which was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), PET, other biological markers, and clinical and neuropsychological assessments can be combined to measure the progression of mild cognitive impairment (MCI) and early AD. AD subjects had a Mini-Mental State Examination (MMSE) ranging between 20 and 26 (inclusively), Clinical Dementia Rating (CDR) equals 0 or 1 and met criteria for probable AD according to the National Institute of Neurological and Communicative Disorders and Stroke– Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA)[28]. Participants were classified as MCI if had MMSE ranging between 24-30, CDR 0.5 (with the memory box score being 0.5 or greater), largely intact general cognition and functional performance, and could not meet criteria for dementia according to NINCDS-ADRDA. (For further details see [29])

Our study population was derived from two data sets. The first dataset was used for crosssectional analyses and included participants that had measures of plasma NfL, CSF NfL and [¹⁸F]FDG PET. The second dataset comprised participants who had longitudinal plasma NfL (0-24 months) and [¹⁸F]FDG PET (for detailed description see Supplementary figure 1). The population was divided in two groups; cognitively unimpaired (CU; n_{cross} =81, n_{longit} = 302) and cognitively impaired (CI; n_{cross} = 162, n_{longit} =713). The CI group consists of individuals clinically defined as having MCI or AD dementia. For the classification of the longitudinal data, the most recent diagnosis assigned to a participant was used. For the cross-sectional analysis, group classification was based on the diagnosis given at the time of plasma or CSF collection. The ADNI inclusion/exclusion criteria are described in detail at www.adni-info.org (accessed October 2018). All enrolled participants or authorized representatives provided informed consent, approved by ADNI centre's respective Institutional Review Boards.

5.4.2 Plasma measurements

Plasma NfL concentration was measured at the Clinical Neurochemistry Laboratory, University of Gothenburg, Mölndal, Sweden by board-certified laboratory technicians with the data here included generated using a single batch of reagents. An in-house immunoassay on the single-molecule array (Simoa) platform, with a 4-fold dilution, as previously described [16]. For cross-sectional data, data acquisition spanned fourteen analytical runs and all the samples ranged between the limits of quantification (LOQ, lower limit = 2.2 ng/L; LOQ, upper limit = 1620 ng/L). For the low-concentration control sample (LCS; 14 ng/L), the intra-assay coefficient of variation was 11.0% and the inter-assay coefficient of variation was 11.1%, whilst for the high-concentration quality control sample (HCS; 137 ng/L), the corresponding coefficients of variation were 8.8% and 9.6%, respectively. For longitudinal data, the LOQ were 6.7 ng/L and 1620 ng/L. The LCS was 11.0 ng/L and the HCS was 173.9 ng/L, with the respective intra-assay coefficients of variation being 6.2% and 4.9%. A single sample was excluded due to not being within the LOQ.

5.4.3 CSF measurements

CSF was sampled by lumbar puncture and NfL levels were quantified in a subset of the participants, at a single time point, using a commercial ELISA (NF-light; UmanDiagnostics) following the protocol provided by the manufacturer. Samples were run in singlicates and using one batch of reagents. Each ELISA plate included internal QC samples with high and low CSF NFL concentrations placed in duplicate both in the beginning and end of the plate. The calibration curve acceptance followed strict criteria as described elsewhere[30]. Intra-assay coefficients of variation were <10%.

5.4.4 MRI/ PET

MRI and PET acquisitions followed the ADNI protocols (http://adni.loni.usc.edu/methods). The MRI T1-weighted images underwent initial preprocessing with intensity normalization and gradient un-warping. They were then processed using the CIVET image-processing pipeline and registered using a nine-parameter affine transformation and nonlinearly spatially normalized to the MNI 152 template. [¹⁸F]FDG PET images were preprocessed to have an effective point spread function of full-width at half-maximum of 8 mm. Subsequently, linear registration and nonlinear normalization to the MNI 152 template were performed with the linear and nonlinear transformation derived from the automatic PET to MRI transformation and the individual's anatomical MRI coregistration. [¹⁸F]FDG standardized uptake value ratio (SUVR) maps were generated using the pons as reference region [31]. More details regarding the image-processing pipeline can be found elsewhere [31, 32]. Here, we used the [¹⁸F]FDG scan with the closest acquisition date to the plasma collection.

In order to classify subjects as A β positive (+) or negative (-), brain amyloid burden was estimated using [¹⁸F]florbetapir PET. The global SUVR values were obtained from the ADNI and the detailed protocols on PET acquisition and processing are available online (http://adni.loni.ucs.edu). The cut-off used here was >1.11, as suggested in the ADNI protocol.

5.4.5 Statistical analysis

The R programing language (version 3.4.3) [33] was used to perform all non-imaging statistical analyses. Linear models and Spearman's rank correlation coefficients were used for demographic comparisons and for cross-sectional analyses, adjusting for age, sex and group

(CUxCI), where appropriate. We performed linear mixed effect regression models (LME), using the *nlme* package, to compare the progression in plasma NfL between groups: (1) CU vs CI and (2) CU A β - vs CU A β +, CI A β - and CI A β +. The LME included plasma NfL as the dependent variable, the interaction between the independent variables time and group, the covariates sex and age at baseline NfL and a random intercept. The 95% confidence intervals were estimated based on the estimated fitted value across the distribution from thousand simulations of the model, that includes all variations except theta. All tests mentioned above were 2-sided with a significance level of P < .05.

LME was also performed at the voxel level to check the association between plasma NfL and [¹⁸F]FDG. Two models were applied using VoxelStats[34]. Firstly, the model tested the association between [¹⁸F]FDG and plasma NfL, adjusting for baseline age, sex, collection time point and time difference between the plasma measurement and the [¹⁸F]FDG acquisition, with a random intercept and accounting for the repeated measures of the longitudinal data. In the second model, we examined the longitudinal association between the interaction of plasma NfL with time in relation to [¹⁸F]FDG, also adjusting for age, sex and time difference between NfL and [¹⁸F]FDG. Here, time was treated as a categorical variable. Both models were performed in the CU and CI groups. Voxel-based linear models were also used to examine the cross-sectional association of both plasma NfL and CSF NfL with [¹⁸F]FDG. The models were corrected for age at biofluid collection, sex and time interval between biofluid and PET acquisition. These analyses were also performed within the CU and CI groups. Random Field Theory (RFT) [35] was used to correct the resulting T parametric maps for multiple comparisons, as this method considers the resolution of the imaging data and the spatial correlation between voxels. Hypothesizing that [¹⁸F]FDG and NfL (plasma or CSF)[36] would

show a negative association, we performed one-tailed hypothesis tests with a type I error α =0.05. Degrees of freedom are indicated in the text.

5.5 Results

5.5.1 Demographics characteristics

Demographic and clinical characteristics are summarized in Table 1. A total of 243 participants were included in the cross-sectional analysis, from whom 62% were males and the average age was 75.0 (SD = 6.6) years old. This population included 81 CU and 162 CI subjects, with no difference in age (t=1.06; P=0.28), sex (χ^2 =2.11; P=0.14) or education (t=0.09; P=0.92) found between groups. As expected, the CI group had more APOE- ε 4 carriers (χ^2 =28.27; P<0.001) and lower MMSE scores (t=10.59; P<0.001), as compared to the CU group. Additionally, CI subjects showed higher levels of NfL, adjusted for age and sex, using both CSF (t=4.80; P<0.001; Figure 1A) and plasma (t=4.98; P<0.001), but not with sex, education or APOE- ε 4 status. Plasma NfL was associated with age (t=5.39; P<0.001) and sex (t=0.29; P=0.77). Contrarily, CSF NfL was associated with both age (t=5.05; P<0.001) and sex (t=2.21; P=0.02). Sex differences in CSF NfL were due to higher NfL concentrations in females, specifically in the CU group (t=2.70; P=0.008). There was no difference in CSF NfL levels between sex groups in CI.

The longitudinal component of the analysis consisted of 1015 participants (average follow-up time=9.2 months), and there were no bias toward sex (% Males = 54.9%), though males were older ($M_{(males)} = 74.9$; SD = 7.4; *t*=5.47; *P*<0.001). The CU group consisted of 302 subjects

(42.3%) while the CI group had 713 subjects. There was no difference in age between the groups (t=0.83; P=0.40), but the CU group had more females (χ^2 =12.4; P=0.001) and more years of education (t=3.56; P<0.001), as compared to the CI group. As expected, the CI group had more APOE- ε 4 carriers (χ^2 =42.89; P<0.001), more A β + subjects (χ^2 =104.95; P<0.001) lower MMSE (t=12.03; P<0.001) scores and higher levels of plasma NfL ($t_{(1010)}$ = 5.03; P<0.001) at baseline (Figure 2A). Plasma NfL was highly associated with age in the whole population and within groups (t_{all} =17.98; t_{CU} =10.19; t_{CI} =14.99; P<0.001), but was not associated with sex (t=0.88; P=0.37), education (t=1.08; P=0.27) or APOE- ε 4 status (t=1.11; P=0.26) when adjusting for diagnosis and covariates. The LME showed a significant difference between CI and CU groups (t=4.93; P<0.001) but no difference in the slopes (t=1.43; P =0.15; Figure 2B). When segregating the groups according to A β status (Figure 2C), there was a significant difference in the slope between the reference group (CU A β -) and both CU A β + (t=2.36; P=0.01) and CI A β + subjects (t=2.34; P=0.01).

5.5.2 Plasma and CSF NfL show similar association with brain glucose metabolism.

Cross-sectionally, plasma NfL showed correlation with CSF NfL (Spearman r=0.57, P<0.001; Figure 2C) which remained significant (t=6.43, P<0.001) after adjusting for age, sex and diagnosis. As expected, CSF-plasma correlation was present within both groups ($r_{(CU)}=0.50$, P<0.001; $r_{(CI)}=0.53$, P<0.001). Voxel-wise analysis in the CI group revealed a significant association between plasma NfL and [18 F]FDG SUVR ($t_{(154)}<-3.14$; P<0.05) in the hippocampus and insula, bilaterally (Figure 1D). Similarly to plasma NfL, CSF NfL was associated with glucose hypometabolism in the hippocampus and insula, although predominantly in the right hemisphere ($t_{(156)}$ <-3.14; P<0.05). CSF NfL also showed a diffuse association with [¹⁸F]FDG in the frontal regions of the brain which did not present a good overlap with what was observed with plasma NfL. In the CU group, the topography of the hypometabolism associated with CSF and plasma NfL was very similar (Figure 1E), being restricted to the right hippocampus. These results, however, did not survive multiple comparison correction.

5.5.3 Plasma NfL is more associated with glucose metabolism in A β -positive individuals.

The voxel-wise LME revealed a link between high plasma NfL levels and reduced [¹⁸F]FDG uptake in the frontal and temporal regions, as well as in the posterior cingulate cortices and occipital regions among CI A β + subjects ($t_{(572)}$ <-3.10; P<0.05; Figure 2D). Within the CI A β -group, these associations were substantially reduced ($t_{(305)}$ <-3.11; P<0.05). In the CU A β + group, NfL and [¹⁸F]FDG were significantly associated ($t_{(117)}$ <-3.16; P<0.05) in small focal cortical clusters in the posterior cingulate, parietal and temporal lobes. Among CU A β -individuals, this association was reduced and more localized in the parietal cortices ($t_{(286)}$ <-3.11; P<0.05; Figure 2E).

5.5.4 The longitudinal association of plasma NfL with brain metabolic changes over 24 months is only present in A β positive individuals.

When testing the interaction between plasma NfL and time in the CI A β + group, NfL associations with [¹⁸F]FDG were higher over 24 months than at the baseline ($t_{(566)}$ <-3.09; $P \le 0.05$) in the posterior cingulate, frontal and temporal cortices (Figure 3). However, the associations between plasma NfL and [¹⁸F]FDG SUVR did not differ between time points in the CI A β -, CU A β + or CU A β - groups.

5.6 Discussion

The main findings from this study are three-fold; (I) plasma and CSF NfL demonstrate a similar pattern of association with glucose metabolism in CI individuals, (II) higher concentrations of plasma NfL are associated to lower glucose metabolism more strongly in A β + individuals and, (III) the association of plasma NfL tracks metabolic decline over-time only in A β + CI subjects.

There has been an increasing focus on blood NfL (plasma or serum) due to the potential prognostic and diagnostic value in AD. Although their ubiquitous and unspecific increase in many neurological conditions limit their diagnostic value, they have potential as a biomarkers of disease progression. Therefore, associations between NfL with established biomarkers of AD (*e.g.* [¹⁸F]FDG) are fundamentally important to interpret NfL plasma levels. Unlike other potential blood biomarkers, the correlation of NfL in blood and CSF has been consistently high across various studies [14, 16, 37]. Likewise, in this study we found an excellent correlation between NfL quantified in the plasma and CSF compartments. Remarkably, utilising a voxel-

wise approach, we have shown that the association between regional glucose metabolism with either plasma or CSF NfL levels converge to the same regions. The fact that these associations were present in both CI and CU groups further reinforces the potential use of plasma NfL as a surrogate measure for CSF NfL or neurodegeneration.

Longitudinally, we have shown that there is a group effect of NfL progression, in which CI participants displayed higher concentrations of plasma NfL over 24 months as compared to CU participants. However, the NfL rate of change was not statistically different between these two groups. In-line with a recent report [19], group stratification based on A β status showed that A β + subjects present greater changes in plasma NfL over time as compared to CU A β -subjects. This finding suggests that increases in plasma NfL are able to detect A β -related neuronal injury at an early stage. This is also supported by previous studies in familial AD [12, 13], Down syndrome [38-40] and unbiased proteomics in CU A β + individuals [41].

Regarding the anatomical distribution, to our knowledge, this is the first study at the voxellevel showing that within both CU A β + and CI A β + subjects metabolic abnormalities occur in AD-related cortical regions. This supports a framework in which NfL combined with a biomarker of A β might convey Alzheimer's disease related neurodegeneration. Indeed, among A β - subjects, the association between plasma NfL and [¹⁸F]FDG did not conform to patterns seen in AD, suggesting that neuronal injury was due to other causes than AD.

The present findings also revealed that increases in the association between plasma NfL and glucose metabolism over 24 months were only detected in $A\beta$ + CI subjects. This result is in line with a previous study that demonstrated that the presence of both $A\beta$ and neurofibrillary tangle (NFT) pathology are necessary to increase brain hypometabolism over a two-year period

[42]. Though we did not have access to tau related data in our study, it is very likely that the majority of A β + participants are also tau positive (T+, i.e. NFT positive). This was evidenced by post-mortem studies showing that over 80% of MCI and AD subjects pathologically classified as probable or definite AD –according to the CERAD criteria– also showed Braak stage \geq 3 [43, 44]. In fact, we have previously shown that plasma NfL are highest in post-mortem cases with Braak stage V/VI [18]. Thus, it is important to highlight that plasma NfL may be able to track neurodegenerative changes that do not necessarily reflect detectable clinical decline in the same period of time.

Certain methodological aspects limit the interpretation of our findings. It is relevant to mention that the period of biofluid collection and PET imaging acquisition was not invariably the same, although models accounted for the time difference between measurements. The reduced sample size of some of the groups could also have an impact on the results as not all the subjects included in the LME models had longitudinal data. Additionally, other important biomarkers of neuronal function/dysfunction such as neurogranin, T-tau and phosphorylated tau were not assessed in the present study. Finally, certain demographic differences were found between the groups; these, however, were accounted for in our statistical models.

In conclusion, this study adds further evidence that plasma NfL is reflective of CSF NfL but more importantly reflects longitudinal neurodegeneration, indexed by FDG PET. In the ADNI cohort, increased plasma NfL in $A\beta$ + participants was associated with advanced glucose hypometabolism, with longitudinal changes confined to CI $A\beta$ + individuals.

5.7 Tables and Figures

	Cross-sectional data		Longitudinal data*	
	CU	CI	CU	CI
No. subjects	81	162	302	713
Age ¹ (mean, SD)	75.6 (5.0)	64.6 (7.2)	73.6 (7.2)	74.1 (7.7)
Males (n, %)	45 (55)	107 (66)	140 (46)*	418 (58)*
Education ¹ (mean, SD)	15.7 (3.0)	15.6 (3.0)	16.6 (2.5)*	15.9 (2.7)*
<i>APOE</i> -e4 (n, %)	18 (22)*	96 (59) [*]	86 (28)*	364 (51)*
MMSE (mean, SD)	$29.0(1.0)^{*}$	26.2 (2.2) [*]	$29.0(1.2)^{*}$	26.4 (3.4)*
Plasma NfL ² (mean, SD)	31.5 (18.9)*	42.5 (24.5) [*]	36.5 (20.5)*	43.5 (22.0)*
CSF NfL ² (mean, SD)	1103.2 (386.9)*	1490.9 (735.4)*	NA	NA
Aβ positive (n, %)	NA	NA	82 (29)*	431 (66)*

Table 5-1 Demographics and key characteristics of the samples.

SD standard deviation; MMSE Mini-Mental State Examination; NA not available.

¹Measured in years; ²Measured in pg/mL. *Statistically different between the groups (p<0.05).

*Based on the first NfL visit.



Figure 5-1 Comparison between CSF and plasma NfL.

Differences in NfL levels are seen between cognitively impaired (CI) and cognitively unimpaired (CU) subjects measured in the CSF (A) and plasma (B). NfL showed a good correlation between plasma and CSF measurements (C). T-statistical parametric maps superimposed on average structural MRI show brain regions where higher NfL levels are associated with lower [¹⁸F]FDG standard uptake value ratios (SUVR) in the CI (D) and CU (E)

groups. T-values that are significant after random field theory (RFT) correction for multiple comparisons are indicated in the text.



Figure 5-2 Longitudinal plasma NfL.

Differences in plasma NfL levels are seen between cognitively impaired (CI) and cognitively unimpaired (CU) subjects at baseline (A). Longitudinal changes are seen between CI and CU groups over time (B); differences were more pronounced when groups were segregated according to A β status (C). T-statistical parametric maps superimposed on average structural

MRI show brain regions where higher plasma NfL levels are associated with lower [¹⁸F]FDG standard uptake value ratios (SUVR in the CI (D) and CU (E) groups classified as $A\beta$ + and $A\beta$ -. T-values that are significant after random field theory (RFT) correction for multiple comparisons are indicated in the text.



Figure 5-3 [¹⁸F]FDG-NfL progression.

T-statistical parametric maps superimposed on average structural MRI show brain regions in CI A β + subjects where the [¹⁸F]FDG-NfL association was greater after 24 months, as compared to the baseline. T-values that are significant after random field theory (RFT) correction for multiple comparisons are <-3.09.

5.8 Supplementary material

Supplementary Figure 5-1. Flowchart depicting how the participants were selected for our study.



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5.10 Author Disclosures

KB has served as a consultant or at advisory boards for Alector, Alzheon, CogRx, Biogen, Lilly, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper. HZ has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg AB, a GU Ventures-based platform company at the University of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg. All other authors have no disclosures relevant to this manuscript.

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Chapter 6 : Discussion

6.1 Summary of results

This thesis contains four studies that relate to two major research lines. In Chapters 2 and 3 we present evidences supporting new genetic markers associated with amyloid pathology in AD. Besides that, Chapters 4 and 5 show that amyloid pathology in AD could be detected using plasma NfL, a biomarker of neurodegeneration. We also show how plasma NfL is associated with other measures of neurodegeneration in the AD spectrum.

6.1.1 Genetic biomarkers of amyloid load

In Chapter 2 we reported the association between amyloid load and a SNP in the *CYP2C19* gene. This association was detected initially using PET and CSF data from ADNI cohort and supported by neuropathological data from ROS-MAP cohorts. We also show that, despite the SNP not being associated with disease status, it is overall associated with reduced cognitive decline in both cohorts. The mediation analysis indicates that the beneficial effect of the SNP on amyloidosis is linked to the cognitive downstream effect above mentioned. In Chapter 3 we used epistasis analysis hoping to unveil additional genetic factors associated with amyloid pathology. This method led to the identification of interacting immune-related genetic variants. When together, the minor alleles of two SNPs, in *IL6r* and *C9* genes, were linked to higher global amyloid load and with increased CSF $A\beta/p$ -tau ratio.

6.1.2 Amyloid-related blood biomarker

In Chapter 4 we showed that amyloid pathology is associated with plasma NfL in the presymptomatic phase of AD, whilst in later stages, when amyloid accumulation is thought to reach a plateau, NfL is linked to NFT pathology. In this study we also showed that NfL-related gray matter atrophy in preclinical phase only present in *APOE*-e4 carriers. In Chapter 5 we provided more evidences of NfL as a biomarker of amyloid-related neurodegeneration by showing how it associates with [¹⁸F]FDG PET in amyloid positive individuals. Longitudinally, metabolic decline over 24 months was only associated with plasma NfL in amyloid positive and cognitively impaired subjects.

6.2 Discussion and future directions

6.2.1 Genetic biomarkers of amyloid pathology

In our two initial studies we have provided supporting evidences that impairments in amyloid clearance pathways may lead to increased brain amyloidosis. As previously introduced, in sporadic AD, despite recent proposition of alternative disease models ²⁰⁸, impairments in amyloid clearance pathways have been thought to be the predominant detrimental effect triggering the cascade of events leading to disease ²⁰⁹. There are several evidences from genetic, *in vivo, postmortem* and *in vitro* studies, presenting mechanisms that may clear A β from the brain ²¹⁰, including (1) phagocytosis, (2) proteolytic degradation, (3) neuronal reuptake as well as transport through brain-blood barrier (BBB) via (4) CSF or (5) capillaries ^{31, 211-214}. The A β phagocytosis is promoted by glial activation, whose role has been reinforced by studies

showing reduction in amyloidosis as a result of A β immunization (for review see ²¹¹. The clearance of A β by proteolytic degradation is mediated by several enzymes working cooperatively from different compartments and metabolizing specific assembly states of amyloid ²¹⁴. Taking this into consideration, we chose to perform our genetic studies based on pathways leading to amyloid clearance.

In genetic studies, although over 30 loci have been associated with AD, only few of them have been also associated with amyloid clearance ^{208, 215}. The *APOE-e4* is the strongest genetic factor associated with AD and its protein has been shown to transport A β through the BBB at a reduced rate as compared to the other APOE isoforms ²¹⁶. Together with *APOE*, the genes *CLU*, *PICALM* and *ABCA7* are potentially involved with A β clearance at the BBB level ²¹⁷⁻²¹⁹. In addition to those, immune-related genes are suspected to lead to microglial dysfunction, modulating A β degradation/aggregation. These genes include *TREM2*, *ABI3*, *CD33* and *PLCy2* ^{172, 173, 220}. Thus, genetic studies have been reinforcing the role of clearance pathways on amyloid build up. However, more studies are needed to understand the complete scenario.

Our findings then corroborate with these lines of evidence. In our first study we proposed the investigation of a poorly explored mechanism by which A β could be cleared or metabolized. As presented in Chapter 2, CYP450 proteins are involved in several cascades that metabolize endogenous compounds ^{221, 222}. Studies have also provided evidences that A β is able to interact with CYPs ^{223, 224}. We then evaluated if amyloid load could be associated with polymorphisms in a few CYP genes. Indeed, we found a SNP in the *CYP2C19* gene that showed a protective effect against amyloid deposition, indexed by different methodologies in two independent cohorts. We could also link the *CYP2C19* polymorphism with amyloid-related cognitive decline in both cohorts.

Based on previous reports we identified two main possible mechanisms by which A β could be affected by CYP2C19 (Figure 6-1). A β was shown to trigger POR , which can activate CYP catabolic activity ²²³. No study has so far delineated pathways showing how CYP catabolic function affects Alzheimer's disease. One possible mechanism can be the catabolism of estrogens by CYP2C19, which would lead to decreased levels of estrogen and, consequently, increased susceptibility to present Alzheimer's disease pathophysiology profile ²²⁵. Based on our results, we proposed that the minor-allele of CYP2C19 SNP could lead to an increased structural incompatibility between the enzyme and its substrate or could cause a reduced expression of this CYP protein in the brain, causing the protective phenotype displayed by minor-allele carriers. Alternatively to this first framework proposed, CYP2C19 epoxygenase function can also affect amyloid- β levels by changing the levels of EET production, which is thought to be beneficial for the central nervous system ²²². When the arachidonic acid is metabolized through other cascades, there is a reduction of EET and an increase in amyloid accumulation and neuroinflammation ²²⁶. The amyloid, in turn, is able to reduce epoxygenase function, reinitiating the cycle ²²⁷. We hypothesize that, in this case, CYP2C19 minor-allele carriers may have an increased expression of this gene, enabling the maintenance of the EETs production and, consequently, its beneficial functions.

We did not find differences in the genotype-phenotype association between diagnostic groups. This suggests that the gene effect has a static or conserved effect throughout the disease process. As expected, based on the biological nature of AD, effect size calculations indicate a modest effect of the reported *CYP2C19* SNP on amyloid load. This corroborates current framework that reinforces the joint use of several genetic biomarkers instead of their individual

Figure 6-1 Hypothetical model showing the relationship between Aβ and CYP2C19 activity.



In the first model (I) we present the mechanism by which $A\beta$ could be associated with CYP2C19 and how it would lead to even more $A\beta$ deposition. In the second (II) model, we propose how the reduced epoxygenase effect (which could include CYP2C19) leads to a feed-forward loop that increases $A\beta$ accumulation.

use in multifactorial diseases like AD ²²⁸. In fact, in biological systems, genes are likely to work in connection between themselves rather than working isolated. This biological reasoning is what motivated the choice of the second study, presented in Chapter 3.

In order to unveil new associations between amyloidosis and genetic biomarkers we tried an alternative approach: epistasis analysis. Based on the well-known participation of immune factors related to the clearance of amyloid, we selected some candidate genes from the literature and then checked whether their interactive effects would have any impact on amyloid deposition.

Indeed, we showed that amyloid load is related to specific combinations of genotypes from the *IL6r* and *C9* genes, regardless of the diagnosis. When both minor alleles of the associated SNPs are together, subjects are found to have more amyloid pathology. In contrast, when the major allele of the *C9* SNP is found together with the minor allele of *IL6r* SNP, subjects display the lowest amyloid burden of all genotype combinations. Results were initially shown using amyloid PET as a proxy of the amyloid load and then confirmed using CSF Aβ/p-tau ratio.

Our findings support a link between immune related genes suggesting that their pathways can be interconnected or that their cumulative effect is somehow potentiated causing increased amyloid burden. In fact, previous reports have shown that IL6 is able to stimulate *C9* mRNA expression in *post-mortem* human astrocytes and neuroblastoma cells, supporting a link between them ^{229, 230}. This is probably the major strength of the method, to be able to detect or suggest new genetic factors or pathways that lead to a specific phenotype. However, this is also a limitation of the method. The fact that statistical associations were found does not guarantee that biological interactions exist, which means that the interpretation of how these proteins are in fact associated with each other in the tissue environment is merely speculative and demand further molecular analyses.

As a whole, our results then shed light on other candidate genes and pathways affecting amyloid clearance and as consequence, amyloid build up in AD. Moreover, we reinforced the utility of using genetic information not only to predict future outcomes but also to discover biological factors involved in promoting pathological changes. In addition, the use of intermediate phenotypes of the disease, as done here with the AD-related amyloidosis, increases the statistical power to detect associations. Due to the "closer" relationship between
gene and proteins, genetic variants are better associated with endophenotypes representing specific disease pathophysiological processes than to clinical phenotypes. Specifically in the case of AD, in which misdiagnosis corresponds to approximately 10-30% of the clinical cases, not using the diagnostic status as outcome measure also increases statistical power. Thus, the use of the pathological phenotype rather than clinical diagnosis is also another strength of our study.

Notwithstanding, genetic studies also encounter limitations intrinsic to the method. Despite the relatively static nature of the genetic sequence, gene expression is susceptible to several modulations that can happen at gene level (1), with epigenetic modifications, at the transcriptional level (2), with the alternative splicing generating different isoforms of the same gene and, at the translational level and post-translational level (3), with modifications that lead to alterations in the protein structure. The complexity of this machinery imposes challenges in genetic studies, as these normally consider the associations between the DNA sequences and protein levels or pathological phenotypes. Moreover, studies have to take into consideration the genetic background of the population analyzed, as this can be a confounding factor in the analysis ²³¹. On top of that, in cases of complex diseases such as AD, where several mechanisms interplay to cause the pathophysiological phenotypes, genetic studies will not find one or few causative genes of the disease. Instead, several genes with small effect sizes are expected to act in combination and thus lead to pathological changes and disease presentation ^{164, 165}, what brings a methodological challenge.

Thus, to be able to use the genetic information as biomarker for AD and enable personalized risk assessment, researchers have focused on creating genetic risk scores (GRS). GRS provide a cumulative risk derived from joining contributions of the many genetic variants associated

with a disease. This personalized score would predict one's odds of being affected by a particular trait. Multiple studies have attempted to create GRS to predict AD ^{232, 233}, but further investigation is needed to verify if the method can be applied in the clinical practice²³⁴. Another possibility of using genetic data for disease prediction, or detection, is to combine together the information of different types of biomarkers, such as genetic and blood for example. Considering the improved feasibility of this approach, we decided to proceed with our studies following similar line of investigation.

6.2.2 Amyloid-related blood biomarker

Regarding fluid biomarkers, the most studied and, so far, most reliable biomarker of amyloid pathology is CSF A β_{1-42} . However, the application of this biomarker in the context of AD is not simple. Although CSF A β_{1-42} has a good predictive power and is able to differentiate AD and MCI from CN ^{133, 135, 235, 236}, several neurodegenerative disorders have presented reduced A β_{1-42} levels similarly to what is described in AD (for review see²³⁷) what reduces the specificity of this biomarker. One alternative to this limitation is the use of CSF A $\beta_{1-42}/A\beta_{1.40}$ ratio. In addition to correcting for individual baseline differences ¹⁴⁰, this ratio is reduced in AD but not in controls or other conditions ²³⁷. This composite biomarker also demonstrates good performance in predicting AD pathology in MCI subjects ¹⁴⁰. Yet, the major constraints about the use of CSF A β_{1-42} and A $\beta_{1-42}/A\beta_{1-40}$ are the inter-laboratory variability (1), that challenges the definitions of broadly used cut offs, and the difficulty of routine and repetitive collection of CSF in primary care centers (2).

Blood biomarkers are then warranted to overcome the need of collecting CSF. Until present days blood amyloid biomarkers were not successful, showing a lack of correlation with the corresponding CSF analog ²³⁸⁻²⁴⁰. Reasons for that are being evaluated and it is speculated if the lack of association is due to limitations in the sensitivity of the current blood assays or if peripheral A β originate from sources other than the nervous central system ¹⁹. Alternatively, the marked technological improvement in methods of protein quantification has allowed untargeted proteomic detection in plasma, which has been used to discover proteins linked to a particular phenotype. This approach also enables the composition of a "protein panel", which is a set of proteins found to be associated with the trait under investigation. Then, similar to genetic risk scores, the plasma biomarker panel is a classifier that provides a personalized phenotypic status. Specifically regarding amyloid pathology, a study identified a plasma protein classifier, with 10 protein features plus *APOE*-e4 count and age, that well predicted chances of CU and CI individuals being amyloid positive based on amyloid PET status (produced a testing AUC_{CU} of 0.891, specificity = 0.77, sensitivity = 0.78) ²⁰⁷. Despite the very promising use of this tool, this panel would probably need refinement, simplification, and undoubtedly validation in independent cohorts.

The plasma biomarker panel, from the study just mentioned above ²⁰⁷, identified 10 proteins in the plasma of CU subjects that were associated with amyloid status. Included in these "potential" early biomarkers of amyloid pathology was the NfL protein. The NfL is a neuronal protein mostly located in large axonal fibers ²⁴¹ and has been reported to be associated with neurodegeneration in several conditions such as Huntington's disease, posterior supranuclear palsy, amyotrophic lateral sclerosis, frontotemporal dementia and others ^{242, 243}. In the context of AD, CSF and plasma NfL are in higher concentrations in MCI and AD subjects as compared to control and have been correlated with CSF A $\beta_{1.42}$ ^{148, 149, 151, 244, 245}. Moreover, a longitudinal study with *APP* and *PSEN1* mutation carriers provided further support to the use of NfL as biomarker of early neurodegeneration in presymptomatic AD ^{150, 246}. Considering the potential of NfL as an early biomarker of AD and amyloid pathology, we sought to contribute to current knowledge and investigate how plasma NfL relates to amyloid pathology, indexed by PET imaging, as well as with other AD-related imaging biomarkers in conjunction with AD's largest genetic factor, the *APOE*-e4 allele.

In Chapter 4 we evaluated the relationship between amyloid load, indexed by PET, and plasma NfL and. Using data from two cohorts, which also means two different radiotracers, we showed that NfL is associated with amyloid PET in regions previously reported to have amyloid deposition in AD. Interestingly, this association was only present in cognitively unimpaired subjects (CU). When we checked the association between NfL and tau PET an opposite pattern was revealed: the NfL-NFT association was detected only in cognitively impaired (CI) individuals and in regions that typically present NFT pathology in AD. Overall, these results indicate that early neurodegeneration, represented by NfL, is initially caused by amyloidosis. In later stages, amyloid accumulation reaches a plateau, and probably because of the reduced variation of this biomarker, the associations with the continuously increasing NfL levels are lost. Because tau PET goes in the opposite direction (is barely detected in CU and continues to increase in CI over time ²⁴⁷), the relationship with NfL is only detected when cognitive impairments are observed. These results are supported by the amyloid cascade hypothesis and by the biomarker model of AD, that suggest amyloid pathology as the main initiator of neurodegenerative events.

In the same study we also used longitudinal data to investigate how plasma NfL relates with gray (GM) and white matter (WM) atrophy. We showed that NfL is associated with gray matter atrophy in both CU and CI groups in regions that resemble the tau PET pattern, reinforcing the

closer link between NFT and neurodegeneration ^{248, 249}. Interestingly, we saw that in CU NfL was only associated with GM atrophy in *APOE-e4* carriers whilst in CI both carriers and noncarriers displayed reduced GM volume (Figure 6-2). This finding suggests that *APOE-e4* is indexing amyloid pathology in CU but not necessarily in CI. In fact, *APOE-e4* carriage shows to induce earlier and faster amyloid deposition, what is clearly evidenced in CN ²⁵⁰. In CI this relationship is not so straightforward as approximately half of the subjects are *APOE-e4* carriers but all of them have ongoing neurodegeneration ²⁵¹. Longitudinally, as compared to baseline, we saw yearly increases in NfL-related GM atrophy in CI but in CU a difference was only seen at month 48.

NfL was also associated with WM atrophy in both CU and CI, but much more extensively in the latter group. Longitudinally, WM atrophy seem to start in periventricular regions in CU, reaching temporal regions after 48 months. Distinctively, WM atrophy in seen primarily in temporal regions and then progressively spreads to superior brain regions being widespread after 48 months. Interestingly, if CU and CI WM results are observed in conjunction, they nicely seem to compose a continuum of age and then disease-related atrophy. Moreover, considering that NfL levels are understood to reflect primarily axonal damage 241 , the fact that WM results were more extensive than the GM ones suggests that WM loss precedes GM damage. Previous reports support this concept as CSF A β changes were shown to predict WM but not GM integrity in CU subjects at risk for AD $^{252, 253}$.

Continuing with this line of investigation, in Chapter 5 we evaluated for the first time the relationship between plasma NfL and voxel-wise [¹⁸F]FDG PET as surrogate of neurodegeneration in amyloid positive and negative subjects. We first reported that, cross-sectionally, CSF and plasma NfL present very similar associations with [¹⁸F]FDG. This



Figure 6-2 Associations between plasma NfL and amyloid PET or GM volume.

Visual comparison of the voxel-based associations found with plasma NfL in CU subjects. The two firsts T-statistical maps show brain regions where plasma NfL is associated with amyloid PET in CU subjects from two cohorts. The third map shows regions where NfL is associated with gray matter atrophy in CU *APOE-e4* carriers.

reinforces previous findings that CSF and blood measurements are correlated and thus represent the same phenomena ²⁵⁴. Additionally, using longitudinal data, NfL was associated with reduced glucose uptake, predominantly in amyloid positive subjects, in both CI and CU groups. In CI, affected areas corresponded to regions where NfL was linked to amyloid and tau PET in our previous study, which means AD-related areas. Over time, increasing hypometabolism over 24 months was only detected in CI amyloid positive subjects.

Figure 6-3 Associations between plasma NfL and imaging markers of neurodegeneration .



Visual comparison of the voxel-based associations found with plasma NfL. The first T-statistical map shows brain regions where plasma NfL is associated with gray matter atrophy in CU *APOE-e4* carriers. The other two maps show regions where NfL is associated with [¹⁸F]FDG PET in amyloid positive CU and CI subjects.

As a whole, findings from Chapter 5 support that, in AD spectrum, NfL reflects neurodegeneration associated to amyloid and NFT pathology. Specially for the CU, the fact that NfL was associated with [¹⁸F]FDG mostly in amyloid positive individuals probably reflects the same relationship initially found between NfL and GM atrophy in *APOE-e4* carriers in the previous study. These results also add the knowledge that NfL might be not only indexing tissue atrophy but also reflecting, at some extent, cell death, taking into consideration that regions associated with [¹⁸F]FDG do not perfectly overlap those associated with GM and WM loss.

6.3 Conclusion

The studies included in this thesis shed light in the complexity of defining biomarkers for amyloid pathology. Several amyloid-therapy trials for AD have failed partly because of the flawed choice of the target population but also because of the lack of precise measurements of pathological changes over time. As previously discussed, it is unfeasible and costly to use PET and CSF biomarkers for population enrichment and monitoring drug effect in clinical trials. The use of genetic and blood biomarkers are ideal in this scenario as they are much less expensive and more accessible. Additionally, the early detection of the population at risk of developing AD is essential to prevent the irreversible neuronal damage present in symptomatic phases.

In this context, our studies corroborated with the current knowledge by investigating possible biomarkers reflecting amyloid pathology. In our initial reports, in **Chapters 2 and 3**, although the amyloid-associated genetic variants discovered here did not have effect enough to be used as isolated biomarkers, they provided evidences of more complex mechanisms of amyloid clearance that deserve further investigation. Understanding the pathways involved with the trait of interest allows the investigation and development of new biomarkers.

Findings from **Chapters 4 and 5** confirmed the use of plasma NfL as biomarker of neurodegeneration and they indicated that NfL reflects amyloid pathology in early stages of the AD spectrum. We also corroborated with the current literature proposing the use of combinations of biomarkers in AD as we suggest that plasma NfL, together with APOE-e4 carriage status, could indicate cognitively unimpaired individuals with amyloid-related

neurodegeneration, which merits further investigation for possible use in population enrichment in clinical trials. Overall, blood-based measures offer great potential for detection of brain pathological changes, improving definition of more accurate diagnosis and enabling better performance of drug therapies, which hopefully will bring an effective treatment for AD.

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