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# An overview of blood-based biomarkers in AD

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[Date]

# An Overview of Blood-Based Biomarkers in AD

## PhD Thesis

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**Declaration**

I certify that this thesis does not, to the best of my knowledge and belief:

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**Abstract (500 words)**

Alzheimer's disease (AD) is the most common form of dementia in the elderly whose main neuropathological features are the presence of extracellular senile plaques in the brain and the intracellular accumulation of hyperphosphorylated tau filaments. However, a relatively cheap and non-invasive method for the diagnosis of AD remains elusive. Recent studies have indicated that cerebral biochemical changes take place decades before the clinical onset of the disease, but current methodologies, brain scan (PET amyloid imaging) and cerebrospinal fluid (CSF) analysis, are unsuited for community-wide screening. Brain scanning methods non-invasively assess amyloid load but are extremely expensive and cannot be used for clinical routine analyses. Conversely, CSF analysis measuring specific AD-related biomarkers (Aβ and tau) are invasive and require trained personnel. While these methodologies are considered the best options for identifying individuals at risk for AD, neither technique is suitable for community-wide screening. These problems could be solved by developing an AD-related blood-based biomarker panel that could reflect the amount of amyloid deposition in the brain, identifying individuals at risk for AD, thereby bypassing the need for current methodologies. This blood analysis would be a cheap and non-invasive community screen, and thus suitable for use in clinical pathology laboratories.

In this thesis, I am presenting my work on specific blood-based AD-related biomarkers. The first part of the thesis is focused on High Density Lipoprotein (HDL) subclasses and its protein cargo. Previous studies have indicated that small HDL subclass is linked to anti-inflammatory and anti-oxidative features, while many of the molecules associated to HDL, have been related to AD. My initial analysis has indicated that protective small HDL subclass is reduced in AD and positively correlate with cognitive functions. Furthermore, I have also isolated HDL particles and shown that HDL composition is changed in AD, with a significant increase of cholesterol/ApoA-I and ApoD/ApoA-I ratios and reduced ApoA-II/ApoA-I ratio.

Additionally, cholesterol/ApoA-I ratio is also positively associated to increased ventricular volume, while ApoA-II/ApoA-I and ApoJ/ApoA-I ratios are positively associated with grey matter volume, hippocampal volume and are negatively associated with ventricular volume. Taken together, these data indicate that plasma HDL composition is related to brain volumetric data and has the potential to be used as part of a broader AD-related biomarker panel.

In the second part of this thesis, in collaboration with colleagues, I have evaluated the levels of specific AD-related biomarkers (Aβ1-42, Aβ1-40, t-tau, p-tau181, glial fibrillary acidic protein (GFAP) and neurofilament light chain (NFL)) in two different cohorts. Our results have indicated that Aβ1-42/Aβ1-40 ratio, p-tau181 and GFAP levels are reliable biomarkers for identifying individuals with ongoing brain amyloidosis and could also be used in a broader AD-related biomarker panel.

While additional studies are necessary, these early data indicate that the development of a specific biomarker panel for AD is achievable. This discovery would allow for the detection of individuals at risk for AD before the clinical onset of the disease, thereby allowing for early medical intervention(s).

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**List of publications**

**Chapter 2: Current and Prospective Treatments for Alzheimer's Disease (and Other Neurodegenerative Diseases). Pedrini S**, Morici M, Martins RN. Neurodegeneration and Alzheimer's Disease: The role of Diabetes, Genetics, Hormones, and Lifestyle, Chapter 14, 391-442, May 2019.

**Chapter 3: High-density lipoprotein-related cholesterol metabolism in Alzheimer's disease. Pedrini S**, Chatterjee P, Hone E, Martins RN. J Neurochem. 2021 Oct;159(2):343-377. doi: 10.1111/jnc.15170. Epub 2020 Oct 27. Review.

**Chapter 4: Plasma High Density Lipoprotein Small Subclass is Reduced in Alzheimer's Disease Patients and Correlates with Cognitive Performance. Pedrini S,** Hone E, Gupta VB, James I, Teimouri E, Bush AI, Rowe CC, Villemagne VL, Ames D, Masters CL, Rainey-Smith S, Verdile G, Sohrabi HR, Raida MR, Wenk MR, Taddei K, Chatterjee P, Martins I, Laws SM, Martins RN; AIBL Research Group. J Alzheimers Dis. 2020;77(2):733-744.

**Chapter 5: Plasma high density lipoprotein cargo proteins are altered in Alzheimer's disease and is associated with regional brain volume. Pedrini S**, Doecke J, Hone E, Wang P, Thota R, Bush AI, Rowe CC, Dore V, Villemagne VL, Ames D, Rainey-Smith S, Verdile G, Sohrabi HR, Raida MR, Wenk MR, Taddei K, Ehrlich M, Gandy S, Masters CL, Chatterjee P, and Martins RN and the AIBL Research Group. J Neurochem. 2022 Oct;163(1):53-67.

**Chapter 6: Diagnostic and prognostic plasma biomarkers for preclinical Alzheimer's disease.** Chatterjee P, **Pedrini S**, Ashton NJ, Tegg M, Goozee K, Singh AK, Karikari TK, Simrén J, Vanmechelen E, Armstrong NJ, Hone E, Asih PR, Taddei K, Doré V, Villemagne VL, Sohrabi HR, Zetterberg H, Masters CL, Blennow K, Martins RN. Alzheimers Dement. 2022 Jun;18(6):1141-1154.

**Chapter 7: Plasma Aβ42/40 ratio, p-tau181, GFAP, and NfL across the Alzheimer's disease continuum: A cross-sectional and longitudinal study in the AIBL cohort.** Chatterjee P#, **Pedrini S**#, Doecke JD, Thota R, Villemagne VL, Doré V, Singh AK, Wang P, Rainey-Smith S, Fowler C, Taddei K, Sohrabi HR, Molloy MP, Ames D, Maruff P, Rowe CC, Masters CL, Martins RN and the AIBL Research Group. Alzheimer's Dement. 2022 (In press). # These authors contributed equally

**Other publications published or submitted during the thesis period not included in this thesis**

**Plasma glial fibrillary acidic protein as a marker for amyloid-β associated cognitive deterioration: A study in the Dominantly Inherited Alzheimer Network.** Chatterjee P, Vermunt L, Gordon BA, **Pedrini S**, Boonkamp L, Armstrong NJ, Xiong C, Singh AK, Li Y, Sohrabi HR, Taddei K, Molloy M, Benzinger TLS, Morris JC, Karch CM, Berman S, Chhatwal J, Chui H, Cruchaga C, Graff-Radford NR, Day G, Farlow M, Fox NC, Goate AM, Hassenstab J, Lee J, Levin J, McDade E, Mori H, Noble J, Perrin R, Salloway S, Sanchez-Valle R, Schofield P, Levey A, Jucker M, Masters CL, Fagan AM, Bateman RJ, Martins RN, Teunissen C. Alzheimer's Dement. 2022 (Submitted).

**Plasma glial fibrillary acidic protein is associated with 18F-SMBT-1 PET: two putative reactive astrogliosis biomarkers for Alzheimer's disease.** Chatterjee P, Doré V, **Pedrini S**, Krishnadas N, Thota R, Bourgeat P, Ikonomovic MD, Rainey-Smith SR, Burnham SC, Fowler C, Taddei K, Mulligan R, Ames D, Masters CL, Fripp J, Rowe CC, Martins RN, Villemagne VL. J Alzheimers Dis. 2022 (Submitted).

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**The Association Between Alzheimer's Disease-Related Markers and Physical Activity in Cognitively Normal Older Adults. Pedrini S**, Chatterjee P, Nakamura A, Tegg M, Hone E,

Rainey-Smith SR, Rowe CC, Dore V, Villemagne VL, Ames D, Kaneko N, Gardener SL, Taddei K, Fernando B, Martins I, Bharadwaj P, Sohrabi HR, Masters CL, Brown B, Martins RN. Front Aging Neurosci. 2022 Mar 28;14:771214.

**Plasma Amyloid-Beta Levels in a Pre-Symptomatic Dutch-Type Hereditary Cerebral Amyloid Angiopathy Pedigree: A Cross-Sectional and Longitudinal Investigation.** Chatterjee P, Tegg M, **Pedrini S**, Fagan AM, Xiong C, Singh AK, Taddei K, Gardener S, Masters CL, Schofield PR, Multhaup G, Benzinger TLS, Morris JC, Bateman RJ, Greenberg SM, van Buchem MA, Stoops E, Vanderstichele H, Teunissen CE, Hankey GJ, Wermer MJH, Sohrabi HR, Martins RN, The Dominantly Inherited Alzheimer Network. Int J Mol Sci. 2021 Mar 13;22(6):2931.

**Plasma glial fibrillary acidic protein is elevated in cognitively normal older adults at risk of Alzheimer's disease.** Chatterjee P, **Pedrini S**, Stoops E, Goozee K, Villemagne VL, Asih PR, Verberk IMW, Dave P, Taddei K, Sohrabi HR, Zetterberg H, Blennow K, Teunissen CE, Vanderstichele HM, Martins RN. Transl Psychiatry. 2021 Jan 11;11(1):27.

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**Plasma neurofilament light chain and amyloid-β are associated with the kynurenine pathway metabolites in preclinical Alzheimer's disease.** Chatterjee P, Zetterberg H, Goozee K, Lim CK, Jacobs KR, Ashton NJ, Hye A, **Pedrini S**, Sohrabi HR, Shah T, Asih PR, Dave P, Shen K, Taddei K, Lovejoy DB, Guillemin GJ, Blennow K, Martins RN. J Neuroinflammation. 2019 Oct 10;16(1):186.

**Ultrasensitive Detection of Plasma Amyloid-β as a Biomarker for Cognitively Normal Elderly Individuals at Risk of Alzheimer's Disease.** Chatterjee P, Elmi M, Goozee K, Shah T, Sohrabi HR, Dias CB, **Pedrini S**, Shen K, Asih PR, Dave P, Taddei K, Vanderstichele H, Zetterberg H, Blennow K, Martins RN. J Alzheimers Dis. 2019;71(3):775-783.

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**Statement of contribution of others**

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# Chapter 1

## Background

## 1.1 Alzheimer's Disease

Alzheimer's Disease (AD), which is the most common form of dementia in the elderly, is a progressive neuropathological disease that was first reported by Alois Alzheimer in 1907 in his article "Uber eine eigenartige Erkankung der Hirnrinde" and subsequently translated [1]. AD symptoms include memory loss, altered behaviour and hallucination among others. In the later stages of the disease individuals are in constant need of care because of their inability to perform daily tasks. The disease is characterized by two major neuropathological features, (a) the presence of the extracellular deposition of amyloid β (Aβ) in the brain in the form amyloid plaques and (b) the presence of intracellular accumulation of hyperphosphorylated tau filaments [2]. Overtime, the continuous accumulation of Aβ and hyperphosphorylated tau leads to neuronal death and brain atrophy. Unfortunately, there is no cure for AD.

## 1.2 Sporadic AD vs Familial AD

AD can be classified into two main categories, sporadic AD, with a late onset (>65 year old) and familial AD, with an early onset (<65 year old). Sporadic AD is by far the most common form of the disease, accounting for more than 95% of all cases of AD. While there are no know causes for this form of the disease, many risk factors have been associated to its onset. Among the known risk factors, diseases such as diabetes and metabolic syndrome, reduced physical activity, diets rich in fat/cholesterol and cardiovascular diseases are the most common [3-9]. Genetically, the presence of the APOE ε4 allele is also the most common risk factor for AD [10-12].

With regards to the familial AD, specific mutations on three genes (APP, PS1 and PS2) have been associated with the onset of the disease at an earlier age. For APP, more than 60

mutations have been described, however not all mutations are considered pathogenic, but usually, those mutations that are associated with familial AD are found near the 3 major cleavage sites of Aβ (α-, β- and γ-secretases) and are usually associated with increased production of the toxic Aβ42 form, albeit in some case the mutations affect the extent of Aβ oligomerization and/or the rate of Aβ degradation. Among the mutations that have been associated with familial AD, Swedish (KM670/671NL), Flemish (A692G), Arctic (E693G), Dutch (E693Q), D694N (Iowa), V717F (Indiana) and V717I (London) [13-19]. In addition, many of the AD-predisposing mutations also increase the extent of Cerebral Amyloid Angiopathy (CAA).

Mutations in the PS1 gene are by far more frequent and aggressive, causing around the 75% of familial AD cases with an age of onset as early as 40 years [20]. On the other hand, mutations in the PS2 gene are rare, representing only the 5% of all familial AD cases and also less aggressive, with an age of onset of 60 years [20]. More than 300 PS1 and 80 PS2 mutations have been discovered, and as for APP, not all mutations are considered pathogenic. However, unlike for APP, mutations in the PS1 and PS2 genes are spread across the entire molecule and not confined in a specific region. Pathogenic mutations, like those used in animal models such as P117L, M139T, M146L, H163R, A246E and L286V (for PS1) and N141I (for PS2) which represent a minimal part of all PS1 and PS2 mutations, tend to alter the production of Aβ42 and to affect the Aβ42/Aβ40 ratio [21-24].

Animal models of the disease used in laboratory experiments include either include a single or a combination of all major APP/PS1/PS2 mutations. Among the most common and widely used mice models are 5xFAD (APP: KM670/671NL, I716V, V717I; PS1: M146L, L286V), APPDutch (APP: E693Q), APP23 and Tg2576 (APP: KM670/671NL), APP/PS1 (APP: KM670/671NL; PS1:A246E), APP/PS1 (APP: KM670/671NL; PS1: Δexon9), TgCRND8

(APP: KM670/671NL, V717F), APP/PS2 (APP: KM670/671NL; PS2: N141I) and TgSwDI (APP: KM670/671NL, E693Q, D694N).

## 1.3 Processing of APP and generation of toxic Aβ

Aβ is a proteolytic fragment, usually 40-42 amino acids long, generated from a longer trans-membrane protein – the Amyloid Precursor Protein (APP). APP is a trans-membrane protein that is coded on chromosome 21 and, through alternative splicing, three different isoforms, APP695, APP751 and APP770, are generated. While APP751 and APP770 are ubiquitously expressed, APP695 is preferentially expressed in the brain [25-27]. Overall, APP is cleaved via two major pathways. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, at a site in the middle of the Aβ sequence; this is followed by the γ-secretase cleavage. This pathway does not generate Aβ, instead produces secreted/soluble APP cleaved at the α-cleavage site (sAPPα), the small proteolytic fragment p3, and a C-terminal fragment (often referred to as the APP intracellular domain or AICD). In the alternative amyloidogenic pathway, APP is first cleaved by β-secretase (known as β-site APP cleavage enzyme-1 BACE-1), and the subsequent cleavage by γ-secretase generates the Aβ peptide and sAPPβ (as well as the C-terminal fragment) and this is considered the pathological amyloidogenic pathway (see figure 1). In general, therapies aimed at these three secretases are aiming to increase α-secretase activity and/or inhibit the β or γ-secretase activities. As already indicated, Aβ is a 4.5 kDa peptide and is the main component of the amyloid plaques in AD brain. Aβ42 displayed high tendencies of aggregating in neurotoxic species and is therefore extremely relevant in the progression of AD (Burdick et al., 1992, Jarrett et al., 1993). It is known that Aβ42 monomers, sequentially aggregates before becoming senile plaques. These intermediate aggregating steps include the formation of small oligomers, high molecular

weight oligomers, protofibrils and fibrils [28-32]. Recent findings have highlighted the importance of small oligomers, rather than senile plaques, as effectors of neurotoxicity in the brain [33, 34]. Their role in the disease has been investigated and it has been reported that small oligomers (mostly dimers, trimers and dodecamers) are responsible for neuronal dysfunction, play an extensive role in neurotoxicity and synaptic degeneration and their levels correlate with the progression of the disease [35-40]. Studies in transgenic mice have supported the importance of small oligomers promoting neurotoxicity, by indicating that mice expressing oligomers display synaptic degeneration and neuronal death in the absence of senile plaques [41]. To further confirm that oligomeric assembly plays an important part in the early stage of the disease, other studies performed on transgenic mice reported that Aβ oligomers are associated with cognitive defects [42, 43]. Reduction of oligomer production also reduces the synaptotoxicity and increases long-term potentiation [32, 44]. Additionally, the assembly of Aβ oligomers, which is followed in the long term by the formation of senile plaques, have been reported to increase the extent of inflammation in the areas surrounding senile plaques, with increased activation of astrocytes and microglia [45-49]. These inflammatory events eventually increased the presence of inflammatory molecules, such as cytokines and chemokines, in the proximity of the plaques, causing further neuronal damage [50-54]. For these reasons, several studies in mouse models of AD involving therapeutic peptides that targeted Aβ oligomerization, reported in some cases improved cognitive functions [55-64].

### 1.3.1 α-secretase

In the non-amyloidogenic pathway, α-secretase is the enzyme responsible for cleaving the APP molecule at the position Lys612-Leu613 of the APP695 protein (corresponding to the sequence Lys16-Leu17 of the Aβ fragments) [65]. Such cleavage, which prevents Aβ

formation by cutting the hypothetical Aβ fragment in the middle, then generates sAPPα and C83 (or CTFα). Accordingly, in AD individuals carrying the Swedish mutation, reduced levels of sAAPα were observed [66]. The subsequent cleavage of C83 by γ-secretase then generates p3 and AICD (Figure 1 – non amyloidogenic pathway) [67]. Initially, three members of the A Disintegrin And Metalloproteinases (ADAM) members were thought to be α-secretases, ADAM-9, ADAM-10 and ADAM-17 [68-72]. However, further studies indicated that ADAM-10 is the main enzyme responsible for the cleavage of APP in neurons and studies in transgenic mice confirmed the importance of ADAM-10 as α-secretase involved in APP cleavage [73-76].

### 1.3.2 β-secretase

In the amyloidogenic pathway, β-secretase (β-site APP-cleaving enzyme – BACE) is the enzyme responsible for cleaving the APP molecule at the position Met596-Asp597 (corresponding to the sequence Met-Asp1 of the Aβ fragments) [77-79]. This cleavage, which does not cut Aβ in the middle of its sequence, generates sAPPβ and C99 (or CTFβ). Subsequently, γ-secretase cleavage of C99 then generates Aβ and AICD (Figure 1 – amyloidogenic pathway). In accordance, increased β-secretory activity was observed in AD patients [80, 81]. Albeit BACE exists in two isoforms (BACE1 and BACE2) which shares more than 60% of homology, BACE2 involvement in Aβ generation is minimal as its expression in the brain is relatively low [82]. In transgenic mouse model of AD, BACE deficiency restored memory deficits and led to negligible Aβ production [83, 84].

### 1.3.3 γ-secretase

γ-secretase is the final enzyme in APP processing responsible for the C83 and C99 (CTFα and CTFβ, respectively), to generate p3 and AICD from C83 and Aβ and AICD from C99. The enzyme is composed of 4 different subunits: Presenilins (PS1 or PS2), Presenilin

Enhancer 2 (PEN2), Anterior Pharynx-Defective 1 (APH1) and Nicastrin [85]. While the γ-secretase activity is associated to PS1 and PS2, the other members of the γ-secretase complex (Nicastrin, APH1 and PEN2) are necessary for the stabilization of the complex and for the complete enzyme activity [86-89]. In animal models, expression of mutated PS genes (with mutations causing familial AD) increased Aβ42 expression compared to animals expressing wild type PS genes [90, 91].

## 1.4 The importance of early diagnosis

Currently, over 50 million people worldwide are affected with dementia, of which, as mentioned, Alzheimer's disease represent the most common form. This number is expected to increase to ~131 million by 2050. In Australia, dementia is the second leading cause of death. In addition, the increasing number of individuals affected by the disease would spike up even more the enormous costs for care that are an incredible financial burden weighting on families and governments. As for any disease, early diagnosis would allow for early therapies, which would, at the very least, improve the quality of life of affected individuals and delay their placement in aged-care facilities. Besides an obvious improvement about individual lifestyle, delayed placement in aged-care facilities would incredibly reduce the financial burden associated to it. The potential for preventive therapy based on early diagnosis has been demonstrated in several clinical trials. As of today, early diagnosis can only be performed by brain imaging or analysis of CSF biomarkers, which do manifest ~2-3 decades prior to symptom onset [92]. However, current methodologies are unsuited for community-wide screening for a variety of reasons. Brain imaging can be obtained by scanning methods (e.g., structural and functional MRI or positron emission tomography (PET) of cerebral metabolism with 18F-fluorodeoxyglucose (FDG) and amyloid tracers like

11C-Pittsburgh Compound-B), but they are expensive and cannot currently be used for routine clinical examination. On the other hand, CSF biomarker analysis, which could provide an accurate assessment of AD-related biomarkers, relies on CSF collection by lumbar puncture, an invasive technique that requires trained personnel and carries and inherent risk. It goes without saying that unveiling a blood-based biomarker panel would eventually lead to the development of a which is sorely missing. Such development would close the existing gap and would lead to the development of a cheap, reliable and non-invasive blood-based preclinical diagnostic assay that could detect AD biomarker changes and could be routinely used in clinical labs for identifying those individuals at risk for AD in which early biochemical changes have started but clinical symptoms are yet to appear. By detecting early changes, we would be able to begin preventative therapies long before the onset of the clinical symptoms, when they are likely to be more effective. Early therapies would eventually have two extremely significantly outcomes. The first is that early intervention will delay the onset of the disease in affected individuals, which would therefore greatly improve their quality of life by allowing them to carry on their daily routine for longer and with better results. The second, is such delayed onset of the disease will in turn delay the placement of individuals in nursing homes, greatly reducing all care-associated costs and relieving the financial burden associated with it.

# Chapter 2

## Current and Prospective Treatments for Alzheimer's Disease (and Other Neurodegenerative Diseases)

**Current and Prospective Treatments for Alzheimer's Disease (and Other Neurodegenerative Diseases). Pedrini S**, Morici M, Martins RN. Neurodegeneration and Alzheimer's Disease: The role of Diabetes, Genetics, Hormones, and Lifestyle, Chapter 14, 391-442, May 2019.

Chapter 2 has been published as a book chapter by Wiley in *Neurodegeneration and Alzheimer's Disease: The Role of Diabetes, Genetics, Hormones, and Lifestyle,* and is not available in this version of the Thesis.

The published chapter is available at:

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# PART I

# HDL as a POTENTIAL BLOOD BIOMARKER in ALZHEIMER'S DISEASE

# Chapter 3

## High Density Lipoprotein-related cholesterol metabolism in Alzheimer's Disease

**High-density lipoprotein-related cholesterol metabolism in Alzheimer's disease. Pedrini S**, Chatterjee P, Hone E, Martins RN. J Neurochem. 2021 Oct;159(2):343-377. doi: 10.1111/jnc.15170. Epub 2020 Oct 27. Review.

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REVIEW

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# High-density lipoprotein-related cholesterol metabolism in Alzheimer's disease

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## Abstract

High-density lipoproteins (HDL) are a heterogeneous class of molecules whose main function is to remove excess cholesterol through a mechanism called reverse transport, in which cholesterol is transported from peripheral organs and from arterial foam cells to the liver, where it is subsequently eliminated with bile. While its ability to eliminate excess cholesterol has always been viewed as its main feature, its beneficial effects go beyond this single effect. Many of the proteins that are associated with HDL are responsible for anti-oxidant and anti-inflammatory properties. These proteins that are associated with HDL during its generation and remodelling, are referred to as 'protein cargo', which has been extensively analysed by mass spectrometry analysis in healthy and diseased individuals. In this review, we discuss the pathway that leads to HDL formation and its subsequent remodelling and catabolism with regards to the possible involvement of HDL 'protein cargo' in Alzheimer's disease.

KEYWORDS

Alzheimer's Disease, Amyloid-β, Cholesterol metabolism, HDL, lipid transport

## 1 | INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia in the elderly and the number of affected individuals is set to increase in the next decades. The two main features of AD are represented by extracellular amyloid plaques of amyloid β (Aβ) in the brain parenchyma and intracellular neurofibrillary tangles of tau protein. In the past decades several lines of evidence have indicated that high-density lipoproteins (HDL) are strongly involved in the aetiology and the progression of the disease, although the overall analysis of HDL levels and their association with cognitive functions somehow lacked consensus. Many of the proteins associated with HDL are also linked to AD and Aβ formation/catabolism, some displaying protective features while others do not, it is not surprising the association between HDL levels and AD provided confounding results. It is therefore possible that components of HDL 'protein cargo', more than the HDL levels alone, are responsible for neuroprotection and affect Aβ formation and/or deposition.

**Abbreviations:** ABCA, ATP-binding cassette transporter A; AD, Alzheimer's disease; ADAM10, a disintegrin and metalloproteinase 10; Apo, apolipoprotein; APP, amyloid precursor protein; Aβ, amyloid β; BACE1, β-site APP - cleaving enzyme 1; BBB, blood–brain barrier; CAA, cerebral amyloid angiopathy; CDK5, cyclin-dependent kinase 5; CE, cholesteryl esters; CETP, cholesteryl ester transfer protein; CNS, central nervous system; CSF, cerebrospinal fluid; DKK1, Dickkopf WNT signalling pathway inhibitor 1; EL, endothelial lipase; Erk, extracellular signal-regulated kinase; GWAS, genome-wide association studies; HDL, high-density lipoprotein; HL, hepatic lipase; LC/ESI-MS/MS, liquid chromatography/electrospray ionization tandem mass spectrometry; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LpL, lipoprotein lipase; LTP, long-term potentiation; LXR, liver X receptors; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; MCI, mild cognitive impaired; MMSE, mini mental state examination; MS, mass spectrometry; PAI-1, plasminogen activator inhibitor 1; PLTP, phospholipid transfer protein; PON-1, paraoxonase-1; PPARγ, peroxisome proliferator-activated receptor-γ; PS1, Presenilin1; RXR, retinoid X receptors; S1P, sphingosine-1-phosphate; SAA, serum amyloid A; SR-BI, scavenger receptor-BI; TG, triglycerides; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; VLDL, very low-density lipoprotein; WT, wild-type.

  

## 2 | HDL METABOLISM

### 2.1 | Reverse cholesterol transport and generation of HDL

In reverse cholesterol transport the formation of HDL particles is initiated by the cholesterol transporter ATP-binding cassette transporter A1 (ABCA1), which catalyses the first step in HDL formation, where free cholesterol is added to free Apolipoprotein A-I (ApoA-I) of the nascent HDL. ABCA-1 is a protein that belongs to the ABC family of transporters, it is localized at the cell surface (Neufeld et al., 2001) and it is ubiquitously expressed (Fan et al., 2013; Haghpassand, Bourassa, Francone, & Aiello, 2001; Jeon et al., 2015; Kielar et al., 2001), whereas the intestine and liver are the main sources of plasma ApoA-I (Sliwkowski & Windmueller, 1984). To a minor extent, another member of the ABC transporters, ABCA7, which shows 54% homology with ABCA1 (Kaminski et al., 2000), has been linked to HDL formation. However, compared to ABCA1, ABCA7 seems to be more efficient in regulating the efflux of phospholipids rather than cholesterol (Abe-Dohmae et al., 2004; Hotta, Abe-Dohmae, Taguchi, & Yokoyama, 2015; Quazi & Molday, 2013; Wang et al., 2003). ApoA-I is the main apolipoprotein of HDL in plasma and induces the translocation of cholesterol and phospholipids in the cytosolic lipid-protein particle with phospholipase C involvement (Ito, Li, Nagayasu, Kheirollah, & Yokoyama, 2004; Ito, Nagayasu, Kato, Sato, & Yokoyama, 2002; Ito, Nagayasu, Kheirollah, Abe-Dohmae, & Yokoyama, 2011). ApoA-II is fused together with the nascent lipoprotein A-I (LpA-I) particle to generate LpA-I/A-II molecules, which carry > 95% of plasma ApoA-II (Clay, Pyle, Rye, & Barter, 2000; Gao, Yuan, Jayaraman, & Gursky, 2012; Gauthamadasa et al., 2010). Following this initial step, lecithin:cholesterol acyltransferase (LCAT) converts the free cholesterol into cholesteryl esters (CE) by mediating the transfer of a fatty acid to the hydroxyl group of the cholesterol (Jonas, 2000). This reaction takes place on the surface of HDL particles (Sorci-Thomas, Bhat, & Thomas, 2009) and requires ApoA-I as an activator. However, while ApoA-I is the major activator of LCAT on HDL, other apolipoproteins can also activate it, though to a lesser degree (Steyrer & Kostner, 1988). Conversely, ApoE is the major activator of LCAT activity on low-density lipoprotein (LDL) (Zhao, Thorngate, Weisgraber, Williams, & Parks, 2005). LCAT is mainly produced by the liver, although astrocytes have also shown the capacity to produce it in the brain (Hirsch-Reinshagen et al., 2009; Warden et al., 1989).

### 2.2 | Remodelling of HDL

Upon transformation of free cholesterol into cholesteryl esters, the nascent discoidal HDL molecule becomes a more stable spherical $HDL_3$ beginning a remodelling process that eventually culminates in the generation of larger $HDL_2$ particles. During this process in which several enzymes are involved, HDL-associated proteins are also affected as some of them are specifically associated with $HDL_3$ or

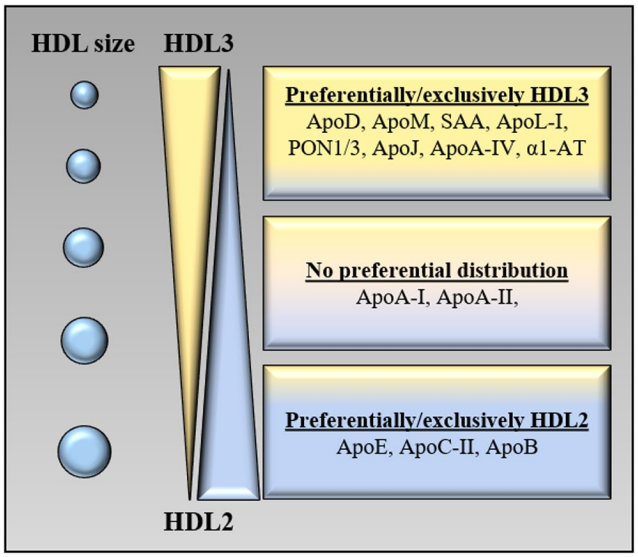


**FIGURE 1** Distribution of high-density lipoproteins (HDL)-associated protein into different HDL subclasses. Expression of proteins on HDL particles is different depending upon the maturation stage of HDL, with some proteins preferring either HDL2 or HDL3 subclasses, whereas other proteins not displaying any preference

$HDL_2$ or to different extents with both particles (Figure 1). There are indications that along this remodelling process, as the HDL particles become larger, LCAT loses its activity, which can be attributed to the ability of sphingomyelin to inhibit LCAT binding to the lipoprotein, as well as a direct inhibition from the product itself (Bolin & Jonas, 1996). Phospholipid transfer protein (PLTP) (a) mediates the fusion of small HDL particles into a larger one (accompanied by the release of smaller particles) (Lusa, Jauhiainen, Metso, Somerharju, & Ehnholm, 1996; Rye & Barter, 1986) through a process that also involves the release of ApoA-I from HDL and whose conversion rate is affected by the ApoA-I/ApoA-II ratio (Jauhiainen et al., 1993; Pussinen, Jauhiainen, & Ehnholm, 1997; Pussinen, Jauhianinen, Metso, Tyynela, & Ehnholm, 1995; Tu, Nishida, & Nishida, 1993) and (b) mediates the transfer of phospholipids between ApoB-rich lipoproteins to HDL (Tall, Krumholz, Olivecrona, & Deckelbaum, 1985). The enlargement of HDL requires the intrinsic phospholipid transfer activity of PLTP and is promoted by triglyceride enrichment in the HDL core (Huuskonen et al., 2000; Jauhiainen et al., 1993; Rye, Jauhiainen, Barter, & Ehnholm, 1998). Additionally, in the reverse cholesterol pathway, PLTP may act as a free cholesterol acceptor upon interaction with ABCA1, acting as an intermediary from the cell to the lipoproteins (Oram, Wolfbauer, Vaughan, Tang, & Albers, 2003). Cholesteryl ester transfer protein (CETP) transfers the CE to the surface of ApoB-containing lipoproteins, mostly LDL and very low-density lipoprotein (VLDL), in exchange for triglycerides (Deckelbaum et al., 1982). This action is viewed as lowering HDL while increasing LDL, suggesting that cholesterol is secreted from the liver after being taken up by LDL receptors via this pathway, rather than through the scavenger receptor-BI. As for PLTP activity, CETP activity is also affected by the apolipoprotein content, as it is reduced in the

presence of ApoA-II compared to ApoA-I alone (Lagrost, Persegol, Lallemant, & Gambert, 1994). While CETP is mainly produced in the liver and adipose tissue (Radeau et al., 1995; Swenson, Simmons, Hesler, Bisgaier, & Tall, 1987), PLTP is ubiquitously expressed (Albers et al., 1995; Guo et al., 1999; Jiang et al., 1998; Vuletic et al., 2003). Hepatic lipase (HL), primarily synthesized in the liver (Semenkovich et al., 1989; Stahnke, Sprengel, Augustin, & Will, 1987) and endothelial lipase (EL), mainly synthesized in the endothelial cells but also in several other tissues (Jaye et al., 1999), belong to the triacylglycerol lipase family. Their function is to hydrolyse phospholipids and triglycerides (HL) or mostly phospholipids (EL) on HDL, although they have different specificity for phospholipids (Duong et al., 2003). In the case of HL, its action on HDL particles is followed by the release of ApoA-I (Clay, Newnham, & Barter, 1991). By this action, HL and EL are viewed as negative regulators of plasma HDL (Edmondson et al., 2009; Jansen, Verhoeven, et al., 1997; Singaraja et al., 2013; Tilly-Kiesi et al., 2004) as hydrolysis of their respective substrates reduces the levels of large $HDL_2$ with formation of smaller $HDL_3$ and pre-β HDL (Mowri, Patsch, Smith, Gotto, & Patsch, 1992; Patsch, Prasad, Gotto, & Bengtsson-Olivecrona, 1984), although some reports indicate that the remnant-$HDL_2$ particles generated by the action of HL possess different features from $HDL_3$ particles (Guendouzi et al., 1999). Additionally, EL in synergy with serum amyloid A (SAA), reduces the formation of nascent HDL through a mechanism that inhibits ABCA1-mediated ApoA-I lipidation (Wroblewski et al., 2011). The presence of ApoA-II on HDL particles increases their affinity for HL, however, there is no consensus about the ApoA-II effects on HL activity as some reports indicate increased activity, whereas other reports describe ApoA-II presence as inhibitory (Boucher et al., 2004; Hime, Barter, & Rye, 1998; Mowri, Patsch, Gotto, & Patsch, 1996; Mowri et al., 1992). The presence of ApoE on HDL instead increases the HL activity (compared to ApoA-I/HDL) in an isoform-dependent manner, with ApoE2 being the most potent activator (Hime, Drew, Hahn, Barter, & Rye, 2004). The same effect of ApoA-II can be seen for EL, in which albeit ApoA-II inhibited the phospholipase activity of EL, in the presence of ApoA-I, the concomitant presence of ApoA-II may either increase or decrease EL phospholipase activity compared to ApoA-I alone (Broedl, Jin, Fuki, Millar, & Rader, 2006; Jahangiri et al., 2005). Studies in animal models also support the notion that HL and EL are negative regulators of HDL (Braschi et al., 1998; Dichek et al., 1998; Ishida et al., 2003; Ma et al., 2003; Mezdour, Jones, Dengremont, Castro, & Maeda, 1997; Otera et al., 2009). Interestingly, in a double knock-out transgenic mouse model ($HL_{ko}/EL_{ko}$), HDL levels were higher than in either single knock-out models, indicating additive, rather than overlapping, effects on HDL metabolism (Brown et al., 2010).

### 2.3 | Catabolism of HDL

Upon enlargement, HDL molecules can in turn be taken up by the liver by the scavenger receptor BI protein, facilitated by HL (Lambert et al., 1999; Ohashi, Mu, Wang, Yao, & Chen, 2005) where cholesterol is eliminated through the bile (Kozarsky et al., 1997). Scavenger receptor-BI (SR-BI) is a scavenger receptor mainly expressed in liver and steroidogenic tissue whose main functions are to (a) bind HDL particles and internalize cholesteryl esters, (b) provide cholesterol for steroid synthesis (Acton et al., 1996; Kinoshita et al., 2004; Rigotti et al., 1996) and (c) facilitate cholesterol efflux from cells (Ji et al., 1997; Jian et al., 1998; Truong, Aubin, Falstrault, Brodeur, & Brissette, 2010). It is still under debate whether SR-BI binds preferentially to ApoA-I rich or ApoA-II rich lipoprotein (de Beer et al., 2001; Pilon et al., 2000), however, it has a higher affinity for large cholesterol-rich α-HDL molecules rather than lipid-free ApoA-I or pre β-HDL (Liadaki et al., 2000; Lund-Katz, Liu, Thuahnai, & Phillips, 2003). Animal models over-expressing or depleting/inactivating SR-BI gene were observed to have reduced HDL levels for the former and increased HDL levels for the latter (Ji, Wang, et al., 1999; Kozarsky et al., 1997; Rigotti et al., 1997; Varban et al., 1998). Another receptor that has been linked to HDL catabolism is the $P2Y_{13}$, a G-coupled receptor that mediates the endocytosis of HDL from the hepatocyte surface by generation of ADP (Jacquet et al., 2005; Martinez et al., 2003). The use of two agonists of $P2Y_{13}$ (AR-C69931MX and CT1007900) increased the hepatic HDL uptake and increased bile secretion (Goffinet et al., 2014; Jacquet et al., 2005). The use of a partial agonist of $P2Y_{13}$, Cangrelor, increased biliary secretion and HDL uptake in wild type and $SR\text{-}BI_{ko}$ mice, but had no effect on $P2Y_{13ko}$ mice, indicating that $P2Y_{13}$ plays a role in HDL catabolism that is independent of the action of SR-BI (Fabre et al., 2010). CD36 (another member of the SR Class B family) has also been suggested to be a receptor for HDL (as well as for VLDL, LDL and modified LDL) (Calvo, Gomez-Coronado, Suarez, Lasuncion, & Vega, 1998). As for SR-BI, its affinity for HDL is affected by the presence of ApoA-II (de Beer et al., 2004). In $CD36_{ko}$ animals, there is a significant decrease in hepatic HDL and CE uptake, whereas cultured cells over-expressing CD36 showed increased CE uptake and HDL internalization (Brundert et al., 2011) (Figure 2).

## 3 | HDL COMPOSITION

### 3.1 | Mass spectrometry as primary method

Over the previous decades, the role of HDL has been revised and repeated studies have indicated that HDL particles are not solely responsible for reverse cholesterol transport, but are also involved in other important cellular processes, such as mediators of inflammation and/or oxidation. These additional properties are because of the presence of several HDL-associated proteins (referred as HDL-protein cargo), which are added to and removed from HDL during HDL generation and remodelling (discussed in the next section). While some of these proteins are similarly expressed on HDL subclasses throughout the whole maturation process, others are specifically expressed only in specific stages of HDL maturation, conferring HDL-specific subclass-related features. Mass spectrometry (MS) analysis has been one of the chosen methods for the

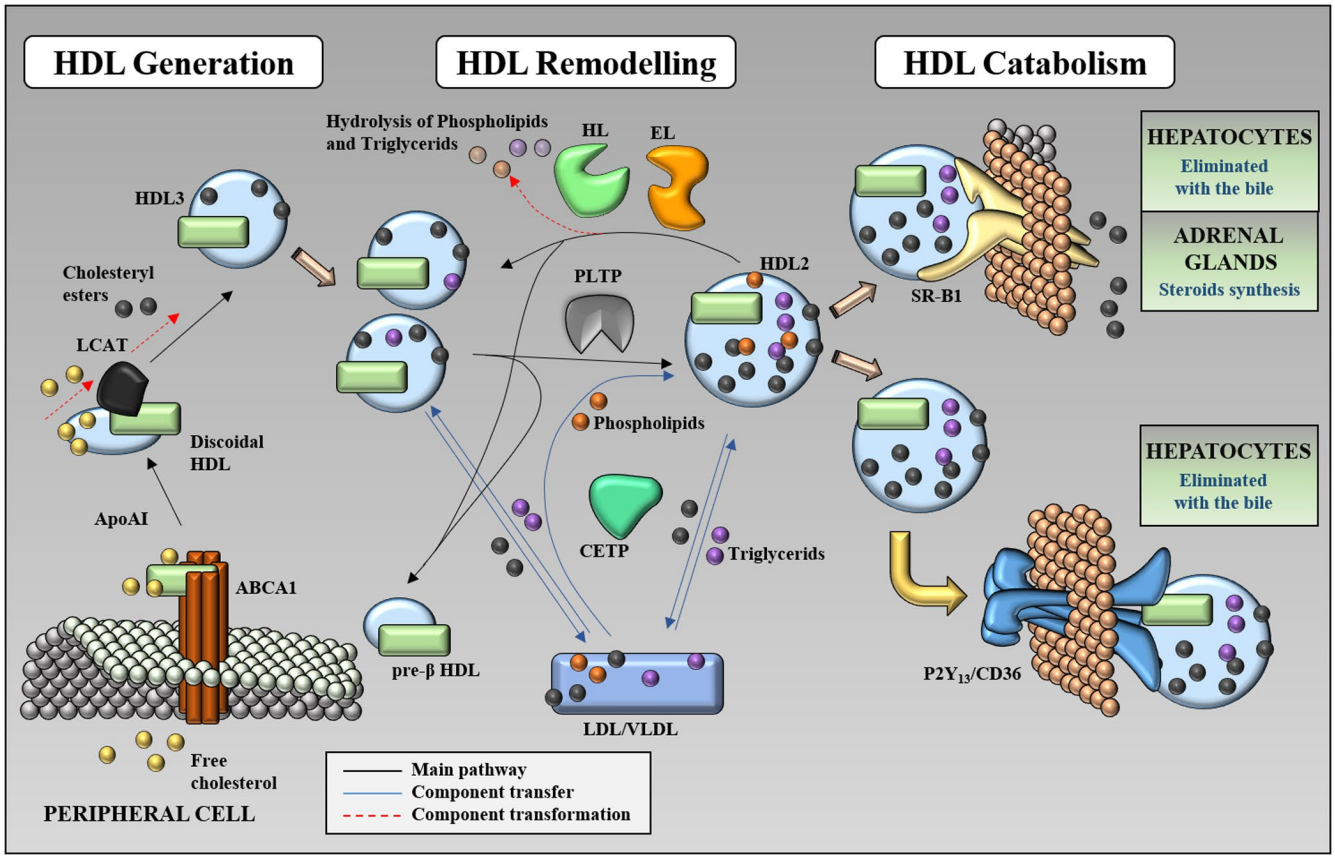


**FIGURE 2** Graphic representation of high-density lipoproteins (HDL) pathway. Changes occurring in HDL particles during generation, remodelling and catabolism and the enzymes involved in such modifications

analysis of HDL protein cargo. Early reviews have compared findings from several studies with regard to the proteins associated with HDL, indicating substantial variability across these studies (Ronsein & Vaisar, 2019; Shah, Tan, Long, & Davidson, 2013; Yassine et al., 2014). Interestingly, as reported in one of the most recent reviews, more than 500 proteins have been detected across all studies, However, only two proteins (ApoA-I and ApoL-I) were consistently identified in all studies, whereas only 21 proteins were identified in at least the 75% of all studies (Ronsein & Vaisar, 2019). This disparity in results can be ascribed to the different methods used to isolate HDL (several variants of ultracentrifugation and/or immunoaffinity), using the MS analysis itself (whether isolated HDL particles are assessed by matrix-assisted laser desorption/ionization time of flight mass spectrometry or liquid chromatography/electrospray ionization tandem mass spectrometry), but other factors, likely related to the cohorts used, may also contribute to these discrepancies.

## 3.2 | Mass spectrometry analysis of HDL in health and disease

Many studies which have assessed HDL composition have analysed healthy controls only, whether as individuals or as pooled samples. Studies that have assessed by MS analysis the HDL protein cargo in distinct HDL subclasses, namely $HDL_2$ and $HDL_3$, have unveiled a specific pattern in which some proteins are similarly expressed in $HDL_2$ and $HDL_3$, whereas others are preferentially associated with either one subclass. Although these studies were performed using different HDL isolation methods and different MS analyses, it appears there is a consensus about the preference of several proteins to HDL subclasses. For instance major apolipoproteins such as ApoA-I and ApoA-II appear to be similarly expressed on both $HDL_2$ and $HDL_3$, whereas ApoE displayed a small, but not exclusive preference for larger $HDL_2$, like ApoC-I. On the other hand, for paraoxonase-1 (PON-1), PON-3, ApoJ, ApoL-I, ApoA-IV and α1-anti-trypsin overall results indicated their preference for smaller $HDL_3$, in some case an exclusive preference (Davidson et al., 2009; Gordon, Deng, Lu, & Davidson, 2010; Heller et al., 2005; Holzer et al., 2016; Karlsson, Leanderson, Tagesson, & Lindahl, 2005). While these studies have reported that HDL cargo composition is affected by remodelling, other studies have reported that several diseases can affect HDL. In Coronary Heart Disease patients, one study reported increased levels of ApoA-IV, ApoE, ApoC-IV, PON and Complement factor C3 specifically in $HDL_3$ subclass, whereas another indicated increased levels of HDL-associated fibrinogen, along with increased levels of SAA, and complement factor C5. In parallel, the levels of -synuclein, ApoC-I, ApoC-II and Fatty Acid Binding Protein were decreased. The increase in SAA on HDL is, however, common to other diseases such as Acute Coronary Syndrome (along with Complement factor C3), End-Stage Renal Disease (along with ApoC-II, Surfactant Protein B

and α1-microglobulin) and Liver Disease (along with ApoE). In Liver Disease, in which HDL particles tend to shift towards larger $HDL_2$ subclass, reduced levels of ApoA-I, ApoA-II, ApoC-I, ApoC-II and PON were also reported (Alwaili et al., 2012; Trieb et al., 2016; Vaisar et al., 2007; Weichhart et al., 2012; Yan et al., 2014). Interestingly, aging is also a factor in modulating HDL protein cargo by increasing the levels of SAA and reducing the levels of ApoE (Holzer et al., 2013).

## 4 | HDL AND THE BRAIN

### 4.1 | Brain HDL versus plasma HDL

Brain HDL differs substantially from plasma HDL as it is generated in situ, rather than transported across the blood–brain barrier (BBB). This is a consequence of the inability of cholesterol to cross the BBB which therefore requires an alternative mechanism for HDL production. While in plasma the main apolipoprotein is ApoA-I, brain-resident cells, such as astrocytes and neurons, generate HDL in which ApoE, and to a minor extent ApoJ, are the main apolipoproteins (Boyles, Pitas, Wilson, Mahley, & Taylor, 1985; Cordero-Llana et al., 2011; Fagan et al., 1999; Laping et al., 1994; Morgan et al., 1995; Oropeza, Wekerle, & Werb, 1987; Pasinetti, Johnson, Oda, Rozovsky, & Finch, 1994; Pitas, Boyles, Lee, Foss, & Mahley, 1987; Xu et al., 2006). Further evidence that brain-resident cells produce their own HDL in situ came from the analysis of HDL particles from liver-transplanted individuals, in which plasma HDL particles post-surgery had the ApoE phenotype of the donor, whereas the cerebrospinal fluid (CSF) HDL maintained the ApoE phenotype of the recipient (Linton et al., 1991) (Figure 3a). Additionally, a recent study in 22 non-demented individuals indicated a significant correlation between plasma and CSF levels of ApoA-I, ApoJ and ApoC-III, whereas no correlation was found between plasma and CSF levels of ApoE, also indicating brain synthesis of ApoE (Koch et al., 2017). In contrast, ApoA-I which is not produced in the brain, can be transported in the CSF across the BBB at the choroid plexus (Stukas, Robert, et al., 2014) or through an SR-BI-mediated process as suggested in an *in vitro* BBB model (Balazs et al., 2004) (Figure 3b). Upon generation of discoidal HDL from astrocytes (LaDu et al., 1998), the particles undergo remodelling and HDL particles found in the CSF acquire a spherical structure similar to the structure of plasma HDL. This remodelling is under the control of the same enzymes that control the remodelling in plasma, all of which are expressed in the CNS, with the sole exception of HL (Albers, Tollefson, Wolfbauer, & Albright, 1992; Albers et al., 1995; Collet, Francone, Besnard, & Fielding, 1999; Demeester et al., 2000; Doolittle, Wong, Davis, & Schotz, 1987; Gander et al., 2002; Hirsch-Reinshagen et al., 2009; Paradis et al., 2004; Vuletic et al., 2003; Yamada et al., 1995). A detailed study indicated that four different classes of HDL are present in the CSF (Lp ApoA-I, Lp ApoE, Lp ApoA-I/ApoE and Lp noApoA-I/noApoE). Although ApoA-I and ApoE may or may not be present, all four subclasses contain ApoA-IV, ApoD and ApoJ. Interestingly, ApoA-II is present only in Lp ApoA-I but absent in Lp ApoA-I/ApoE (Koch et al., 2001). These differences in the main apolipoprotein also reflect differences in cholesterol and phospholipid content and in size, as Lp ApoE are bigger than Lp ApoA-I (Koch et al., 2001; Pitas, Boyles, Lee, Hui, & Weisgraber, 1987). Another study indicated the presence of three different lipoprotein groups in the CSF, with the main two being Lp ApoA-I and Lp ApoE, depending on the major apolipoprotein of the two, with also the presence of small amounts of ApoA-IV, ApoD and ApoJ. A third group in which the

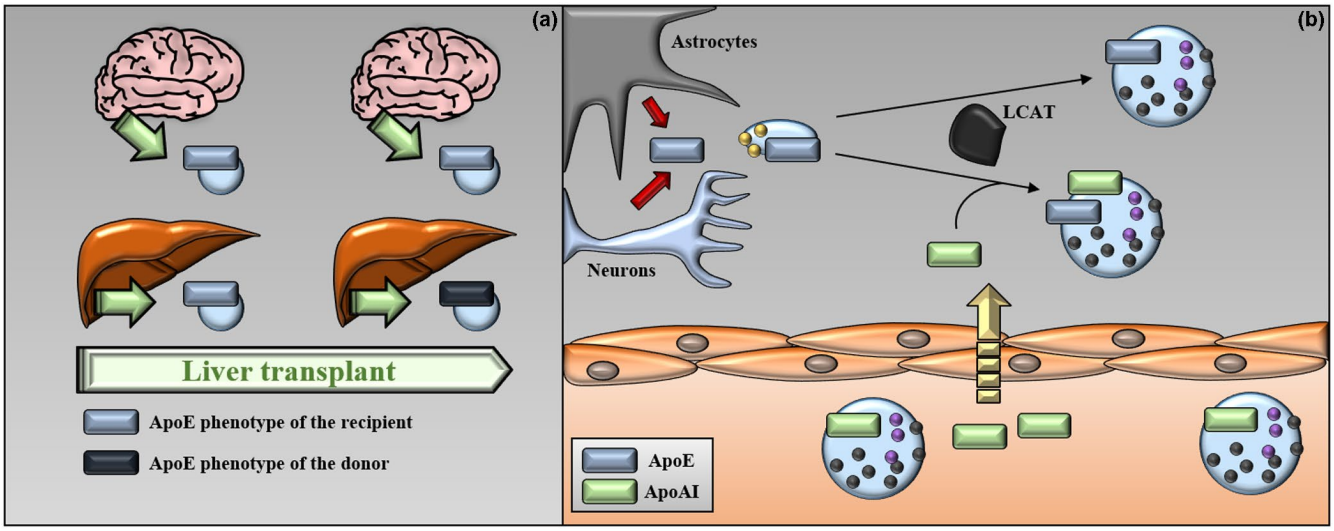


**FIGURE 3** High-density lipoproteins (HDL) generated in the brain and periphery are different. (a) Brain-resident cells produce their own HDL, which was demonstrated by HDL particle analysis from liver-transplanted individuals in which plasma HDL particles post-surgery have the ApoE phenotype of the donor, whereas cerebrospinal fluid HDL maintained the ApoE phenotype of the recipient. (b) Although ApoA-I is not produced in the brain, it can be transported across the blood–brain barrier and be present in small amounts on brain-generated HDL, in which the main apolipoprotein is ApoE

major apolipoprotein was Lp ApoA-IV was also present and this subgroup of lipoproteins was bigger in size compared to the other two (Borghini, Barja, Pometta, & James, 1995). However, there is no ApoE isoform-related difference in the composition of Lp ApoE (Rebeck et al., 1998). As the brain is a rather isolated environment, it prevents cholesterol entry from the blood stream, but it also prevents excess cholesterol in the brain to cross the BBB. Unlike in the bloodstream, where cholesterol is eliminated through the SR-BI (or alternative receptors) on the hepatocytes as CE, the major elimination route in the brain involves cholesterol conversion to 24-hydroxycholesterol by the action of 24S-hydroxylase. Because of its higher solubility, 24-hydroxycholesterol can then cross the BBB into the bloodstream where it is picked up by circulating lipoproteins and delivered to the liver for the elimination process (Lütjohann et al., 1996).

### 4.2 | HDL effects on brain regions and cognitive functions and their implications for AD

When evaluating HDL with regard to brain volume and cognitive functions, several reports have associated higher HDL levels with better cognitive outcomes and/or increased brain volume. High serum HDL levels have been associated with greater hippocampal volume, whereas low serum levels were associated with a greater risk of dementia in a study in which serum LDL and serum total cholesterol levels did not show any association (Wolf et al., 2004). Further studies indicated that high plasma HDL levels were associated with a reduced risk for AD, albeit plasma HDL levels were not associated with the risk for mild cognitive impairment (MCI) (Reitz et al., 2008, 2010). Another study showed that serum HDL levels inversely correlated with brain amyloid β (Aβ) deposits, whereas showing a positive correlation between brain Aβ deposits and serum LDL levels (Reed et al., 2014). In other studies, low serum HDL levels were associated with increased white matter changes (Crisby, Bronge, & Wahlund, 2010) or a higher probability of memory deficits (Singh-Manoux, Gimeno, Kivimaki, Brunner, & Marmot, 2008). Serum HDL levels were also associated with grey matter volume and better cognitive functions or higher mini mental state examination scores (Atzmon et al., 2002; Bates et al., 2017; Ward et al., 2010). Recent publications have summarized the association between brain lipids and dementia and suggested that HDL and ApoE levels were inversely associated with dementia (Koch & Jensen, 2016; Wellington & Frikke-Schmidt, 2016). However, a few reports from The Rotterdam Scan Study failed to confirm the protective role of HDL (dan Heijer, Hofman, Koudstaal, & Breteler, 2005; van Velsen et al., 2013). Anti-oxidant and anti-inflammatory HDL properties are the key features for the protective role of HDL, as oxidized HDL (oxHDL) and oxidized LDL (oxLDL) have been consistently linked to increased neuronal death (Keller, Hanni, & Kindy, 2000; Keller, Hanni, & Markesbery, 1999; Sugawa, Ikeda, Kushima, Takashima, & Cynshi, 1997; Vaisar et al., 2007). Metabolic syndrome, which is a risk factor for dementia, is associated with high oxidative stress and altered HDL anti-oxidant and anti-apoptotic properties (Hansel et al., 2004; de Souza et al., 2008). It is important to note that the majority of HDL protective effect, whether by reducing amyloid deposition or by modulating cognitive effects, are associated with brain areas mostly affected in AD, such as hippocampus and cortex (Scahill, Schott, Stevens, Rossor, & Fox, 2002).

### 4.3 | The glymphatic and the meningeal lymphatic systems

The glymphatic system is a recently identified system, equivalent to the lymphatic system in the periphery, that has the function of clearance of waste as well as the distribution of several compounds in the brain parenchyma (Benveniste et al., 2019; Jessen, Munk, Lundgaard, & Nedergaard, 2015). The CSF produced in the choroid plexus is directed to the subarachnoid space where it enters the brain parenchyma in the perivascular space surrounding arteries and it is in contact with the astrocytic end feet on the outer side. The presence of Aquaporin-4 on the astrocytic end feet facilitates the CSF/ISF exchange into the brain parenchyma leading towards the diffusion of particles into the brain (Iliff et al., 2012). The bulk flow of ISF collects waste products and eventually exits the parenchyma through the perivascular space surrounding the veins where the fluid, through the meningeal lymphatic system (a network of lymphatic vessels that drains fluids through meninges) is then directed towards the cervical lymphatic system (Hershenhouse, Shauly, Gould, & Patel, 2019; Da Mesquita, Fu, & Kipnis, 2018). The glymphatic/meningeal lymphatic waste collection system is mostly active during sleep and reduced functions have been observed in aged mice (Iliff et al., 2013; Kress et al., 2014; Xie et al., 2013). As Aβ clearance in the brain is mediated by many pathways, including the glymphatic and meningeal lymphatic systems, sleep deprivation and reduced glymphatic and meningeal lymphatic functions have been linked to reduced waste clearance with consequent increased brain Aβ deposition (Peng et al., 2016; Shokri-Kojori et al., 2018; Tarasoff-Conway et al., 2015; Wang et al., 2019; Xu et al., 2015). However, the glymphatic system is not only responsible for the clearance of waste, but through perivascular space surrounding arteries, but not veins, it transports small lipophilic molecules and distributes ApoE in the brain parenchyma. The diffusion of ApoE is isoform-specific, with ApoE2 diffusing more than ApoE4, whereas ApoE3 diffuses at an intermediate level (Achariyar et al., 2016; Rangroo Thrane et al., 2013).

## 5 | HDL IN AD PATHOLOGY

Some of the HDL-associated proteins described in this section are not solely carried on HDL, but also on other lipoproteins such LDL and/or VLDL. As we have previously reported, several lines of evidence indicated that HDL molecules are protective in AD and while it would be intuitive to associate apolipoprotein protective effects with HDL, we cannot exclude that some of these protective effects may not be mediated through HDL.

**TABLE 1** Effects of ABCA1 depletion in animal models

| Reference | Model | ABCA1 | ApoE | APP | | Aβ | Other effects |
|---|---|---|---|---|---|---|---|
| | | | | Processing | Levels | | |
| Koldamova, Lefterov, et al., (2005) and Koldamova, Staufenbiel, & Lefterov, (2005) | APP23 | | | | | | |
| | APP23/ABCA1$^{-/-}$ | ↓↓↓ | ↓ Levels | ↔ | ↔ | ↑ Insoluble Aβ40–42, ↑ Aβ deposition, ↑ CAA. | |
| Wahrle, 2005 | PDAPP | | | | | | |
| | PDAPP/ABCA1$^{-/-}$ | ↓↓↓ | ↓ Levels (3-mth old mice), ↔ levels (12-mth old mice). | ↔ | ↔ | ↑ Insoluble Aβ40–42 (12-mth old mice), ↑ Soluble Aβ40 (3-mth old mice), ↑ Aβ deposition (albeit *n.s.*), ↑ CAA. | |
| Hirsch-Reinshagen, 2005 | Tg-SwDI/B | | | | | | |
| | Tg-SwDI/B/ABCA1$^{-/-}$ | ↓↓↓ | ↓ Levels | | ↔ | ↔ Insoluble Aβ40–42, ↑ Amyloid deposition | |
| | APP/PS1 | | | | | | |
| | APP/PS1/ABCA1$^{-/-}$ | ↓↓↓ | ↓ Levels | | ↔ | ↔ Insoluble Aβ40–42 | |
| Lefterov, 2009 | APP23 | | | | | | |
| | APP23/ABCA1$^{+/-}$ | ↓ | ↓ Levels | | | ↑ Insoluble Aβ40, ↑ Aβ oligomers (A11 Ab) | ↓ Cognitive performances |
| Fitz et al., (2012) | APP/E3 | | | | | | |
| | APP/PS1/E3/ABCA1$^{+/-}$ | ↓ | ↔ Levels | ↔ | ↔ | ↔ Insoluble Aβ40–42, ↔ Aβ deposition | ↔ Cognitive performances |
| | APP/E4 | | | | | | |
| | APP/PS1/E4/ABCA1$^{+/-}$ | ↓ | ↓ Levels | ↔ | ↔ | ↑ Insoluble Aβ40–42, ↑ Aβ deposition, ↓ Aβ clearance | ↓ Cognitive performances, |

*Note:* Summary of main effects on levels of ABCA1, ApoE, APP, Aβ and cognitive performance in ABCA1 knock out animal models (↓ decrease, ↔ stable, ↑ increase).

Abbreivations: ABCA, ATP-binding cassette transporter A; APP, amyloid precursor protein; Aβ, amyloid β; CAA, cerebral amyloid angiopathy; PS1, Presenilin1.

**TABLE 2** Effects of ABCA1 over-expression in transgenic animal models

| Reference | Model | Treatment | ABCA1 | ApoE | APP | | Aβ | Other effects |
|---|---|---|---|---|---|---|---|---|
| | | | | | Processing | Levels | | |
| Koldamova, Lefterov, et al., (2005) and Koldamova, Staufenbiel, & Lefterov, (2005) | APP23 | T0901317 | ↑ | ↔ Levels | ↑ sAPPα, ↓ sAPPβ, ↔ CTFs | ↔ | ↓ Soluble Aβ40–42 | |
| Wahrle et al., (2005) | PDAPP/ABCA1wt | none | | | | | | |
| | PDAPP/ABCA1Tg | | ↑ | ↓ Levels, ↔ mRNA | ↔ | ↔ | ↑ Aβ40–42 (3-mth old mice), ↓ Aβ40–42 (12-mth old mice) | |
| Riddell et al., (2007) | Tg2576 | TO901317 | ↑ | ↑ Levels | ↔ | ↔ | ↓ Aβ42 | ↑ contextual memory |
| Jiang et al., (2008)8 | Tg2576 | GW3965 | ↑ | ↑ Levels | ↔ | ↔ | ↓ Aβ40–42, ↓ amyloid plaques | ↑ contextual memory |
| Donkin et al., (2010) | ♀APP/PS1 | GW3965 | ↑ | ↑ Levels | ↑ CTFs (high dose, albeit *n.s.*) | ↔ | ↓ Amyloid burden | ↑ object recognition |
| | ♀APP/PS1/ABCA1$^{-/-}$ | GW3965 | *n.a.* | ↔ Levels | ↑ CTFs (high dose, albeit *n.s.*) | ↔ | ↑ Amyloid burden | ↔ object recognition |
| Fitz et al., (2010) | APP23 | HF diet | | | | | | |
| | | HF diet/ T0901317 | ↑ | ↑ Levels | ↔ | ↔ | ↔ Aβ40–42, plaques, ↓ Aβ oligomers, ↓ amyloid plaques | ↑ memory retention, ↑ spatial learning |
| Fitz et al., (2014) | APP23 | T0901317 | ↑ | ↑ Levels | ↔ | | ↓ Amyloid plaques (albeit *n.s.*), ↔ Aβ40–42 (brain), ↓ Aβ42 (ISF) | ↑ Spatial memory |

*Note:* Summary of main effects on ApoE, APP, Aβ and cognitive performance in animal models with ABCA1 over-expression (↓ decrease, ↔ stable, ↑ increase).

Abbreviations: ABCA, ATP-binding cassette transporter A; APP, amyloid precursor protein; Aβ, amyloid β; PS1, Presenilin1.

### 5.1 | Involvement of ABCA1 in AD pathology and lipidation of ApoE

HDL generation is a complex process that involves several proteins and enzymes. As several reports have indicated that HDL displayed protective features, changes in any of the proteins carried onto HDL or on any of the enzymes that are responsible for HDL generation/remodelling may also alter Aβ deposition and cognitive functions associated with such changes. The role of ABCA1 in initiating HDL formation in the periphery by lipidating ApoA-I, is paralleled in the CNS by its role in lipidating astrocyte-secreted ApoE, which has been suggested to be a key factor that modulates Aβ processing and degradation (Jiang et al., 2008). In vitro and in vivo studies have confirmed that the absence of ABCA1 in the brain (or in brain-derived primary cultures) alters CNS ApoE metabolism and reduced its lipidation state (Hirsch-Reinshagen et al., 2004; Wahrle et al., 2004). Animal models of AD lacking ABCA1 (APP23/ABCA1$^{+/+,-/-}$, PDAPP/ABCA1$^{+/+,+/-,-/-}$, APPswe/PS1Δ9/ABCA1$^{+/+,+/-,-/-}$ and Tg-SwDI-B/ABCA1$^{+/+,-/-}$) reported that brain soluble ApoE levels were greatly reduced and Aβ deposition was increased, possibly through mechanisms involving Aβ clearance and degradation as APP processing was unaffected (Hirsch-Reinshagen et al., 2005; Koldamova, Staufenbiel, & Lefterov, 2005; Wahrle et al., 2005). Interestingly, in an AD mouse model heterozygous for ABCA1 (APPswe/hApoE3/PSΔ9/ABCA1$^{+/-}$ and APPswe/hApoE4/PS1Δ9/ABCA1$^{+/-}$) increased memory deficits and increased Aβ deposition were present only in mice with human ApoE4, but not with human ApoE3. This may be a consequence of plasma HDL levels, significantly higher in human ApoE3 mice compared to human ApoE4 mice (Fitz et al., 2012). In the APP23 transgenic mouse heterozygous for ABCA1 (APP23/ABCA1$^{+/+,+/-}$), memory deficits correlated with the levels of oligomeric Aβ, but not with the levels of insoluble Aβ (Lefterov et al., 2009). In vivo effects of ABCA1 depletion are summarized in Table 1. Conversely, brain over-expression of ABCA1 in PDAPP mice (PDAPP and PDAPP/ABCA1$^{Tg}$) showed decreased Aβ deposition and fewer Aβ plaques. These results may be a consequence of increased ApoE lipidation observed in this study (Wahrle et al., 2008). Up-regulation of ABCA1 can also be achieved through stimulation of nuclear hormone liver X receptors and retinoid X receptors with the ligands T0901317 and 22-hydroxycholesterol for the former and 9-*cis*-retinoic acid and 13-*cis*-retinoid acid for the latter. In vitro experiments have indicated that up-regulation of ABCA1 through these pathways increased ApoE lipidation and was followed by decreased Aβ secretion (Koldamova et al., 2003; Koldamova, Lefterov, et al., 2005), whereas in vivo experiments (APP23, Tg2576 and APPswe/PS1Δ9) employing T0901317 or GW3965 (another liver X receptors agonist) increased ApoE lipidation, improved memory deficits and reduced Aβ plaques and soluble and insoluble Aβ deposition, possibly though mechanisms that modulate Aβ clearance (Donkin et al., 2010; Fitz et al., 2010, 2014; Jiang et al., 2008; Koldamova, Lefterov, et al., 2005; Riddell et al., 2007) (Table 2). The improvement of the ApoE lipidation state, which is suggested to be one of the factors that modulate Aβ deposition, is specifically a more prominent process in the brain, as ApoE is the major apolipoprotein in brain-derived HDL compared to plasma-derived HDL, where ApoA-I is the major constituent. However, despite an increased expression of ABCA1 in the hippocampus of AD patients (Akram, Schmeidler, Katsel, Hof, & Haroutunian, 2010; Kim et al., 2010), ABCA1-mediated cholesterol efflux is reduced in the disease, although the reduced levels of CSF-ApoE or ApoA-I from AD patients used in these studies may be the direct cause of reduced cholesterol efflux (Khalil, Berrougui, Pawelec, & Fulop, 2012; Yassine et al., 2016). These results were recently confirmed in another study in which ABCA1- and ABCG1-mediated cholesterol efflux was impaired in CSF from AD patients, but not from non-AD demented patients (Marchi et al., 2019). Regardless, these data highlight the central role that reverse cholesterol transport plays in the disease and the importance of ABCA1.

### 5.2 | ApoA-I and inhibition of Aβ toxicity

ApoA-I has shown to display protective features in the central nervous system as it redistributes lipids to damaged membranes and plays a role in regenerating and remyelinating nerves and axons after injury (Boyles et al., 1989; LeBlanc et al., 1989; Posse de Chaves, Rusinol, Vance, Campenot, & Vance, 1997). In light of these protective generic effects, it was therefore suggested that ApoA-I may protect against AD. In in vitro experiments, the discovery that the direct interaction of ApoA-I with APP inhibits Aβ aggregation and toxicity (Koldamova, Lefterov, Lefterova, & Lazo, 2001) was then confirmed in other reports that have indicated the protective role of ApoA-I, by reducing the toxicity mediated by the carboxy-terminal fragment of APP (Maezawa et al., 2004) and, by direct interaction with Aβ, preventing the amyloid-induced toxicity, through a mechanism that likely involved inhibition of Aβ protofibril formation (Paula-Lima et al., 2009). Animal studies have also supported previous findings, suggesting a protective role of ApoA-I in the disease. Over-expression of ApoA-I in a triple transgenic mouse model (APPswe/PS1Δ9/hApoA-I$^{Tg}$) resulted in reduced cognitive deficits and reduced cerebral amyloid angiopathy (CAA), despite unaltered Aβ deposition. These findings may be associated with reduced neuroinflammation, as HDL-associated Paraoxonase (PON) activity is increased in plasma, therefore increasing HDL anti-oxidative and anti-inflammatory properties (Lewis et al., 2010). Cognitive functions in transgenic mice (APPswe/PS1Δ9) were also improved upon treatment with an ApoA-I mimetic peptide (in combination with pravastatin, although pravastatin was given at concentrations that were not affecting Aβ processing), and such improvements were associated with decreased Aβ load, decreased glial activation and inflammatory markers. However, decreased Aβ deposition in the brain was not a consequence of altered APP processing (Handattu et al., 2009). Depletion of ApoA-I (PDAPP/ApoA-I$^{+/+,+/-,-/-}$) significantly reduced cholesterol levels in the brain and plasma, while increasing plasma levels of ApoE, although brain ApoE and Aβ levels were unaffected (Fagan et al., 2004). In a different AD transgenic model (APPswe/PS1Δ9/ApoA-I$^{+/+,-/-}$), depletion

of ApoA-I increased CAA and Aβ aggregation and worsened cognitive defects without affecting APP processing, soluble Aβ and Aβ deposition and brain ApoE levels (Lefterov et al., 2010). It appears that ApoA-I does not alter APP processing and Aβ deposition, but rather modulates mechanisms that reduce Aβ toxicity. High serum ApoA-I levels have also been associated with a reduced risk of dementia in the Honolulu-Asia Aging Study and dementia risk was even lower in individuals with no ApoE4 allele (Saczynski, White, Peila, Rodriguez, & Launer, 2007). Also, overall plasma and CSF levels of ApoA-I are reduced in AD and correlate with the severity of the disease (Johansson et al., 2017; Liu et al., 2006; Merched, Xia, Visvikis, Serot, & Siest, 2000; Shih et al., 2014). To further confirm the protective role for ApoA-I, an inverse correlation was found between serum ApoA-I levels and white matter lesions in elderly (Yin et al., 2014) and low plasma ApoA-I levels (as well as ApoH) increased the risk for cognitive decline (Song et al., 2012). A schematic representation of the effects of ABCA1 and ApoA-I is illustrated in Figure 4.

### 5.3 | The $ABCA1_{ko}$, $ApoA\text{-}I_{ko}$, $ApoE_{ko}$, ApoA-I/$ApoE_{dko}$ paradigm

A further step towards understanding the role of ABCA1, ApoA-I and ApoE was taken when a double ko (ApoA-I/$ApoE_{dko}$) mouse model was generated, with the goal to mimic the results obtained in the $ABCA1_{ko}$ mouse model (Fitz et al., 2015) and compare them to the single $ApoA\text{-}I_{ko}$ and $ApoE_{ko}$ and wild-type (WT) models. All these models were generated on an AD background (APPswe/PS1Δ9/$ABCA1_{ko}$, APPswe/PS1Δ9/$ApoA\text{-}I_{ko}$, APPswe/PS1Δ9/$ApoE_{ko}$ and APPswe/PS1Δ9/ApoA-I/$ApoE_{dko}$). The $ABCA1_{ko}$ lacks ApoA-I and has extremely reduced levels of ApoE, therefore the ApoA-I/$ApoE_{dko}$ should have confirmed such findings. Surprisingly, the ApoA-I/$ApoE_{dko}$ results more closely reflected those obtained in the single $ApoE_{ko}$ model. Parenchymal Aβ deposition in the hippocampus and in the cortex and brain soluble and insoluble levels of Aβ, which were either increased or unaffected in $ABCA1_{ko}$ and $ApoA\text{-}I_{ko}$ compared to WT, were extremely reduced in ApoA-I/$ApoE_{dko}$ and

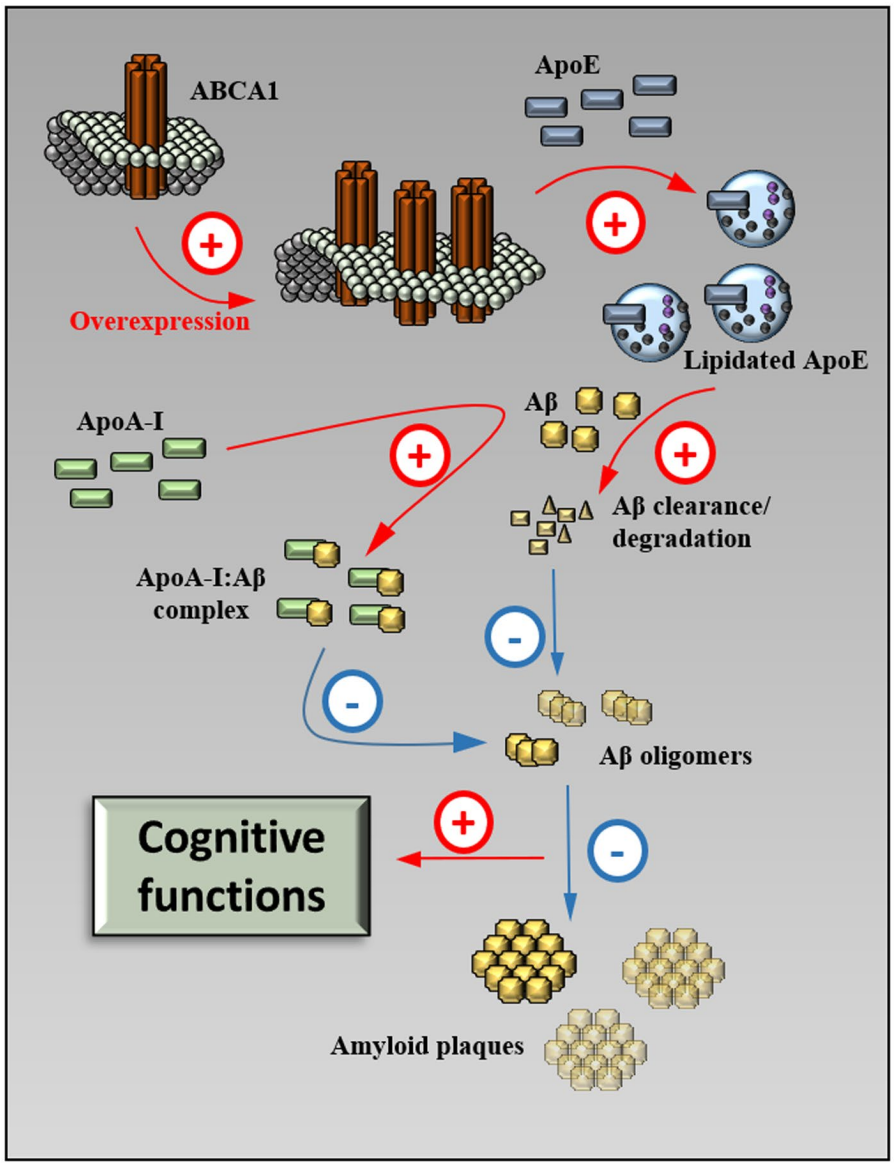


**FIGURE 4** ATP-binding cassette transporter A (ABCA1) and ApoA-I provide protection through different pathways. ABCA1 protection is associated with greater ApoE lipidation, which enhances amyloid β (Aβ) degradation, whereas ApoA-I protection is associated with a direct effect on Aβ in the formation of ApoA-I:Aβ complexes. Both pathways converge at the end by reducing the formation of toxic Aβ oligomers with improved cognitive functions

were comparable to the levels of $ApoE_{ko}$. These results were supported by an increased clearance of Aβ in $ApoE_{ko}$ and in ApoA-I/$ApoE_{dko}$. Of the possible causes for such unexpected observations, the authors proposed that the lack of ABCA1 is paralleled by the virtual absence of ApoE-HDL in the CNS that alters the clearance of Aβ and reduces the Aβ efflux from the brain. However, the authors also acknowledge that these hypotheses do not fully explain the reduced Aβ deposition in the ApoA-I/$ApoE_{dko}$ compared to $ABCA1_{ko}$. A possible explanation could come from the lipoprotein profile. Because of the absence of CE activity, cholesterol in mice is mostly associated with HDL rather than VLDL/LDL (Agellon et al., 1991; Jiao, Cole, Kitchens, Pfleger, & Schonfeld, 1990). In this study, plasma of WT mice display almost only HDL, whereas $ApoE_{ko}$ and ApoA-I/$ApoE_{dko}$ genotypes were associated with the highest levels of VLDL/LDL (ApoA-I/$ApoE_{dko}$ also had the highest levels of Aβ42 in plasma). This would suggest that high plasma levels of VLDL/LDL would in turn increase the Aβ efflux from the brain through a mechanism named 'peripheral sink' (DeMattos et al., 2001) as suggested by the authors themselves. Conversely, the levels of oligomeric Aβ, increased in $ABCA1_{ko}$ compared to WT, in ApoA-I/$ApoE_{dko}$ were not decreased. However, the $ABCA1_{ko}$ and the ApoA-I/$ApoE_{dko}$ models both displayed memory deficits and altered neuronal architecture compared to WT, which would exclude the presence of Aβ oligomers as the cause of such deficits, as the oligomer levels in the ApoA-I/$ApoE_{dko}$ were comparable to WT (the study also excludes the presence of Aβ as possible cause for memory deficits as mice not harbouring the APP transgene display the same memory deficits). This altered neuronal morphology (which is noticeable only in the CA1 region of the hippocampus, but not in the CA2 region) may instead be associated with the absence of HDL-containing ApoE in the CNS (although data on $ApoE_{ko}$ are not available). These different behaviours underline a more complex role of lipoprotein and apolipoproteins associated with them, as the absence of ApoE on HDL may be responsible for the altered neuronal structure, but the brain Aβ levels may be more closely related to the VLDL/LDL levels in plasma. This ApoE effect in mice had already been reported ($APP^{V717F+/-}$/$ApoE^{+/+,+/-,-/-}$, PDAPP/$ApoE^{+/+,+/-,-/-}$, APPsw/$ApoE^{+/+,+/-,-/-}$,), as $ApoE_{ko}$ mice have shown decreased levels of plaques, indicating that murine ApoE is necessary for plaque formation. However, the addition of human ApoE transgene on an ApoE null background ($APP^{V717F+/-}$/$ApoE^{+/+,-/-}$, $APP^{V717F+/-}$/$hApoE3^{+/-}$ and $APP^{V717F+/-}$/$hApoE4^{+/-}$) reduces the plaque even further than the ApoE null mouse model (Bales et al., 1997, 1999; Holtzman et al., 1999, 2000).

## 5.4 | ApoE isoforms and the detrimental role of ApoE4 in AD

The role of ApoE in AD has been extensively studied over 20 years. In humans, this 299-aminoacid protein is present in three different isoforms that differ at amino acid residue positions 112 and 158 of the protein. While ApoE2 has cysteine at positions 112 and 158 and ApoE3 differs by having arginine at position 158, ApoE4 has arginine at both positions (Huang & Mahley, 2014). Structurally, in ApoE4 the Arg-112 causes the Arg-61 to extend away from the helix and interact with the Glu-255 to confer a compact structure to ApoE4. In contrast, in the human ApoE3, Cys-112 does not cause Arg-61 to protrude, limiting its binding to the Glu-255 and allowing the molecule to have a more relaxed structure (Zhong & Weisgraber, 2009). This structural difference between ApoE3 (or ApoE2) and ApoE4 may also be one of the main reasons of the different distribution of ApoE isoforms on different lipoproteins, as ApoE4 is preferentially associated with Very Low-Density Lipoproteins (VLDL), whereas ApoE3 is more closely associated with HDL (Nguyen et al., 2010). This different distribution may also be a reason for the higher predisposition of ApoE3 to form complexes with ApoA-II, which can then be directed towards HDL in a more efficient way (Gregg et al., 1986; Weisgraber, 1990). The alteration of this binding domain by substituting the Arg-61 with a Thr-61 in ApoE4 also induces Aβ production similar to the levels obtained with ApoE3 rather than ApoE4 stimulation (Ye et al., 2005). It is widely accepted that the presence of the allele ApoE4 is a major risk factor for late-onset AD and causes the onset of the disease at an earlier age (Corder et al., 1993; Strittmatter et al., 1993). The hypotheses behind this different behaviour are multiple and affect several biological processes in the CNS. Many of the ApoE4-related effects are associated with several aspects of Aβ. For instance in vitro, in human brains and in murine models of AD (PDAPP, 5xFAD), ApoE4 is associated with increased Aβ production, accumulation and oligomerization (Bales et al., 2009; Hashimoto et al., 2012; Koffie et al., 2012; Ye et al., 2005; Youmans et al., 2012). However, ApoE4-treated hippocampal neurons failed to be protected against Aβ oligomer insults and this was not true for ApoE3-treated neurons, which were protected through mechanisms involving over-expression of Protein Kinase Cε (Sen, Alkon, & Nelson, 2012). The stimulation with Aβ oligomers also affects long-term potentiation (LTP) more strongly in ApoE4 mice than in ApoE2 or ApoE3 mice (Trommer et al., 2005). Reduced clearance, either by reducing the expression of an Aβ-degrading enzyme, Insulin-Degrading Enzyme or by other mechanisms has also been associated with the presence of ApoE4 (Castellano et al., 2011; Cook et al., 2003; Deane et al., 2008; Du, Chang, Guo, Zhang, & Wang, 2009). Lipidation of ApoE, mentioned previously, plays an important role in ApoE binding to Aβ for its subsequent degradation, is also affected by APOE genotype, with ApoE3 having a higher affinity for Aβ than ApoE4 (Tokuda et al., 2000). Comparatively, degradation of Aβ is also affected in astrocytes from ApoE4 mice that eliminate Aβ plaques less effectively than astrocytes from ApoE3 mice (Simonovitch et al., 2016). Finally, in an AD mouse model (Tg2576/hApoE3 and Tg2576/hApoE4), the expression of ApoE4 induces CAA at a greater rate than ApoE3 (Fryer et al., 2005). However, not all ApoE4-related effects can be associated with Aβ and its downstream effects on neurons. For instance, ApoE3 binds to and slows Tau phosphorylation, in stark contrast with ApoE4 which does not bind Tau, promoting its phosphorylation and destabilizing microtubules

(Strittmatter et al., 1994). Several other studies have evaluated the role of tau with regard to ApoE4. Increased Tau phosphorylation has been more prominent in ApoE4, but not in ApoE3 mice and correlated with the presence of ApoE C-terminal truncated fragments, which are reported to induce cognitive deficits and neurodegeneration. Indeed, ApoE4 is more prone to such internal cleavage than ApoE3, hence the increased neurotoxicity associated with it. It is, however, interesting that such cleavage was present only in neuron-specific ApoE (under the Neuron-Specific Enolase promoter) but not in astrocyte-specific ApoE (under the Glial Fibrillary Acidic Protein promoter) and the accumulation of cytotoxic fragments was more prominent in Alzheimer-related affected areas, such as hippocampus and cortex, rather than in an unaffected area such as cerebellum (Brecht et al., 2004; Harris et al., 2003). Such phosphorylation of Tau has been associated with several pathways, including the extracellular signal-regulated kinase and Calpain-cyclin-dependent kinase 5 signalling pathways (Harris, Brecht, Xu, Mahley, & Huang, 2004; Zhou et al., 2016). In a tauopathy mouse model (P301S/ApoE2, P301S/ApoE3, P301S/ApoE4, P301S/ApoE$^{-/-}$), the presence of ApoE4 was associated with increased brain atrophy and increased astrocytic activation and the damage caused by the treatment of P301S neurons with recombinant ApoE isoforms was more prominent with ApoE4 (Shi et al., 2017). For instance in a Tau-dependent fashion, the presence of ApoE4 (but not ApoE3) reduced the number of GAD67- and Somatostatin-positive interneurons in the dentate gyrus area and preceded cognitive deficits (Andrews-Zwilling et al., 2010). The presence of ApoE4 also affected other processes in the brain which were related to impulse transmission between neurons. In ApoE4/4 mice attenuation of excitatory transmission was higher than in ApoE3/3, but was partially rescued by ApoE2 in ApoE2/4 mice. However, the dendritic length in neurons was lower in the presence of ApoE4, regardless of the presence of ApoE2 (Klein, Mace, Moore, & Sullivan, 2010). These results were in accordance with a previous research which indicated that spine density and dendritic arborization in cortical neurons in ApoE4 mice were reduced (Dumanis et al., 2009). ApoE4 also failed to protect against age-dependent neurodegeneration, as indicated by the loss of synaptophysin-positive pre-synaptic terminals and MAP2-positive neuronal dendrites, which was not detected in ApoE3 mice (Buttini et al., 1999). In addition, another study indicated that neurogenesis is reduced in ApoE4 knock-in mice, compared to ApoE2 and ApoE3 mice (Koutseff, Mittelhaeuser, Essabri, Auwerx, & Meziane, 2014). APOE genotype has also been repeatedly reported to affect the hippocampal volume in MCI and AD, with the ApoE4 strongly associated with increased atrophy and reduced hippocampal volume (Agosta et al., 2009; Hostage, Roy Choudhury, Doraiswamy, & Petrella, 2013; Manning et al., 2014; Moffat, Szekely, Zonderman, Kabani, & Resnick, 2000; Tang, Holland, Dale, & Miller, 2015). Since lower plasma ApoE levels were also associated with smaller hippocampus (Teng et al., 2015), whereas higher plasma levels were associated with lower brain amyloidosis (Koch et al., 2018), APOE genotype may orchestrate such effects as individuals carrying the ε4 allele are known to display lower levels of ApoE. More detailed reading of these and other ApoE effects are reviewed elsewhere (Huang & Mahley, 2014;Mahley& Huang, 2012; Yu, Tan, & Hardy, 2014). Murine ApoE differs substantially from human ApoE, despite having an arginine at the position 112, like human ApoE4. The reason of this altered behaviour lies on the amino acid at the position 61 which is an arginine in humans but a is threonine in mice (and in most other species as well), which does not lead to the ApoE4-like compact structure (despite the presence of Arg-112 and Glu-255), instead its behaviour resembles the human ApoE3 (Zhong & Weisgraber, 2009).

### 5.5 | ApoJ and Aβ clearance

ApoJ (or Clusterin) is an atypical apolipoprotein which is associated with HDL, produced in a variety of tissues and has chaperon activity (Calero et al., 2000; de Silva, Harmony, Stuart, Gil, & Robbins, 1990; de Silva, Stuart, et al., 1990). Although there is consensus ApoJ is increased in AD and it is a binding partner for Aβ (Ghiso et al., 1993; Matsubara, Frangione, & Ghiso, 1995), the main role of ApoJ in AD is rather contradictory, as there is no agreement whether its role in the disease is more protective or more detrimental. The ApoJ:Aβ complex is known to interact with the Low-Density Lipoprotein receptor-related protein 2 (Megalin), which mediates the efflux and the clearance of Aβ from and to the brain (Bell et al., 2007; Hammad, Ranganathan, Loukinova, Twal, & Argraves, 1997; Zlokovic et al., 1994, 1996). Several reports have indicated that ApoJ levels are increased in plasma and brain regions in AD that are specifically associated with senile plaques (Gupta et al., 2016, 2017; Howlett, Hortobagyi, & Francis, 2013; Lidstrom et al., 1998; Miners, Clarke, & Love, 2017). Although high levels of ApoJ in plasma were associated with higher hippocampal volume and slower brain atrophy rates in MCI, higher entorhinal cortex atrophy rates were associated with higher plasma and CSF ApoJ levels in AD, corroborating other findings in which higher plasma ApoJ levels were associated with faster cognitive decline (Desikan et al., 2014; Jongbloed et al., 2015; Koch et al., 2018; Thambisetty et al., 2010, 2012). Primary neurons treated with Aβ increased intracellular ApoJ levels, and this intracellular ApoJ increase was also observable in a transgenic model of AD (APPswe/PS1Δ9) (Wang et al., 2017). The fact that ApoJ is a binding partner for Aβ on the surface of HDL (Koudinov, Matsubara, Frangione, & Ghiso, 1994) and that this binding, unlike the ApoE-Aβ binding, is not altered by the ApoJ lipidation state (Calero et al., 1999), still leaves unanswered questions about the role of ApoJ in the disease as contradictory findings have been described. ApoJ has shown the capacity to bind to the Aβ in vitro, reducing its toxic effects (Cascella et al., 2013; Matsubara, Soto, Governale, Frangione, & Ghiso, 1996; Narayan et al., 2011, 2012; Yerbury et al., 2007). It also appears that the protective effects of ApoJ relies on its capacity to alter Aβ aggregation by inhibiting its primary and secondary nucleation (Beeg et al., 2016). On the other hand, Aβ

modulates its toxicity by up-regulating intracellular ApoJ and activating Dickkopf WNT signalling pathway inhibitor 1, whereas these effects were reduced in primary neurons devoid of ApoJ (Killick et al., 2014; Robbins et al., 2018).

## 5.6 | Synergistic effects surrounding ApoE and ApoJ

ApoE and ApoJ are the two main apolipoproteins expressed in the central nervous system and have been related to the transport and the clearance of Aβ. We have previously discussed that murine ApoE is necessary for plaque formation as AD mice lacking ApoE do not have Aβ deposition. In these transgenic mice ($APP^{V717F+/-}$/$ApoE^{+/+,+/-,-/-}$), the reduction of Aβ deposits in the cortex and the hippocampus is APOE dose-dependent, with the heterozygous ApoE carriers ($ApoE^{+/-}$) showing intermediate deposition. Accordingly, such reduced deposition was also paralleled by reduced activated glia associated with Aβ deposition (Bales et al., 1997, 1999). Interestingly, a mouse model of AD ($APP^{V717F+/+}$, PDAPP/$ApoJ^{+/+,+/-,-/-}$) lacking ApoJ displayed reduced fibrillar Aβ and neuritic dystrophy compared to the mice expressing ApoJ, although fibrillar Aβ and neuritic dystrophy are independent of each other. It is noteworthy that despite comparable Aβ deposition, regardless of the expression of ApoJ, PDAPP/$ApoJ^{-/-}$ have markedly reduced Thioflavin-S positive amyloid plaques in the cortex and in the hippocampus, which indicates a less 'compact' structure (DeMattos et al., 2002). Another report indicated that APP/PS1 mice lacking ApoJ (APPswe/PS1Δ9/$ApoJ^{+/+,-/-}$) also displayed reduced Aβ deposition in the brain parenchyma. However, such decreased brain amyloid deposition was counter-balanced by a noticeable amyloid vascular deposition with CAA (Wojtas et al., 2017). Together, these results suggested that both ApoE and ApoJ were facilitating the fibrillization of Aβ in the brain and the consequent neuronal damage associated with it. However, a successive study unexpectedly indicated that in absence of both ApoE and ApoJ (PDAPP/ApoE/$ApoJ_{dko}$) there was a consistent increase in Aβ deposition, suggesting that ApoE and ApoJ have additive effects in regulating Aβ deposition. This effect was, as mentioned, rather unexpected since lacking either ApoE or ApoJ led to a reduction of Aβ deposition. Interestingly, the neuritic dystrophy surrounding the plaques was similar in PDAPP/$ApoE^{+/+}$/$ApoJ^{+/+}$ as it was in the PDAPP ApoE/$ApoJ_{dko}$, suggesting that while both molecules are involved in Aβ deposition, the Aβ-related damage is independent of their expression (DeMattos et al., 2004).

## 5.7 | ABCA7 and phagocytosis

In line with previous experiments suggesting ABCA1 to be a protective factor in Aβ generation, in vitro over-expression of ABCA7 also reduced the secretion of Aβ without affecting APP processing (Chan et al., 2008). Furthermore, depletion of ABCA7 in three mouse models of AD (J20/$ABCA7^{+/+,-/-}$, CRND8/$ABCA7^{+/+,-/-}$ and APPswe/PS1Δ9/$ABCA7^{+/+,-/-}$) reported increased levels of cerebral Aβ (Kim et al., 2013; Sakae et al., 2016; Satoh, Abe-Dohmae, Yokoyama, St George-Hyslop, & Fraser, 2015). It is important to note, however, that the primary mechanism behind the protective features of ABCA7 towards Aβ metabolism might not be an increased ApoE lipidation state as it is for ABCA1 (Jiang et al., 2008). Rather, several reports indicated that the protective effects of ABCA7 are mediated by other mechanisms, as depletion of ABCA7 increased APP endocytosis (Satoh et al., 2015) and reduced efficiency in taking up Aβ oligomers (Fu, Hsiao, Paxinos, Halliday, & Kim, 2016; Kim et al., 2013). This would also be consistent with the fact that the primary functions of ABCA7 are related to phagocytic activity (Iwamoto, Abe-Dohmae, Sato, & Yokoyama, 2006; Jehle et al., 2006). However, increased levels of β-secretase have also been suggested as a potential mechanism behind Aβ increase (Sakae et al., 2016).

## 5.8 | LCAT and PLTP

Although the majority of the molecules involved in AD, most notably ApoA-I, ApoE and ABCA1, are involved in the initial steps of HDL generation, other molecules downstream in the HDL generation and remodelling have also been associated with AD, though to a much lesser extent. Interestingly, in a murine model of AD (APPswe/PS1Δ9/$LCAT^{+/+,-/-}$) deficiency of LCAT had no effect on Aβ metabolism and on cerebral and vascular amyloid load (Stukas, Freeman, et al., 2014), despite greatly reduced levels of ApoA-I in plasma and CSF. This is counterintuitive, considering that ABCA1 and ApoA-I deficiency have been associated with increased Aβ levels and worsening of cognitive defects. This may be explained by the fact that amyloid clearance is affected by reduced lipidation of ApoE, therefore deficiency in ABCA1 reduces ApoE lipidation, but LCAT deficiency does not, and while ApoA-I levels are reduced, a small amount of it is still present, mostly in the pre-β form. However, LCAT activity in the CSF of AD patients has been reported to be significantly lower than in the CSF of controls (Demeester et al., 2000). Downstream in the remodelling process, PLTP deficiency has been shown to alter Aβ processing and to increase memory deficits by altering APP turnover, steering APP towards β- and γ-secretases. It was also suggested that PLTP itself could be a carrier of APP to the cell surface, as it was reported that APP and PLTP displayed interaction. Additionally, in transgenic animals lacking PLTP (APPswe/PS1Δ9/$PLTP^{+/+,-/-}$) increased brain expression of β-site APP - cleaving enzyme 1 and PS1 were also reported, whereas PLTP deficiency did not affect a disintegrin and metalloproteinase 10 (Tong et al., 2015).

## 5.9 | HDL receptors

Both HDL receptors belonging to the SR Class B family (SR-BI and CD36) have shown to play a central role in Aβ processing. It has been widely demonstrated that activated microglia surround amyloid plaques in the AD brain, secreting a variety of pro-inflammatory molecules which overtime are harmful for the brain. However,

activated microglia has also shown Aβ phagocytosis capacity. As the binding of Aβ to microglia is a key step in glial activation, several receptors have been indicated as potential ligands for Aβ. SR-BI has shown the capacity to bind fibrillar Aβ and generate reactive oxygen species production in neonatal microglia (Husemann, Loike, Kodama, & Silverstein, 2001). Additionally, in mouse models of AD (J20/SR-BI$^{+/+,+/-}$), reduction in SR-BI increased amyloid deposition and CAA, and worsened cognitive and memory performance through an ApoA-I and ApoE-independent mechanism (Thanopoulou, Fragkouli, Stylianopoulou, & Georgopoulos, 2010). Similar to SR-BI, CD36 binding to Aβ induces the release of $H_2O_2$ in microglia and macrophages and this release is partially inhibited by antibodies against CD36 (Coraci et al., 2002). Activation of the pro-inflammatory cascade upon Aβ binding was subsequently confirmed by another group who reported microglial activation through Src kinase family members (Moore et al., 2002). Furthermore, activation of microglia and the ensuing phagocytosis in macrophages was caused by the binding of Aβ to a complex involving CD36, CD47 and α6β1-integrin. This, in turn, generated the release of pro-inflammatory molecules through a tyrosin-based signalling cascade (Bamberger, Harris, McDonald, Husemann, & Landreth, 2003; Koenigsknecht & Landreth, 2004). In mouse models of AD (APPswe/PS1Δ9 and J20), up-regulation of CD36 through the use of peroxisome proliferator-activated receptor-γ agonists such as Rosiglitazone, Pioglitazone and DSP-8658, was shown to improve memory deficits and to reduce amyloid plaques by enhancing microglial uptake (Escribano et al., 2010; Yamanaka et al., 2012). However, CD36 has also been shown to mediate vascular damage through a mechanism that involves ROS production from NADPH oxidase (Park et al., 2011).

## 5.10 | ApoA-II and ApoA-IV

ApoA-II and ApoA-IV have also been linked to neuroprotection and, as for ApoA-I, their involvement is associated with mechanisms that do not affect APP processing. **ApoA-II** displayed different properties when interacting with ApoE, as it forms complexes with ApoE2 and ApoE3 but not with ApoE4. In cell culture supernatant, ApoE2/ApoA-II and ApoE3/ApoA-II complexes were able to increase cell viability by binding to Aβ, reducing its internalization. These protective features were missing in ApoE4-treated cells because of the absence of ApoE4/ApoA-II complexes (Yamauchi et al., 2000). Although we had indicated in the previous chapter that in CSF ApoA-II is present only in Lp ApoA-I, but absent in Lp ApoA-I/ApoE and here we report different effects of ApoA-II/ApoE complexes, it is important to remember that these data were obtained in vitro and, more importantly, plasma HDL composition is different than CSF HDL-like particles. **ApoA-IV**, just like ApoA-I, is increased at the site of repair in peripheral nerve injuries, although this increased concentration is because of a transfer of ApoA-IV from plasma, rather than increased synthesis (Boyles, Notterpek, & Anderson, 1990). A transgenic mouse model of AD lacking ApoA-IV (5xFAD/ApoA-IV$^{+/+,-/-}$) displayed increased cerebral Aβ paired with increased spatial learning deficits. As APP processing is unaffected by the ablation of ApoA-IV, data strongly suggest that ApoA-IV involvement is more associated with Aβ clearance (Cui, Huang, He, Zhang, & Luo, 2011).

## 5.11 | Other Apolipoproteins

ApoC-III is a 79-aminoacid apolipoprotein, synthesized in the liver and the intestine and carried on VLDL and HDL (Brewer, Shulman, Herbert, Ronan, & Wehrly, 1974). ApoC-III has been associated with hypertriglyceridemia through mechanisms that involved increased TG-rich VLDL production and reduced TG catabolism by lipoprotein lipase inhibition (Sundaram et al., 2010; Wang, McConathy, Kloer, & Alaupovic, 1985). ApoC-III binds Aβ and its plasma levels were lower in healthy controls with the familial history of AD and AD patients, suggesting that it could be used as an early marker for AD (Shih et al., 2014). Additionally, CSF levels of ApoC-III (as well as ApoD and ApoH) positively correlated with atrophy rates in the inferior and middle temporal cortex (Mattsson et al., 2014). **ApoD** belongs to the lipocalin superfamily and is more frequently associated with HDL, but unlike the majority of other apolipoproteins which are mainly produced in the liver and intestine, ApoD is ubiquitously expressed, although it appears that the brain is one of the regions with the highest ApoD expression (Drayna et al., 1986; Eichinger, Nasreen, Kim, & Skerra, 2007; Seguin, Desforges, & Rassart, 1995). ApoD is a key molecule in triglyceride metabolism as high plasma levels have been associated with reduced levels of triglycerides in mice, through mechanisms that increase lipoprotein lipase activity, therefore increasing TG catabolism rather than reducing its synthesis (Perdomo et al., 2010). It has also been proposed that ApoD is up-regulated and has protective features against oxidative stress, both in vitro and in vivo (Bajo-Graneras et al., 2011; Ganfornina et al., 2008; He et al., 2009). In light of these findings, several studies have reported increased levels of ApoD in CSF, hippocampus and prefrontal cortex of AD patients, strongly suggesting that neurotoxic biochemical changes, such as augmented inflammation and oxidative stress, could in turn activate protective defence mechanisms such as ApoD up-regulation. Additionally, in brain regions specifically affected in AD such as the hippocampus and prefrontal cortex, increased levels of ApoD were reported and correlated with the levels of lipid peroxidation products (Bhatia et al., 2013; Glockner & Ohm, 2003; Terrisse et al., 1998; Thomas et al., 2003). In postmortem brain, the increase in ApoD in the frontal and temporal cortex was observed in AD patients, but not in Frontotemporal Dementia patients who had ApoD levels comparable to controls (Bhatia, Kim, Shepherd, & Halliday, 2019). In rats, increased mRNA levels of ApoD in the hippocampus were also observed after entorhinal cortex lesions, a region that is strongly affected in AD (Terrisse et al., 1999). Nonetheless, ApoD has also been reported to be a component of amyloid plaques (Desai et al., 2005). Finally, a study in AD transgenic animals (APPswe/PS1Δ9/ApoD$^{-/-}$, APPswe/PS1Δ9/hApoD$^{+/+}$ and PDAPP) have reported increased ApoD mRNA, whereas another has associated the absence of ApoD with an increased number of amyloid

plaque in the hippocampus and the over-expression of ApoD with a reduced number of them (Li et al., 2015; Thomas, Sautkulis, Criado, Games, & Sutcliffe, 2001). These results suggest that ApoD in AD could be physiologically increased to protect agaist the presence of amyloid plaques, whereas its transgenic over-expression would instead reduce the formation of amyloid plaques themselves. **ApoF** is an atypical apolipoprotein that is mainly associated with HDL, although a small fraction is also associated with LDL (He, Greene, Kinter, & Morton, 2008; Koren, McConathy, & Alaupovic, 1982) and is an important modulator of HDL. It acts as Lipid Transfer Inhibitor Protein given its ability to inhibit CE transfer amongst lipoproteins, and this activity appears to be restricted to the LDL-bound ApoF fraction, which as a consequence increases the CE transfer from HDL to VLDL, hence lowering HDL levels (Morton & Greene, 1994; Wang, Driscoll, & Morton, 1999). Accordingly, the over-expression of ApoF reduces HDL in mice, while unexpectedly, ApoF deficiency has no effect (Lagor et al., 2009, 2012). However, there is no current link between ApoF expression/activity and AD. **ApoM** is an apolipoprotein secreted from the kidneys and liver, that is carried by a small fraction of HDL particles (Christoffersen et al., 2006; Zhang et al., 2003). Although there are contradictory data regarding the importance of the absence of ApoM in the formation of pre-β HDL, its presence increased the formation of larger pre-β HDL, which in turn increased the cholesterol efflux, leading to an increased protection against atherosclerosis (Elsoe, Christoffersen, Luchoomun, Turner, & Nielsen, 2013; Mulya et al., 2010; Wolfrum, Poy, & Stoffel, 2005). Additionally, ApoM has also displayed anti-oxidant activity for its capacity to bind oxidized phospholipids, hence enhancing the anti-oxidant activity of HDL itself, and its anti-apoptotic activity by binding to and delivering sphingosine-1-phosphate (S1P) to the vascular endothelium (Christoffersen et al., 2008, 2011; Elsoe et al., 2012; Ruiz, Frej, et al., 2017; Ruiz, Okada, & Dahlback, 2017). Additionally, S1P appears to be more prominent in the $HDL_3$ fraction, which is the fraction more prominently associated with HDL protective features (Kontush, Chantepie, & Chapman, 2003; Kontush et al., 2007). In a proteomic analysis of CSF, ApoM levels were significantly reduced in AD patients compared to controls (Khoonsari et al., 2016), which corroborates other reports of decreased S1P levels in the AD brain (Couttas et al., 2014; He, Huang, Li, Gong, & Schuchman, 2010).

## 5.12 | The anti-oxidant effects of Paraoxonase

Paraoxonase (PON) is an enzyme with paraoxonase/arylesterase/lactonase activity produced in the liver that is mainly associated with HDL, although small quantities can also be found on VLDL (Bergmeier, Siekmeier, & Gross, 2004; Deakin, Moren, & James, 2005). It is associated with ApoJ and is activated by ApoA-I on HDL, steps that are necessary for the full display of its protective features such as increasing cholesterol efflux in in vitro experiments, an effect that is mediated by ABCA1 (Berrougui, Loued, & Khalil, 2012; Gaidukov & Tawfik, 2005; Kelso et al., 1994; Rosenblat, Vaya, Shih, & Aviram, 2005). Conversely, over-expression of ApoA-II appears to be pro-atherogenic because of its capacity to displace PON from HDL (Castellani et al., 1997; Ribas et al., 2004). As for many proteins associated with HDL, each stage of HDL maturation is associated with different proteins and therefore with different properties. PON is mainly expressed in the small $HDL_3$ fraction, which is also the fraction that is mostly associated with anti-oxidant features (Bergmeier et al., 2004; Camont et al., 2013; Kontush et al., 2003). In fact, PON inhibits the generation of lipid peroxides and prevents the damage caused by oxLDL (Garcia-Heredia et al., 2013; Mackness, Arrol, Abbott, & Durrington, 1993; Mackness, Arrol, & Durrington, 1991). Therefore, PON levels/activity in serum or in HDL-isolated fraction were diminished in several diseases, which at least in part reflects the inability of HDL to fully display its anti-oxidant, anti-inflammatory and anti-apoptotic properties (Kappelle, Bijzet, Hazenberg, & Dullaart, 2011; Murakami et al., 2013). Several reports have indicated, not without slight discrepancies among PON enzymes involved, that overall serum PON activity was down-regulated in AD patients (Bacchetti et al., 2015; Cervellati et al., 2015; Paragh et al., 2002). Such decrease in PON activity in AD patients may be, at least in part, the cause behind the up-regulated oxLDL levels in the disease, which in turn may increase the secretion of Aβ from neuronal cells (Bacchetti et al., 2015; Dias et al., 2014).

## 5.13 | Serum Amyloid A (SAA) and HDL remodelling

SAA is an acute-phase protein that travels on HDL and whose levels greatly increase during inflammatory states. As a consequence, SAA has been associated with HDL remodelling, the protective properties of HDL. During inflammatory states, it has been suggested that SAA increases its concentration on HDL by displacing ApoA-I from HDL itself, although this is not true in the absence of an acute phase protein (Coetzee et al., 1986; Hosoai et al., 1999; Parks & Rudel, 1985). SAA capacity to displace apolipoprotein from HDL is not limited to ApoA-I, since in the CSF SAA has been associated with the displacement of ApoE, the main brain-derived HDL apolipoprotein, with this effect being more prominent in ApoE4 individuals (Miida et al., 2006). In turn, this HDL remodelling has been associated with a loss of protective properties, common to several diseases. For instance cholesterol efflux is impaired in HDL derived from inflamed human and mice and it is inversely correlated with the extent of SAA on HDL (Vaisar et al., 2015). Albeit one report indicated that, in order to affect the cholesterol efflux, the concentration of SAA on HDL must be relatively high (>50%) (Banka et al., 1995). Other data from mice (WT and $SAA^{-/-}$) and humans indicated that cholesterol efflux and HDL anti-inflammatory properties are affected by the presence of SAA on HDL, which blocks HDL access to the adipocyte membranes (Han et al., 2016). In patients with End-Stage Renal Disease and Sistemic Lupus erythematosus, HDL displayed reduced anti-inflammatory properties as a consequence of SAA enrichment (Han et al., 2016; Tolle et al., 2012). In metabolic syndrome patients (a condition that is considered a risk factor for AD), high levels of SAA correlated with lower PON activity, suggesting that the SAA-driven

damage on HDL may affect PON (Kappelle et al., 2011). Alteration in HDL particles (mainly $HDL_3$) with reduced anti-oxidative capacity was also reported in Myocardial Infarction patients, where reduced levels of ApoA-I were paralleled by an increase in SAA, lysophosphatidylcholine and phosphatidic acid (Rached et al., 2015). However, in the absence of SAA, an acute phase response does not alter HDL size in mice, but induces changes on HDL such as increased phospholipid and decreased total proteins, which can be a consequence of the absence of CETP in mice (de Beer et al., 2010). In spite of many reports that have indicated that SAA on HDL is one of the key molecules responsible for reduced HDL protective features, a few reports have indicated that the presence of SAA on HDL does not affect anti-oxidative functions of HDL and promote cholesterol efflux (Jayaraman, Haupt, & Gursky, 2016; Sato et al., 2016; van der Westhuyzen, Cai, de Beer, & de Beer, 2005). While SAA seems to play a prominent role in inflammatory states and alters the activities of HDL, little is known about its involvement in AD, despite increased levels in the disease (Elovaara, Maury, & Palo, 1986; Gupta et al., 2017). It is, however, known that the SAA-driven displacement of ApoE from HDL-CSF discussed earlier is more prominent in ApoE ε4 carriers, than in ε2 or ε3 carriers, affecting Aβ clearance and therefore suggesting another possibility by which the ε4 allele of ApoE may play a role in AD (Miida et al., 2006).

### 5.14 | Plasminogen activator inhibitor 1 (PAI-1) and plasmin-related Aβ degradation

PAI-1 belongs to the serpins family (Serine Protease Inhibitors) and is the natural inhibitor of tissue and urokinase Plasminogen Activator (tissue plasminogen activator, tPA and urokinase plasminogen activator, uPA, respectively), which are the two main enzymes responsible to convert plasminogen to plasmin. Plasmin is reportedly responsible for Aβ cleavage, therefore, this pathway is stimulated by the up-regulation of tPA and uPA. These results were also confirmed in several animal models, in which tPA activity is reduced in AD models (CRND8, Tg2576) and inefficiently cleared brain-injected Aβ in $tPA^{-/-}$ or $plasminogen^{-/-}$ mouse models (Melchor, Pawlak, & Strickland, 2003; Tucker, Kihiko, et al., 2000; Tucker, Kihiko-Ehmann, Wright, Rydel, & Estus, 2000). As positive regulation of the tPA-plasminogen pathway leads to Aβ cleavage, molecules involved in its negative regulation should play the opposite role in the disease. As expected, PAI-1 levels are increased in the brain of mouse models of AD. Expectedly, in $PAI\text{-}1^{-/-}$ AD mouse models (APP/PS1Δ9/$PAI^{+/-,-/-}$), there is a decreased brain Aβ load and reduced levels of soluble Aβ, which is also associated with increased tPA activity (Liu et al., 2011). In support of these findings, a recent study reported that the administration of a PAI-1 inhibitor increases tPA, uPA and plasmin activity in the hippocampus of treated mice (APP/PS1Δ9) and reduces Aβ plaques (Akhter et al., 2018). Although the direct association of PAI-1 with HDL is under investigation, its secretion from adipocytes is stimulated by $HDL_3$ in a S1P dose-dependent manner, rather than $HDL_2$ (Lee et al., 2010). One report suggested that plasma PAI-1 levels could be used as diagnostic marker for AD as they are significantly increased in the disease, whereas other reports have not been able to corroborate these results given that levels were unchanged in AD, in both plasma and CSF (Ban et al., 2009; Martorana et al., 2012; Oh, Lee, Song, Park, & Kim, 2014). Additionally, no difference was observed in the tPA and plasminogen levels in the CSF of AD patients (Martorana et al., 2012).

## 6 | THE GENETICS BEHIND HDL AND AD

### 6.1 | ABCA1 and ABCA7

Mutations in all genes involved in HDL generation, remodelling and catabolism may affect HDL levels at different levels, from a mild alteration to a more severe deficiency. Tangier Disease is caused by mutations in the *ABCA1* gene and it is characterized by extremely low levels of HDL and peripheral neuropathy. These mutations in the *ABCA1* gene reduce the cholesterol efflux from macrophages, leading to the formation of foam cells with increased risk of Coronary Heart Disease (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Fitzgerald et al., 2002; Lawn et al., 1999; Marcil et al., 1999; Rust et al., 1999). Although this is a rather rare disease in which HDL is almost absent, other mutations have shown to have a milder effect on HDL levels, either reducing or increasing its levels. Since high HDL levels have been and still are considered protective against AD, mutations in genes that are involved in HDL formation have been evaluated in several studies. Data from one study indicating an increased frequency of the 219K (R219K) mutation in AD (Rodriguez-Rodriguez et al., 2007) were then confirmed in another study, in which females carrying the same mutation had an increased risk of developing AD, whereas males displayed opposite trend (albeit non-significantly) (Sundar, Feingold, Minster, Dekosky, & Kamboh, 2007). This increased risk for AD is in contradiction with other studies which indicated that carriers of the 219K allele have a lower incidence of AD and higher levels of HDL (Wang & Jia, 2007; Xiao et al., 2012). However, other reports indicated that the 219K carrier is associated with lower CSF cholesterol in healthy controls and with a delayed age of onset in AD, but the alleles were not differently distributed in AD compared to healthy controls (Wollmer et al., 2003). Additionally, although one study indicated the allele 1587K (R1587K) to be increased in AD (Chu et al., 2007), two studies evaluated the haplotype combination of three genes (R219K, I883M, R1587K). With the exception of the I883M site, both studies indicated that the haplotypes carrying the 219K and the 1587R (K-x-R) were significantly more present in the AD population (Katzov et al., 2004; Rodriguez-Rodriguez et al., 2007). Two meta-analysis studies focused on the *ABCA1* common variants R219K, I883M and R1587K did not show any significant association with AD (Jiang et al., 2012; Wang et al., 2013). A rare loss-of-function mutation (N1800H) was associated in heterozygous individuals with decreased levels of cholesterol, ApoE and ApoA-I and with an increased risk for AD (Nordestgaard, Tybjaerg-Hansen, Nordestgaard, & Frikke-Schmidt, 2015). Studies in the promoter

region of the *ABCA1* gene also suggested that homozygotes for the TT genotype (C-14T) have a higher risk for AD, whereas the G-17C did not show any association (Rodriguez-Rodriguez et al., 2007; Sundar et al., 2007). Unlike in *ABCA1*, mutations in *ABCA7* have been detected in several genome-wide association studies (GWAS) and *ABCA7* has resulted in one of the genes that most strongly is associated with AD. These mutations detected in GWAS are located in introns (rs3764650 and rs4147929) and exons (rs3752246, G1527A) (Hollingworth et al., 2011; Lambert et al., 2013; Naj et al., 2011). Other meta-analyses have then confirmed the genetic involvement of *ABCA7* with the disease (Almeida, dos Santos, Trancozo, & de Paula, 2018; Ma et al., 2018). To date, the risky G allele of the rs3764650 has been associated with reduced ABCA7 expression, hippocampal atrophy, increased plaque pathology and subsequent cognitive decline (Andrews, Das, Cherbuin, Anstey, & Easteal, 2016; Ma et al., 2018; Ramirez et al., 2016; Shulman et al., 2013; Vasquez, Fardo, & Estus, 2013), whereas the G1527A mutation affected Aβ deposition (Hughes et al., 2014). Three other mutations not included in the GWAS studies (rs3752242 and rs4147912 both intronic and rs3752240 synonimous V915V) have also been associated with altered cerebral Aβ pathology, whereas a fourth one (rs72973581 G215S) is protective and more expressed in controls (Sassi et al., 2016). Finally, other studies reporting that rare loss-of-function mutations were associated with AD (Cuyvers et al., 2015; Steinberg et al., 2015) have strengthened the role of ABCA7 in the disease, indicating that the correct ABCA7 activity/expression is necessary to provide neuroprotection

## 6.2 | Apolipoproteins

Correct interaction between ApoA-I and its ABCA1 is necessary for the generation of functional HDL particles. Mutation of *APOA-I* in the region comprised between the amino acids 218–231 displayed reduced binding to ABCA1 with consequent reduced levels of HDL (Chroni et al., 2003; Fotakis, Kateifides, et al., 2013; Fotakis, Tiniakou, et al., 2013). The correct interaction between ApoA-I and LCAT is also a necessary step in HDL formation, for which the ApoA-I helix domain at the amino acid position 143–164 regulates LCAT activation (Sorci-Thomas et al., 1998). In accordance, mutation of ApoA-I in the region comprised between the amino acids 140–160 limits the interaction of ApoA-I with LCAT and its subsequent activation, resulting in reduced levels of functional HDL (Cho & Jonas, 2000; Koukos, Chroni, Duka, Kardassis, & Zannis, 2007a, 2007b; Roosbeek et al., 2001; Sviridov, Hoang, Sawyer, & Fidge, 2000). The carriers of the AA genotype for the G-75A polymorphism of *APOA-I* have been associated with an earlier age of onset compared to the other genotypes (Vollbach et al., 2005). However, other studies, while linking this polymorphism to cognitive decline and altered HDL levels, failed to identify a genetic association with AD (Helbecque, Codron, Cottel, & Amouyel, 2008; Smach et al., 2011). Several mutations have also been described in *APOA-IV*, although the two most common are a T347S and Q360H aminoacid substitutions (Lohse, Kindt, Rader, & Brewer, 1990a, 1990b, 1991). These mutations have been associated with altered lipid profile, including triglyceride clearance, cholesterol absorbance and overall variability of HDL particles (Gomaraschi et al., 2010; Hockey, Anderson, Cook, Hantgan, & Weinberg, 2001; Jansen, Lopez-Miranda, et al., 1997; Weinberg et al., 2000). Although one study indicated a genetic association between the *APOA-IV* Q360T polymorphism and AD, two other reports failed to confirm this finding (Csaszar, Kalman, Szalai, Janka, & Romics, 1997; Ji, Urakami, et al., 1999; Merched, Xia, Papadopoulou, Siest, & Visvikis, 1998). A study among African-American evidenced that out of four polymorphisms on *APOD* rarely seen among Caucasians, the F36V mutation on the *APOD* gene is associated with lower $HDL_3$ and ApoA-I levels. Interestingly, carriers of the F36V mutation are also increased, albeit non-significantly, in AD patients (Desai, Bunker, Ukoli, & Kamboh, 2002; Desai et al., 2003). Additional studies have reported other *APOD* polymorphisms were associated with AD (F15S, rs1568565 on Int2 and rs1568566 on Int3), however, their association with HDL levels remains unclear (Chen et al., 2008; Helisalmi et al., 2004). Two major genome-wide association studies (GWAS) have indicated a strong linkage between *APOJ* and AD (Harold et al., 2009; Lambert et al., 2009). In these studies, several intronic polymorphisms (rs11136000, rs933188 and rs227990) have been associated with the disease, along with polymorphisms on other genes such as Complement component Receptor 1 (*CR1*) and Phosphatidylinositol Binding Clathrin Assembly Protein (*PICALM*) (Harold et al., 2009; Lambert et al., 2009). Other subsequent studies have then confirmed a strong genetic involvement of *APOJ* in AD, in form of allelic and haplotype variance (Corneveaux et al., 2010; Gu et al., 2011; Jun et al., 2010). Genetic variation on *APOJ* has therefore been associated with increased brain Aβ deposition with reduced memory and faster cognitive decline for the carrier of the C-risky allele of the rs11136000 polymorphism (Pedraza et al., 2014; Tan et al., 2016; Thambisetty et al., 2013). This is in accordance with data on healthy controls, in which carriers of the C-risky allele displayed neural inefficiency and reduced hippocampal volume (Lancaster et al., 2015). Additionally, carriers of the G-risky allele of the rs9331888 polymorphisms have also been associated with increased brain Aβ deposition and reduced hippocampal volume (Tan et al., 2016). However, while the direct effects of these polymorphisms are is not fully understood, a few studies have indicated that altered ApoJ plasma levels and altered ApoJ alternative splicing are among the possibilities (Mullan et al., 2013; Schurmann et al., 2011; Szymanski, Wang, Bassett, & Avramopoulos, 2011; Xing et al., 2012).

## 6.3 | LCAT, PLTP and CETP

On the other end, mutations in the *LCAT* gene, which cause a drop in LCAT activity, may also be responsible for greatly reduced HDL, CE levels and ApoA-I levels. Mutation in *LCAT* is responsible for two similar diseases, named Fish Eye Disease when LCAT activity is partially reduced and Familial LCAT deficiency

when LCAT activity is almost completely absent (Kuivenhoven et al., 1997). Unlike with *APOA-I* in which all mutation affecting the binding to LCAT were clustered in a relatively small portion of the *APOA-I* gene, *LCAT* mutation are spread across the whole molecule. Several reports have indicated that missense mutation (P10Q, G30S, A93T, T123I, R135Q, R135W, T147I, Y156N, R158C, L209P, V309M, T321M, C337Y and T347M), nonsense mutations (Y83STOP and P260STOP) and frameshift mutations (H35 >61Stop and Q376 >416Stop) have all been associated with reduced or absent LCAT activity rather than the inability of LCAT to correctly bind to ApoA-I (Baass et al., 2009; Fotakis, Kuivenhoven, Dafnis, Kardassis, & Zannis, 2015; Funke et al., 1993; Idzior-Walus et al., 2006; Kasid, Rhyne, Zeller, Pritchard, & Miller, 2001; Klein et al., 1992, 1993; Kuivenhoven et al., 1996; Rosset, Wang, Wolfe, Dolphin, & Hegele, 2001; Yang et al., 1997). Mutations affecting *PLTP* have been described in previous studies, in which lower PLTP activity is associated with altered HDL remodelling, most notably smaller HDL particle size but in higher numbers (Aouizerat et al., 2006; Vergeer, Boekholdt, et al., 2010). This is in accordance with the notion that PLTP mediates the fusion of small $HDL_3$ particles into larger $HDL_2$, therefore a reduced PLTP activity is viewed as a limiting factor in such remodelling maintaining HDL particles as $HDL_3$ (Vergeer, Boekholdt, et al., 2010). It is therefore fitting that high PLTP activity, which reduced the number of $HDL_3$ particles, has been associated with an increased risk of cardiovascular diseases (van Haperen et al., 2002; Moerland et al., 2008; Robins, Lyass, Brocia, Massaro, & Vasan, 2013; Schlitt et al., 2003), since $HDL_3$ particles have been described protective against atherosclerosis (Kontush et al., 2003). In GWAS, a *PLTP* polymorphism linked to higher PLTP transcripts was also associated with higher HDL levels (Kathiresan et al., 2009). However, a study in a Japanese population did not report any genetic association between five polymorphisms on *PLTP* and AD (Kuerban, Shibata, Komatsu, Ohnuma, & Arai, 2010). Several *CETP* mutations have been associated with greatly reduced levels of CETP, with consequent reduction in protein activity. Mutations in the *CETP* gene, mostly in Japanese individuals, in the intron 14 (Int14T and Int14A), exon 5 (L151P), exon 9 (R268Stop and R282C) and exon 15 (D442G) led to minimal or null levels of CETP with greatly increased levels of HDL (Brown et al., 1989; Inazu et al., 1990, 1994; Nagano et al., 2002; Teh, Dolphin, Breckenridge, & Tan, 1998). In some instances, ApoA-I levels were also increased, whereas ApoB levels were decreased (Inazu et al., 1990; Teh et al., 1998). An analysis of studies performed between 1970 and 2008 indicated that the C-629A and I405V mutations lead to lower CETP activity, higher HDL and higher ApoA-I levels (Thompson et al., 2008). As it is widely accepted that high levels of HDL provide protection against cardiovascular disease, *CETP* mutations that increase HDL levels are often associated with reduced risk of coronary heart disease (CHD), although results from several studies are controversial (Curb et al., 2004; Guo et al., 2016; Millwood et al., 2017; Moriyama et al., 1998; Robins et al., 2013; Thompson et al., 2008; Zhong et al., 1996). It is therefore fitting that two mutations that reduced CETP activity and also increased HDL and ApoA-I levels (C-629A and I405V) have been associated with reduced memory decline, reduced brain atrophy and reduced risk for AD (Lythgoe et al., 2015; Murphy et al., 2012; Rodriguez et al., 2006; Sanders et al., 2010; Sundermann et al., 2016).

## 6.4 | HL and EL

HDL particles were also affected by mutations in the HL and EL genes, the majority of which, by lowering the enzymes' activity, affected the remodelling of $HDL_2$. Many *HL* mutations have been associated with reduced levels and/or reduced activity of the lipase (Durstenfeld, Ben-Zeev, Reue, Stahnke, & Doolittle, 1994; Knudsen et al., 1996, 1997; Ruel et al., 2003; Tilly-Kiesi et al., 2004). Associated with such reduced activity were various degrees of higher HDL levels, higher $HDL_2$ fractions and higher HDL triglyceride content. Intronic (Intron 1) and promoter region (C-480T) mutations have also been linked to HL deficiency (Brand, Dugi, Brunzell, Nevin, & Santamarina-Fojo, 1996; Jansen, Verhoeven, et al., 1997). However, although one report indicated a genetic association, results are controversial (Laws et al., 2010; Xiao et al., 2012; Zhu, Taylor, Bennett, Younkin, & Estus, 2008). As for *EL*, a variety mutations have been identified in humans, but only a small subset of these have been associated with increased plasma HDL levels, as some of these mutations do not affect EL activity (Razzaghi et al., 2013). Among all mutations, T111I and N396S are two of four mutations (G26S and T298S being the other two) found in individuals with high HDL levels (deLemos, Wolfe, Long, Sivapackianathan, & Rader, 2002). However, several reports have associated high HDL levels with reduced EL activity caused by the N396S mutation (Edmondson et al., 2009; Singaraja et al., 2013; Voight et al., 2012), whereas the mutation T111I, which does not affect EL activity, appears to have questionable effect on HDL levels, if any (Reilly, Foulkes, Wolfe, & Rader, 2005; Vergeer, Cohn, et al., 2010). However, in spite of being associated with higher HDL levels, the Ser carrier in the N396S mutation has been associated with depressive symptoms and greater white matter lesions (Amin et al., 2017).

## 6.5 | HDL receptors

HDL levels are also affected by mutations in the *SR-BI* gene, which is involved in HDL catabolism. First reported in 1999 (Acton et al., 1999), other reports have subsequently confirmed that several mutations in the *SR-BI* gene affect HDL levels, possibly by altering the CE uptake rate in hepatocytes (Brunham et al., 2011; Morabia et al., 2004; Osgood et al., 2003; Vergeer et al., 2011; Zeng, Tang, Ye, Su, & Jiang, 2017). Accordingly, mutations in another HDL receptor, *CD36*, have been associated with altered HDL levels and risk for metabolic syndrome (Love-Gregory et al., 2008). However, the intronic polymorphism rs3211982, which has been associated with AD

(Sery et al., 2017), was not among those associated with metabolic syndrome and HDL levels.

## 7 | CONCLUSIONS

It is clear that the association between AD and HDL goes beyond the simple evaluation of HDL levels, which more often than not, shows minimal or no alteration at all. As of today, there is enough evidence to support the theory that HDL composition plays a more important role than previously assumed, as the delicate balance between protective and dangerous molecules carried during the various stages of HDL maturation may play a prominent role in protecting or predisposing to AD. This HDL alteration of composition, often associated with the $HDL_3$ protective subclass, has already been reported for many other diseases, some of which are considered risk factors for AD. Recently, there has been a consistent commitment towards improving prevention and reducing the risk for AD by acting on lifestyle factors such as healthy diets and increased physical exercise. Among diets, the Mediterranean Diet (rich in fruits, vegetables, bread and other grains, potatoes, beans, nuts and seeds, uses olive oil as a primary fat source and limits the consumption of dairy products, eggs, fish and poultry) has been linked to a reduced rate of AD, but it has also shown the capacity to improve HDL functionality, which is directly associated with the quality of the HDL protein cargo composition. While this does not represent a direct cause-effect link, it would be also unfair to dismiss it as simply random effect. Additionally, the use of synthetic HDL has also been tested as therapeutic agent in acute coronary syndrome and albeit this avenue could be tested in AD, previous data regarding the importance of high HDL levels have provided conflicting results and it appears that cargo composition is more important than absolute levels. In accordance, several HDL-associated proteins have been linked to neurodegeneration and brain atrophy, often in AD-related brain areas such as the hippocampus and cortex, but very rarely to areas that are unaffected in the disease such as cerebellum. Taken together, these data paint a broad picture that indicates a very strong association between HDL, and more importantly HDL-associated cargo with AD. It is therefore necessary a more detailed analysis of HDL-associated proteins to understand their role in the disease, as not only indicated by the detrimental role of ApoE4, compared to ApoE3 or ApoE2, but also by the synergistic interaction between ApoE and ApoJ or the unexpected effects observed in ApoA-I/$ApoE_{dko}$ mice.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Chapter 4

## Plasma high density lipoprotein small subclass is reduced in Alzheimer's disease patients and correlates with cognitive performance

**Plasma High Density Lipoprotein Small Subclass is Reduced in Alzheimer's Disease Patients and Correlates with Cognitive Performance. Pedrini S,** Hone E, Gupta VB, James I, Teimouri E, Bush AI, Rowe CC, Villemagne VL, Ames D, Masters CL, Rainey-Smith S, Verdile G, Sohrabi HR, Raida MR, Wenk MR, Taddei K, Chatterjee P, Martins I, Laws SM, Martins RN; AIBL Research Group. J Alzheimers Dis. 2020;77(2):733-744.

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## 4.1 Abstract

**Background**: The link between cholesterol and Alzheimer's disease (AD) has received much attention, as evidence suggests high levels of cholesterol might be an AD risk factor. The carriage of cholesterol and lipids through the body is mediated via lipoproteins, some of which, particularly apolipoprotein E (ApoE), are intimately linked with AD. as In humans, high density lipoprotein (HDL) is regarded as a "good" lipid complex due to its ability to enable clearance of excess cholesterol via 'cholesterol reverse transport', although its activities in the pathogenesis of AD are poorly understood. There are several subclasses of HDL; these range from the newly formed small HDL, to much larger HDL.

**Objective**: We examined the major subclasses of HDL in healthy controls, mild cognitively impaired and AD patients who were not taking statins to determine whether there were HDL profile differences between the groups, and whether HDL subclass levels correlated with plasma amyloid β (Aβ) levels or brain Aβ deposition.

**Methods**: Samples from AIBL cohort were used in this study. HDL subclass levels were assessed by Lipoprint while Aβ1-42 levels were assessed by ELISA. Brain Aβ deposition was assessed by PET scan. Statistical analysis was performed using parametric and non-parametric tests.

**Results**: We found that small HDL subclass is reduced in AD patients and it correlates with cognitive performance while plasma Aβ concentrations do not correlate with lipid profile or HDL subfraction levels.

**Conclusions**: Our data indicate that AD patients exhibit altered plasma HDL profile and that HDL subclasses correlate with cognitive performances.

# Chapter 5

## Plasma high density lipoprotein cargo proteins are altered in Alzheimer's disease and is associated with regional brain volume

**Plasma high density lipoprotein cargo proteins are altered in Alzheimer's disease and is associated with regional brain volume. Pedrini S**, Doecke J, Hone E, Wang P, Thota R, Bush AI, Rowe CC, Dore V, Villemagne VL, Ames D, Rainey-Smith S, Verdile G, Sohrabi HR, Raida MR, Wenk MR, Taddei K, Ehrlich M, Gandy S, Masters CL, Chatterjee P, and Martins RN and the AIBL Research Group. J Neurochem. 2022 Oct;163(1):53-67.

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ORIGINAL ARTICLE

# Plasma high-density lipoprotein cargo is altered in Alzheimer's disease and is associated with regional brain volume

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## Abstract

Cholesterol levels have been repeatedly linked to Alzheimer's Disease (AD), suggesting that high levels could be detrimental, but this effect is likely attributed to Low-Density Lipoprotein (LDL) cholesterol. On the other hand, High-Density Lipoproteins (HDL) cholesterol levels have been associated with reduced brain amyloidosis and improved cognitive function. However, recent findings have suggested that HDL-functionality, which depends upon the HDL-cargo proteins associated with HDL, rather than HDL levels, appears to be the key factor, suggesting a quality over quantity status. In this

**Abbreviations:** AD, Alzheimer's Disease; Aβ, amyloid-β; BBB, blood-brain barrier; CAA, cerebral amyloid angiopathy; CRP, C-reactive protein; CSF, cerebrospinal fluid; GM, grey matter; HC, healthy control; HC-Conv, healthy control converter; HDL, high-density lipoprotein; HL, hippocampus left; HR, hippocampus right; LD, lipid droplets; LDL, low-density lipoprotein; LTP, long-term potentiation; MMSE, mini-mental state examination; SAA, serum amyloid A; SUV, standardized uptake values; SUVR, standardized uptake value ratio; VLDL, very low-density lipoproteins; WM, white matter.

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report, we have assessed the HDL-cargo (Cholesterol, ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA) in stable healthy control (HC), healthy controls who will convert to MCI/AD (HC-Conv) and AD patients (AD). Compared to HC we observed an increased cholesterol/ApoA-I ratio in AD and HC-Conv, as well as an increased ApoD/ApoA-I ratio and a decreased ApoA-II/ApoA-I ratio in AD. Higher cholesterol/ApoA-I ratio was also associated with lower cortical grey matter volume and higher ventricular volume, while higher ApoA-II/ApoA-I and ApoJ/ApoA-I ratios were associated with greater cortical grey matter volume (and for ApoA-II also with greater hippocampal volume) and smaller ventricular volume. Additionally, in a clinical status-independent manner, the ApoE/ApoA-I ratio was significantly lower in APOE ε4 carriers and lowest in APOE ε4 homozygous. Together, these data indicate that in AD patients the composition of HDL is altered, which may affect HDL functionality, and such changes are associated with altered regional brain volumetric data.

**KEYWORDS**
HDL, cholesterol, Alzheimer's disease, HDL-cargo, ApoE, amyloid-β

## 1 | INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease which is characterized by the extracellular deposition of amyloid β (Aβ) in the brain to form amyloid plaques and by the intracellular accumulation of hyperphosphorylated tau filaments (Masters et al., 2015). These combined events eventually are responsible for neuroinflammation, neuronal death, and reduction of brain volume, ultimately leading to the onset of disease symptoms. Several reports have indicated that diet, high cholesterol, high low-density lipoproteins (LDL) cholesterol, and low high-density lipoproteins (HDL) cholesterol levels are possible AD risk factors. Low cholesterol levels have been linked to Aβ precursor protein (AβPP) processing through the non-amyloidogenic pathway (Buxbaum et al., 2001; Fassbender et al., 2001; Kojro et al., 2001; Simons et al., 1998), while high levels of intracellular cholesterol have been linked to an increase in Aβ deposition in the brain (Burns et al., 2003; Refolo et al., 2000; Wahrle et al., 2002). These results were also supported by studies in rodents indicating that high-fat diets affect brain amyloid levels and brain mass (Levin-Allerhand et al., 2002; Pedrini et al., 2009; Refolo et al., 2000). Many studies have therefore suggested a protective role of HDL for AD, indicating that higher HDL cholesterol levels have been associated with better cognitive outcomes, higher MMSE, a reduced risk for AD (but not to MCI), and increased brain grey matter and hippocampal volume (Atzmon et al., 2002; Bates et al., 2017; Reitz et al., 2008; Reitz et al., 2010; Ward et al., 2010; Wolf et al., 2004). Furthermore, HDL cholesterol levels have been shown to inversely correlate with brain Aβ deposits (Reed et al., 2014). Conversely, low HDL cholesterol levels were associated with white matter changes, a higher probability of memory deficits, or a greater risk of dementia (Crisby et al., 2010; Singh-Manoux et al., 2008; Wolf et al., 2004). However, in spite of several studies indicating a protective role for HDL, other reports failed to confirm such an association (den Heijer et al., 2005; van Velsen et al., 2013). Additionally, discrepancies were also reported when assessing the overall levels of HDL cholesterol, with some studies indicating altered levels of HDL cholesterol between controls and AD, while other studies did not (den Heijer et al., 2005; Isbir et al., 2001; Reitz et al., 2004; Xiao et al., 2012; Zhang et al., 2004). These findings may indicate that HDL particles provide actions that go far beyond the classical role of excess cholesterol elimination by the liver. Many of these effects, such as anti-inflammatory and anti-oxidant, are mediated through HDL-protein cargo, which comprises many apolipoproteins (and several other proteins) that are associated with and form the HDL particles, extensively described in several studies (Ronsein & Vaisar, 2019; Shah et al., 2013; Yassine et al., 2014). For example, ApoA-I, the major constituent of plasma HDL, has shown protective effects in AD by binding to Aβ and its precursor protein (AβPP), reducing Aβ aggregation and Aβ-induced toxicity (Koldamova et al., 2001; Paula-Lima et al., 2009). In animal studies, overexpression of ApoA-I reduced cognitive deficits and cerebral amyloid angiopathy (CAA), despite unaltered Aβ deposition, likely through reduced neuroinflammation (Lewis et al., 2010). Conversely, depletion of ApoA-I increased CAA and Aβ aggregation with consequent worsening of cognitive function without altering APP processing and Aβ deposition (Lefterov et al., 2010). Altogether, it appears that ApoA-I modulates Aβ toxicity through mechanisms other than altering APP processing and Aβ deposition. Whilst high levels of ApoA-I have been associated with a reduced risk of dementia (Saczynski et al., 2007), reduced levels of ApoA-I have been described in AD and correlate with the severity of the disease (Kawano et al., 1995; Liu et al., 2006; Merched et al., 2000). On the other hand, ApoE is the major constituent of CSF HDL and the ε4 isoform is considered the major risk factor for sporadic AD (Corder et al., 1993; Strittmatter et al., 1993). ApoE ε4 isoform has been associated with increased Aβ production, accumulation and oligomerization (Bales et al., 2009; Hashimoto et al., 2012; Koffie et al., 2012; Ye et al., 2005; Youmans et al., 2012) and with reduced Aβ clearance (Castellano et al., 2011;

Cook et al., 2003; Deane et al., 2008; Du et al., 2009). With regard to Aβ degradation mediated by binding to ApoE, ApoE ε3 binds to Aβ more efficiently than ApoE ε4 and astrocytes from ApoE ε4 transgenic mice clear amyloid plaques less efficiently than astrocytes from ApoE ε3 transgenic mice (Simonovitch et al., 2016; Tokuda et al., 2000). Additionally, in an AD mouse models, CAA is induced more prominently by ApoE ε4 compared to ApoE ε3 (Fryer et al., 2005) and Aβ stimulation affected Long Term Potentiation (LTP) more prominently in ApoE ε4 transgenic mice rather than in ApoE ε2 or ApoE ε3 transgenic mice (Trommer et al., 2005). ApoE ε4 has also been strongly associated with increased atrophy and reduced hippocampal volume (Agosta et al., 2009; Hostage et al., 2013; Manning et al., 2014; Moffat et al., 2000; Tang et al., 2015). Finally, since low levels of ApoE and ApoE ε4 levels were found significantly reduced in AD patients (Beffert et al., 1999; Gupta et al., 2011), it is fitting that lower plasma ApoE levels are linked with the smaller hippocampus (Teng et al., 2015), while higher plasma ApoE levels are linked with reduced brain amyloidosis (Koch et al., 2018). ApoA-II is capable to form complexes with ApoE ε2 and ApoE ε3 but not with ApoE ε4 and these complexes (ApoE ε2/ApoA-II and ApoE ε3/ApoA-II) increased cell viability by binding to Aβ and reducing its internalization. Such protective features were absent in ApoE ε4-treated cells because of the absence of ApoEε4/ApoA-II complexes (Yamauchi et al., 2000). An AD transgenic mouse model lacking ApoA-IV displayed increased cerebral Aβ and learning deficits, likely through mechanisms that are associated with Aβ clearance (Cui et al., 2011), while the absence of ApoD was associated with an increased number of amyloid plaque in the hippocampus and, at the opposite, overexpression of ApoD was associated with a reduced number of them (Li et al., 2015). ApoJ forms complexes with Aβ (ApoJ:Aβ) for their interaction with low-density lipoprotein receptor-related protein 2 (Megalin), which mediates the transport and the clearance of Aβ from and to the brain (Bell et al., 2007; Hammad et al., 1997; Zlokovic et al., 1994; Zlokovic et al., 1996). Levels of ApoJ are increased in plasma and brain regions of AD patients, specifically in areas that are loaded with amyloid plaques (Bertrand et al., 1995; Gupta et al., 2016; Gupta et al., 2017; Howlett et al., 2013; Lidstrom et al., 1998; May et al., 1990; Miners et al., 2017). However, it is important to point out that the composition of plasma HDL is different from CSF HDL as not all apolipoproteins associated with HDL are produced by brain-resident cells. In the CSF ApoE and ApoJ are the main apolipoproteins, while ApoA-I, the main constituent of plasma HDL is not produced in the central nervous system. However, damage to the blood-brain barrier (BBB), common in AD, allows ApoC-III, not usually produced in the brain, to be detected in the CSF (Picard et al., 2022), while ApoA-I can be transported across the BBB at the choroid plexus (Stukas et al., 2014). In general, lipid dysregulation in the brain affects AD like many other neurodegenerative diseases. In this regard, lipid droplets (LD) which are cytoplasmatic organelles containing cholesteryl esters which provide energy for cell metabolism and membrane synthesis (Walther & Farese Jr., 2012) were first described by Alois Alzheimer (Alzheimer et al., 1995). In AD, lipid droplet accumulation is an event that takes place before amyloid accumulation, emphasizing the importance of lipid metabolism in the disease (Hamilton et al., 2015). The importance of LD in AD and many other neurodegenerative diseases was recently assessed (Farmer et al., 2020; Ralhan et al., 2021).

Modulation of HDL cargo has been observed and reported in many diseases, such as Coronary Heart Disease, Acute Coronary Syndrome, End Stage Renal Disease and Liver Disease (Alwaili et al., 2012; Trieb et al., 2016; Vaisar et al., 2007; Weichhart et al., 2012; Yan et al., 2014). Aging has also been reported to be a factor in modulating HDL protein cargo (Holzer et al., 2013). In light of these tight connections between HDL-protein cargo and AD and the fact that modulation of HDL protein cargo is a common factor in many other diseases, our goal in this study is to determine if HDL-protein cargo is altered in AD and if specific changes can be directly associated with the extent of brain amyloidosis. If so, alteration of HDL-protein cargo could serve as a direct measurement of amyloid deposits in the brain, providing a diagnostic tool that could indicate healthy controls at risk for AD.

## 2 | MATERIAL AND METHODS

### 2.1 | Participants

The AIBL study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committees of St. Vincent's Health and Austin Health in Melbourne and Hollywood Private Hospital and Edith Cowan University in Perth (Australia; Protocol number HPH215). All volunteers gave written and informed consent before participating in our study. A total of 213 participants were divided into stable healthy controls (HC, $n = 87$, HC for at least 36 months (t1 = 18 months and t2 = 36 months after HDL-cargo analysis (t0))), healthy controls converters (HC-Conv, $n = 38$, HC at the time of HDL-cargo analysis (t0) but converted to MCI/AD within the following 36 months (either at t1 or t2)) and Alzheimer's patients (AD, $n = 88$, from t0 through t2) from the AIBL cohort were used in this study (Figure S1). Exclusion criteria included a history of non-AD dementia, schizophrenia, bipolar disorder, current depression (GDS score above 5/15), Parkinson's disease, uncontrolled hypertension (systolic BP >170 or diastolic BP >100), cancer (other than basal cell skin carcinoma) within the last two years, symptomatic stroke, uncontrolled diabetes, or current regular alcohol use exceeding two standard drinks per day for women or four per day for men (Ellis et al., 2009). The AIBL Study clinical panel meets on a monthly basis to discuss baseline classification for each new patient and to ensure that diagnoses were made in accordance with the NINCDS-ARDA criteria (McKhann et al., 1984; Winblad et al., 2004). Body parameters such as weight, height, blood pressure, and pulse rate were evaluated during the examinations. Blood was drawn from overnight fasting participants and collected to obtain plasma for our analysis. *APOE* status was determined by genotyping cells from whole blood as previously described (Gupta et al., 2011). Total cholesterol, LDL, HDL, and TG levels in plasma were also assessed.

## 2.2 | HDL fractionation

HDL fraction was obtained by precipitating VLDL and LDL fractions using a solution identical to the one reported in the Quantolip® protocol (Reagent A: $Na_2HPO_4x2H_2O$ 8 g/L, $NaH_2PO_4xH_2O$ 11 g/L, $Na_2EDTA$ 1 g/L, Sodium Azide 0.9 g/L, Polyethylenglycol 20,000 95 g/L). Briefly, 30 µl of plasma were mixed with 60 µl of Reagent A and sat for 10 min at RT, followed by 15 min of centrifugation at 2500 g. The supernatant containing the HDL fraction was then collected, aliquoted, and stored at −80°C until analysis. HDL isolation by Polyethylenglycol precipitation has already been used in the past (Kostner et al., 1985).

## 2.3 | HDL and HDL-associated protein measurement in HDL fractions

HDL-cholesterol in HDL fractions (dilution 1:75) was assessed using HDL-Cholesterol Assay Kit (Cell Biolabs) using the manufacturer's instructions.

HDL-cargo proteins in HDL fractions were assessed by Bioplex analysis. Serum Amyloid A (SAA) levels associated with HDL fractions (dilution 1:200) were evaluated using SAA bioplex kit (Merck Millipore) using the manufacturer's instructions. A broad panel of Apolipoprotein (ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, and CRP) levels associated with HDL fractions (dilution 1:10,000) were evaluated using Pro Human Apolipoprotein Panel bioplex kit (Biorad) using the manufacturer's instructions (Jóźwiak et al., 2022; Mlambo et al., 2020).

## 2.4 | Calculations of HDL and HDL-associated proteins

For references and calculation, in this paragraph, we will refer to cholesterol-$HDL_{PL}$ as the cholesterol concentration on HDL in plasma (PL) and to cholesterol-$HDL_{HF}$ as the cholesterol concentration on HDL in the HDL fraction (HF). We will also refer to Apolipoproteins/SAA/CRP as $Protein_{HF}$, for formula convenience, as all proteins were measured in the HDL fraction.

To calculate the correct amount of each Protein (in ng) or cholesterol (in µg) per µg of ApoA-I in the HDL fraction, we used the following formulas: $Protein_{HF}(ng/ml)/ApoA\text{-}I_{HF}(\mu g/ml)$ and $Cholesterol\text{-}HDL_{HF}(\mu g/ml)/ApoA\text{-}I_{HF}(\mu g/ml)$. This calculation indicates the HDL-cargo composition with regard to ApoA-I which is the major and more stable apolipoprotein in HDL particles.

To calculate the correct amount of each Protein (in µg) associated to HDL) in 1 ml plasma, we used the following formula: $Protein_{HF}(\mu g/ml)/Cholesterol\text{-}HDL_{HF}(mg/ml)*Cholesterol\text{-}HDL_{PL}(mg/ml)$.

For general purposes, we will refer to HDL-cargo to the 10 analytes assessed (Cholesterol, ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, SAA) compared to ApoA-I levels.

## 2.5 | PET scan

PET scans consisting of 30 min acquisitions were performed 40 min after injection of 370 MBq $^{11}$C-PiB. PET images were processed using a semi-automatic region-of-interested method as previously described (Villemagne et al., 2011). Standardized uptake values (SUV) for $^{11}$C-PiB were calculated for all brain regions examined. The SUV ratio (SUVR) was calculated by dividing all regional SUV by the cerebellar cortex SUV. However, the centiloid scale was recently proposed to provide a standard quantification of Aβ-PET images. In the centiloid scale, the Aβ burden can be expressed with values ranging from 0 (the typical Aβ burden in young controls) to 100 (the typical Aβ burden in mild AD patients) (Klunk et al., 2015). Centiloid values were generated using CapAIBL as described elsewhere (Bourgeat et al., 2018).

## 2.6 | MRI imaging

Scanning centers either in Melbourne or Perth were used to acquire images using Siemens 3T Trio and Siemens 3T Skyra scanners (Melbourne) or Siemens 3T Verio and Siemens 1.5T Avanto scanners (Perth). The scans also included a 3D MPRAGE (Magnetization Prepared Rapid Acquisition Gradient Echo) image (voxel size 1.2×1×1 mm3, repetition time/echo time = 2300/2.98, flip angle = 9°). A 3D T2-weighted Fluid-attenuation inversion recovery (FLAIR) sequence, included in the image acquisition protocol, was obtained using two different sets of parameters. Gradient Recalled Echo (GRE) images used for SWI (Susceptibility-Weighted Imaging) and QSM (Quantitative Susceptibility Mapping) were also acquired. Full details of protocols and parameters are described elsewhere (Fowler et al., 2021).

## 2.7 | Statistical analysis

The operator was unaware of the experimental groups during the experiments and data collection. The sample size was determined using G-Power, assuming a Cohen's D of 0.5 (medium-size effect). For such analysis, to obtain a significance of $p < 0.05$, $n = 64$ samples were required in both HC and AD groups. Data were assessed for normality using Skewness and Kurtosis analysis and were log-transformed when values were outside of the −2/+2 range. An interquartile range test was used to assess for outliers. Participant demographic and clinical characteristics were compared using either ANOVA or Wilcoxon Signed Ranks test for age, HDL, MMSE, and Brain Aβ deposition, and the Chi-squared test for Gender, Site, *APOE* ε4 allele status, and brain Aβ deposition status. Statistical comparison of biomarker means in different groups was performed using Generalized linear models, unadjusted or adjusted for age, gender, and *APOE* ε4 allele status. When necessary, data were log-transformed to better approximate normal distribution. *p*-values less than 0.05 were regarded as nominally significant, with values less

than 0.005 (Bonferroni adjusted value [0.05/10]) regarded as statistically significant. Partial correlation analysis was run upon adjustment for adjustment for covariates such as age, gender, and *APOE* ε4 allele status. Analyses were carried out using the R Statistical Environment (R Core Team, www.r-project.org, v4.0.2) and SPSS version 27. For the Generalized linear model analyses, outliers were replaced with the median value for each marker. For the correlation analyses, outliers were not replaced.

We applied recursive feature elimination (RFE) to rank the HDL-cargo proteins in differentiating different groups of individuals (AD, HC-Conv, and HC) based on random forest (RF) classifier. RF is among the most robust to noise and missing data machine learning methods and RF is robust to overfitting and redundancy in predictors (Grinberg et al., 2020). RFE first fit the RF model using all the proteins as predictors, where each predictor was then ranked using its importance to the classification performance. At each iteration of the RFE process, top-ranked predictors were retained, the model was refitted and performance was reassessed. Consequently, the proteins and factors that did not improve the classification accuracy were removed, and the top predictors were used to fit the final model. 1000 trees were used to build the RF model and root mean square error (RMSE) was used as the criterion to select the optimal model. Auto tuning was then applied to optimize the parameters of the RF. We implemented RFE with RF classification using caret package v6.0-86 (Kuhn, 2008). This was performed by ten times five-fold cross-validation. This variable selection method provided insight into which variables and/or factors have the most distinguishing power in classifying Alzheimer's patients, thus those proteins and factors can serve as biomarkers for better diagnosis and prognosis. We evaluated all HDL-cargo proteins as potential biomarkers and assessed the 5 most important with the goal to determine the best set of proteins relevant to other factors as diagnostic and prognostic biomarkers.

## 3 | RESULTS

The basic demographics of the study participants are summarized in Table 1. In total, 87 stable healthy controls (HC), 38 HC-Converters (HC-Conv), and 88 Alzheimer's patients (AD) were studied (all participants were 65 years old and older). All 213 samples were evaluated for total HDL cholesterol levels and Mini-Mental State Exam (MMSE) scores, while a smaller subset was assessed for brain amyloid deposition (assessed by PET scan) (Table 1).

The levels of HDL-cargo proteins, along with HDL cholesterol (which refers to the cholesterol associated with HDL particles), were assessed compared to the levels of ApoA-I, which is the main apolipoprotein expressed on HDL particles. Generalized linear model (HC compared to AD) corrected for age, gender, and *APOE* ε4-carrier status are shown in Table 2. Of the 10 HDL-cargo analytes assessed among clinical groups, analyses showed statistically significant increases in cholesterol/ApoA-I ($p < 0.001$ and $p = 0.001$, unadjusted and adjusted, respectively) and ApoD/ApoA-I ($p < 0.001$ for both unadjusted and adjusted) and a significant decrease in ApoA-II/ApoA-I ($p < 0.001$ for both unadjusted and adjusted) in AD participants as compared with controls (Table 2).

We then assessed if such changes in HDL cholesterol and HDL-cargo (compared to ApoA-I) occurred before the conversion from HC to AD or if such changes were mainly associated with the ongoing disease. Generalized linear model corrected for age, gender, and *APOE* ε4-carrier status for HC compared to HC-Conv are shown in Table 3. Unlike the HC vs AD comparisons, only the levels of cholesterol/ApoA-I were nominally significantly (increased in HC-Conv as compared with HC; $p = 0.019$ and $p = 0.031$, unadjusted and adjusted, respectively). All other analytes in the HDL-cargo did not statistically differ between HC and HC-Conv, suggesting that some HDL-cargo remodeling (such as ApoA-II/ApoA-I and ApoD/ApoA-I) takes place once after the clinical onset of the disease (Table 3).

As the cholesterol/ApoA-I was the only analyte that differed between HC and HC-Conv, we investigated if such change took place in the proximity of the clinical onset or years before. As the HC-Conv group includes individuals who converted to MCI/AD at 18 and 36 months, we assessed if the increased ratio of cholesterol/ApoA-I in HC-Conv was affected by a similar magnitude in both HC-Conv groups (i.e. @18 & 36 months, Figure 1). Interestingly, individuals whose conversion to MCI/AD will take place within 18 months and were, therefore, closer to conversion (HC-Conv 18m) had a cholesterol/ApoA-I ratio significantly higher than in HC ($p < 0.001$) and similar to the AD

**TABLE 1** Demographic characteristics, HDL levels, cognitive score, and amyloid brain deposition in HC, HC-Conv, and AD participants

| | HC | HC-Conv | AD | *p* |
|---|---|---|---|---|
| *N* | 87 | 38 | 88 | |
| Age | 73 ± 6 | 75 ± 7 | 77 ± 7 | 0.27 |
| Gender (M/F) | 43/44 | 19/19 | 39/49 | 0.75 |
| Site (Melbourne/Perth) | 45/42 | 19/19 | 58/30 | 0.10 |
| APOE ε4 (no/yes) | 57/30 | 19/19 | 26/62 | **<0.001** |
| HDL Cholesterol (mg/dl) | 64 ± 15 | 68 ± 21 | 65 ± 17 | 1.00 |
| MMSE | 29 ± 1 | 28 ± 1 | 20 ± 4 | **<0.001** |
| Brain Aβ deposition (n/y) | 60/23 | 7/4 | 1/18 | **<0.001** |

*Note*: Values are presented as mean ± SD or as frequency. Analysis was considered significant with $p < 0.05$ (bold).

| | HC (*n*) | AD (*n*) | *p* | *p*[a] |
|---|---|---|---|---|
| µgCholesterol/µgApoA-I | 1.34 ± 0.33 (87) | 1.54 ± 0.38 (88) | **0.000183** | **0.00139** |
| ngApoA-II/µgApoA-I | 293 ± 87 (77) | 246 ± 53 (88) | **6.11e-05** | **0.000403** |
| ngApoC-I/µgApoA-I | 298 ± 73 (75) | 273 ± 66 (85) | **0.0213** | 0.274 |
| ngApoC-III/µgApoA-I | 58.4 ± 20.5 (87) | 56.9 ± 19.8 (88) | 0.617 | 0.531 |
| ngApoD/µgApoA-I | 44.3 ± 8.1 (87) | 49.8 ± 9.2 (88) | **4.36e-05** | **0.000383** |
| ngApoE/µgApoA-I | 13.7 ± 5.3 (87) | 10.4 ± 4.5 (88) | **2.25e-05** | **0.0243** |
| ngApoH/µgApoA-I | 524 ± 155 (87) | 492 ± 141 (88) | 0.149 | 0.126 |
| ngApoJ/µgApoA-I | 46.4 ± 10.4 (87) | 45.1 ± 9.7 (88) | 0.382 | 0.751 |
| ngCRP/µgApoA-I | 5.39 ± 3.36 (87) | 4.63 ± 2.37 (88) | 0.0867 | 0.117 |
| ngSAA/µgApoA-I | 12.9 ± 14.8 (87) | 18.9 ± 20.1 (88) | **0.0284** | 0.107 |

**TABLE 2** Comparison of HDL-cholesterol and HDL-cargo (ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA) expressed as a ratio to ApoA-I in stable HC vs AD

*Note*: Values are presented as mean ± SD. Generalized Linear Model analyses were performed unadjusted (*p*) and adjusted for age, gender, and ApoE ε4-carrier status (*p*[a]). Data are presented as raw values, but statistical analysis was performed on log-transformed data to better approximate normal distribution. Analysis was considered significant with $p < 0.05$ (bold).

[a]Data were adjusted for age, gender, and ApoE ε4-carrier status.

| | HC (*n*) | HC-Conv (*n*) | *p* | *p*[a] |
|---|---|---|---|---|
| µgCholesterol/µgApoA-I | 1.34 ± 0.33 (87) | 1.51 ± 0.38 (38) | **0.0188** | **0.0307** |
| ngApoA-II/µgApoA-I | 293 ± 87 (77) | 272 ± 69 (36) | 0.156 | 0.195 |
| ngApoC-I/µgApoA-I | 298 ± 73 (75) | 291 ± 72 (35) | 0.611 | 0.928 |
| ngApoC-III/µgApoA-I | 58.4 ± 20.5 (87) | 56.9 ± 17.7 (38) | 0.687 | 0.709 |
| ngApoD/µgApoA-I | 44.3 ± 8.1 (87) | 43.8 ± 6.1 (38) | 0.711 | 0.613 |
| ngApoE/µgApoA-I | 13.7 ± 5.3 (87) | 12.6 ± 4.1 (38) | 0.238 | 0.733 |
| ngApoH/µgApoA-I | 524 ± 155 (87) | 516 ± 118 (38) | 0.759 | 0.742 |
| ngApoJ/µgApoA-I | 46.4 ± 10.4 (87) | 44.1 ± 8.8 (38) | 0.211 | 0.43 |
| ngCRP/µgApoA-I | 5.39 ± 3.36 (87) | 4.76 ± 1.18 (38) | 0.128 | 0.334 |
| ngSAA/µgApoA-I | 12.9 ± 14.8 (87) | 9.4 ± 9.4 (38) | 0.113 | 0.152 |

**TABLE 3** Comparison of HDL-cholesterol and HDL-cargo (ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA) expressed as a ratio to ApoA-I in stable HC vs HC-Converters

*Note*: Values are presented as mean ± SD. Generalized Linear Model analyses were performed unadjusted (*p*) and adjusted for age, gender, and ApoE ε4-carrier status (*p*[a]). Data are presented as raw values, but statistical analysis was performed on log-transformed data to better approximate normal distribution. Analysis was considered significant with $p < 0.05$ (bold).

[a]Data were adjusted for age, gender, and ApoE ε4-carrier status.

group (Figure 1). Conversely, individuals whose conversion to MCI/AD will take place in 36 months and therefore were farther from conversion (HC-Conv 36m) had levels similar to HC (Figure 1), suggesting that cholesterol overload on HDL takes place shortly before conversion to AD (within 18 months before conversion).

Additionally, we have analyzed if the levels of HDL-cargo proteins were altered when assessed as levels of HDL-associated protein per ml of plasma (Table S1). However, as many of the HDL-protein cargo are not exclusively expressed on HDL (with the sole exception of ApoA-I), these values do not represent the total amount of circulating protein in plasma, but only the fraction that it is directly associated with HDL particles.

To determine the effect of APOE genotype on HDL-cargo, we then assessed if any HDL-cargo protein was influenced by APOE genotype and clinical classification, or by APOE genotype alone. ApoE/ApoA-I ratio was significantly affected by APOE genotype ($p < 0.001$) alone. However, there was no significant interaction between clinical classification and APOE genotype, indicating that differences in ApoE levels were solely affected by APOE genotype, while ApoE levels within each APOE genotype were not affected by the clinical classification (Figure 2a). When assessed by APOE genotype alone, ApoE/ApoA-I ratio showed the highest levels in APOE ε2/3 ($p < 0.001$ vs ε3/3, ε3/4, and ε4/4), while APOE ε4/4 showed the lowest ($p < 0.001$ vs ε3/3, ε2/4 and ε3/4) (Figure 2b). Of the remaining 9 HDL-cargo analytes, no HDL-cargo protein (compared to ApoA-I) was affected either by APOE genotype alone or by the interaction between clinical classification and APOE genotype, indicating that their levels were independent of APOE genotype or the clinical classification within each APOE genotype (data not shown).

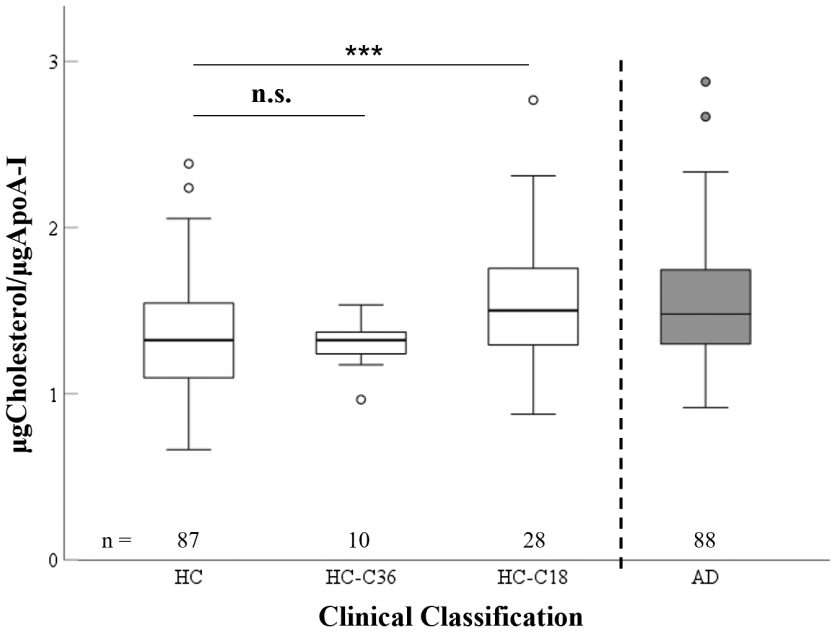


**FIGURE 1** Graphical representation of cholesterol levels on HDL (to ApoA-I levels) in healthy controls (HC), healthy controls who will convert to MCI/AD in 36 months (HC-C36), healthy controls who will convert to MCI/AD in 18 months (HC-C18), and AD participants (AD) (ANOVA df = 2, $F = 5.633$, $0 < 0.01$). *p*-values from pairwise comparisons unadjusted for confounders. Statistical analysis was performed using log-transformed data to better approximate normal distribution. $^{*}p < 0.05$; $^{***}p < 0.001$.

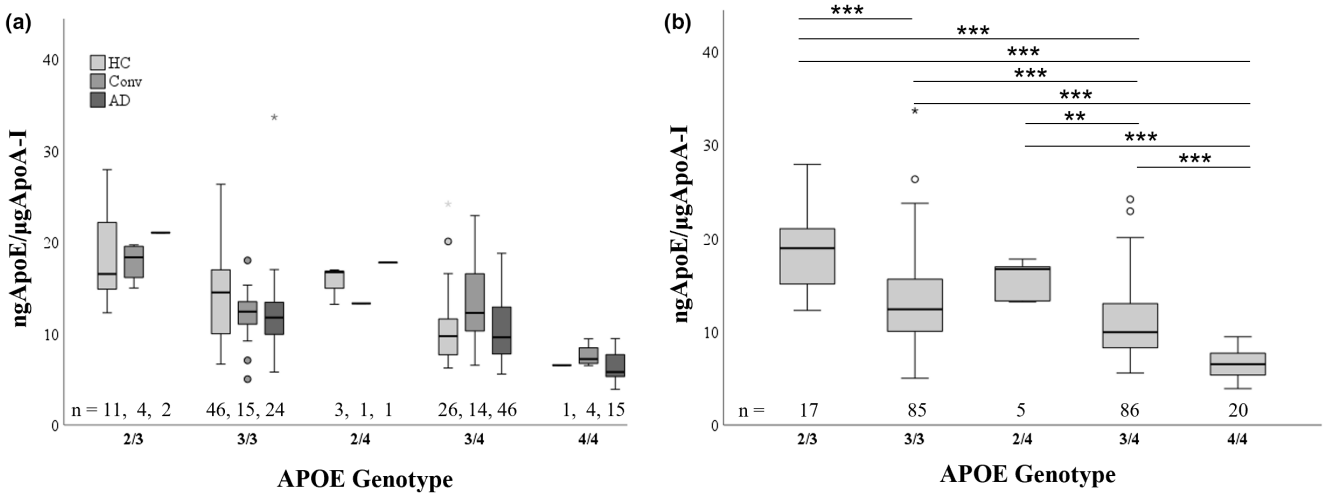


**FIGURE 2** Graphic representation of ApoE levels (to ApoA-I levels) in different APOE genotypes (ε2/3. ε3/3, ε2/4, ε3/4, and ε4/4) in different clinical groups (healthy controls (HC), healthy controls converters (HC-Conv), and AD patients (AD)) (Figure 2a) (ANOVA df = 8, $F = 1.131$, $p = 0.344$). Graphical representation of ApoE levels (to ApoA-I levels) in different APOE genotypes (ε2/3. ε3/3, ε2/4, ε3/4, and ε4/4). *p*-values from pairwise comparisons unadjusted for confounders. Statistical analysis was performed using log-transformed data to better approximate normal distribution. $p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.01$ (Figure 2b) (ANOVA df = 4, $F = 29.236$, $p < 0.001$).

To determine if any of the HDL-cargo analytes correlated with brain amyloid levels in individuals with ongoing brain amyloidosis (HC, HC-Conv, and AD), correlation analyses were carried out (Table 4). In unadjusted correlations, only the levels of cholesterol/ApoA-I significantly correlated with the levels of brain amyloid ($p = 0.036$). However, upon correction for age, gender, and APOE ε4-carrier status, the correlation analysis only approached nominal significance ($p = 0.061$). None of the other HDL-cargo analytes were significantly correlated with brain amyloid levels, regardless of whether the analysis was carried out unadjusted or adjusted for age, gender, and APOE ε4-carrier status (Table 4).

Subsequently, we carried out a correlation analysis (adjusted for age, gender, and APOE ε4-carrier status) to determine if any HDL-cargo protein levels were associated with brain volumetric parameters such as grey matter volume (GM), white matter volume (WM), ventricle volume (Vent), left and right hippocampal volume (HL and HR, respectively) in individuals with ongoing brain amyloidosis (Table 5). Cholesterol/ApoA-I ratio positively and significantly correlated with ventricular volume ($p = 0.043$), while negatively correlated with grey matter volume, albeit this was not quite significant ($p = 0.063$). ApoA-II/ApoA-I ratio positively correlated with grey matter and hippocampal volume ($p < 0.001$, $p = 0.038$ and $p = 0.035$

| | Centiloid t0 (0m) | | | |
|---|---|---|---|---|
| | *r* | *p* | *r*[a] | *p*[a] |
| µgCholesterol/µgApoA-I | **0.314** | **0.036** | 0.292 | 0.061 |
| ngApoA-II/µgApoA-I | −0.041 | 0.790 | −0.030 | 0.850 |
| ngApoC-I/µgApoA-I | 0.228 | 0.142 | 0.163 | 0.314 |
| ngApoC-III/µgApoA-I | 0.065 | 0.685 | 0.020 | 0.900 |
| ngApoD/µgApoA-I | −0.041 | 0.791 | −0.021 | 0.893 |
| ngApoE/µgApoA-I | 0.009 | 0.952 | −0.077 | 0.629 |
| ngApoH/µgApoA-I | −0.086 | 0.575 | −0.060 | 0.707 |
| ngApoJ/µgApoA-I | −0.074 | 0.629 | −0.089 | 0.574 |
| ngCRP/µgApoA-I | −0.186 | 0.222 | −0.189 | 0.229 |
| ngSAA/µgApoA-I | 0.026 | 0.867 | −0.028 | 0.858 |

*Note*: Pearson's correlation unadjusted and adjusted for age, gender ApoE ε4-carrier status was performed between HDL-cargo and brain amyloid data. Analysis was considered significant with $p < 0.05$ (bold).

[a]Data were adjusted for age, gender, and ApoE ε4-carrier status. For all analytes $n = 45$ except for ngApoC-I/µgApoA-I where $n = 43$.

**TABLE 4** Correlation and partial correlation of HDL-cholesterol and HDL-cargo (ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA) expressed as a ratio to ApoA-I with brain amyloid deposition and brain amyloid deposition longitudinal changes in individuals with ongoing brain amyloidosis (PiB+)

**TABLE 5** Partial correlation of HDL-cholesterol and HDL-cargo (ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA) expressed as a ratio to ApoA-I with brain volumetric parameters in individuals with ongoing brain amyloidosis (PiB+)

| | GM t0 | | WM t0 | | Vent t0 | | HL t0 | | HR t0 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *r*[a] | *p*[a] | *r*[a] | *p*[a] | *r*[a] | *p*[a] | *r*[a] | *p*[a] | *r*[a] | *p*[a] |
| µgCholesterol/µgApoA-I | −0.327 | 0.063 | −0.105 | 0.562 | **0.355** | **0.043** | −0.201 | 0.263 | −0.263 | 0.140 |
| ngApoA-II/µgApoA-I | **0.543** | **<0.001** | 0.183 | 0.308 | **−391** | **0.024** | **0.363** | **0.038** | **0.368** | **0.035** |
| ngApoC-I/µgApoA-I | 0.002 | 0.993 | −0.077 | 0.680 | −0.140 | 0.452 | −0.021 | 0.912 | 0.011 | 0.953 |
| ngApoC-III/µgApoA-I | 0.190 | 0.290 | −0.027 | 0.883 | −0.036 | 0.844 | 0.109 | 0.548 | 0.185 | 0.304 |
| ngApoD/µgApoA-I | 0.140 | 0.438 | 0.097 | 0.592 | −0.174 | 0.333 | 0.076 | 0.673 | 0.104 | 0.564 |
| ngApoE/µgApoA-I | −0.067 | 0.712 | −0.184 | 0.306 | −0.005 | 0.977 | 0.229 | 0.200 | 0.093 | 0.605 |
| ngApoH/µgApoA-I | 0.301 | 0.089 | 0.055 | 0.760 | −0.129 | 0.475 | 0.163 | 0.365 | 0.125 | 0.487 |
| ngApoJ/µgApoA-I | **0.380** | **0.029** | 0.033 | 0.855 | −0.324 | 0.066 | **0.373** | **0.033** | 0.309 | 0.080 |
| ngCRP/µgApoA-I | 0.291 | 0.101 | 0.105 | 0.561 | −0.256 | 0.150 | 0.284 | 0.109 | 0.231 | 0.196 |
| ngSAA/µgApoA-I | 0.204 | 0.255 | 0.127 | 0.482 | 0.029 | 0.872 | 0.007 | 0.969 | 0.003 | 0.986 |

*Note*: Pearson's correlation adjusted for age, gender ApoE ε4-carrier status was performed between HDL-cargo and brain volumetric parameters at t0. Analysis was considered significant with $p < 0.05$ (bold).

Abbreviations: GM, grey matter volume; HL, left hippocampal volume; HR, right hippocampal volume; Vent, ventricular volume; WM, white matter volume.

[a]Data were adjusted for age, gender ApoE ε4-carrier status. For all analytes $n = 36$ except for ngApoC-I/µgApoA-I where $n = 34$.

for GM, HL, and HR, respectively), while negatively correlated with ventricular volume ($p = 0.024$). ApoJ/ApoA-I ratio positively correlated with grey matter and left hippocampal volume ($p = 0.029$ and $p = 0.033$, respectively), while right hippocampal volume only trended toward significance ($p = 0.080$). None of the other HDL-cargo analytes were significantly correlated with brain volumetric parameters (Table 5).

On assessing whether MMSE scores were associated with any HDL-cargo analytes in AD patients, MMSE scores were found to decrease with the disease progression. No HDL-cargo analytes were significantly associated with MMSE score or MMSE longitudinal changes, suggesting that HDL-cargo is not involved with altered cognitive impairment changes (Table S2).

RFE variable selection indicated that among all the HDL-cargo protein variables ApoE/ApoA-I and ApoD/ApoA-I have the best distinguishing powers in classifying AD and HC, followed by ApoA-II/ApoA-I, CRP/ApoA-I, and cholesterol/ApoA-I (data not shown).

## 4 | DISCUSSION

Over the past decades, the importance of cholesterol, LDL, and HDL in AD have been extensively studied, and in this regard, it is widely accepted that high cholesterol levels and high LDL levels are considered risk factor for the disease, albeit it appears that such increased risk is present at mid-life and disappeared with increased age (Mielke

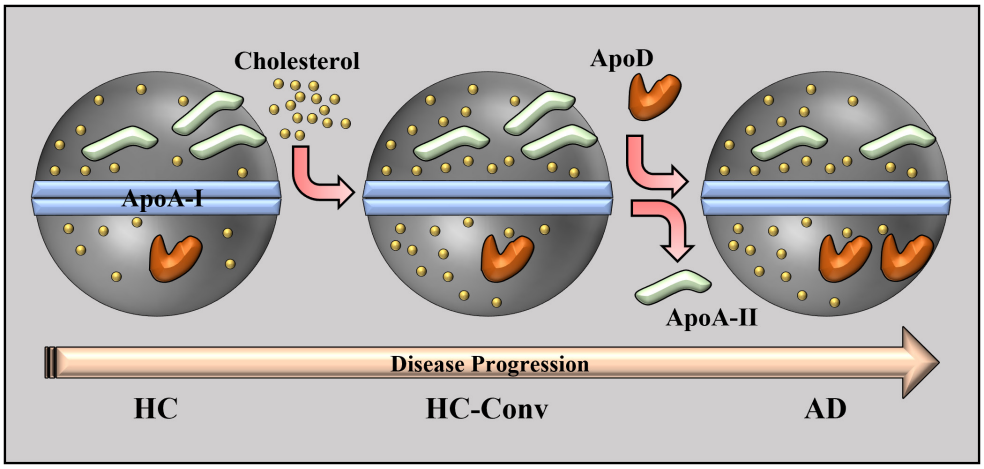


**FIGURE 3** Schematic representation of HDL remodeling in which cholesterol on HDL particles increases before the conversion to AD, while the decrease in ApoA-II and the increase in ApoD take place once the disease has started.

et al., 2005). As such, a clear distinction between the roles of LDL and HDL has indicated that while LDL cholesterol levels were associated with increased brain amyloid deposition (Reed et al., 2014), high HDL cholesterol levels were instead considered protective. This latest notion came from several studies in which HDL cholesterol levels were associated with lower brain amyloid deposition, a reduced risk for AD, better cognitive functions, and higher MMSE scores (Atzmon et al., 2002; Bates et al., 2017; Reitz et al., 2010). It is, however, important to note that recent evidence suggested that HDL functionality, rather than HDL overall levels, determine HDL functions (Rosenson et al., 2016).

In this report, we, therefore, assessed the HDL-cargo composition (Cholesterol, ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ, CRP, and SAA, all measured as ratio to ApoA-I) in stable healthy control, healthy controls who will convert to MCI/AD within 36 months and AD to determine if any of the HDL-cargo analytes were associated to brain amyloid deposition, brain volumetric parameters (cortical grey matter, cortical white matter, ventricular volume, hippocampal volume), and cognitive functions scores (MMSE). We found an increased amount of cholesterol/ApoA-I ratio (in both HC-Conv and AD, compared to HC), increased ApoD/ApoA-I (in AD), and decreased ApoA-II/ApoA-I ratios (in AD). While the cholesterol/ApoA-I ratio varies in the general population and as a function of HDL size and HDL maturation, the increased cholesterol/ApoA-I ratio on HDL particles in our study was unexpected, as the overall plasma levels of HDL-Cholesterol were unchanged in all clinical groups. Since ApoA-I is solely expressed on HDL, our data also indicated that levels of HDL-associated ApoA-I per ml of plasma were significantly lower in AD. Such a decrease can be considered an ApoA-I decrease in plasma and is in accordance with other reports which have indicated lower levels of ApoA-I in AD (Kawano et al., 1995; Liu et al., 2006; Merched et al., 2000). As ApoA-I is also the main (and more stable in number on HDL particles) Apolipoprotein on HDL, and in consideration of its reduced levels in AD, our data suggest that in AD (and in HC-Converters, but only in those who are within 18 months from conversion to MCI/AD), there is a reduced number of HDL particles which are overloaded with cholesterol. This would explain the lower amount of ApoA-I in plasma, but the increased amount of cholesterol on HDL particles (increased cholesterol/ApoA-I ratio) would counterbalance the reduced numbers of HDL particles and explain the absence of different Cholesterol-HDL plasma levels across clinical groups. Accordingly, most of the other HDL-cargo proteins (evaluated in the same assay with ApoA-I), which have shown reduced levels in AD compared to HC per ml of plasma (HDL-associated protein cargo/ml plasma), do not display a different ratio to ApoA-I, suggesting that there is no altered composition of HDL particle, but rather a reduced number.

Overall, the only exception in the composition, along with the Cholesterol/ApoA-I ratio which occurs before the clinical conversion from HC to MCI/AD, are ApoA-II/ApoA-I ratio, which is decreased in AD compared to HC and ApoD/ApoA-I ratio, which is increased in AD compared to controls. However, since both ApoA-II/ApoA-I and ApoD/ApoA-I ratios are not altered in HC-Conv compared to stable HC, it is possible that such changes take place once the disease has started, unlike the cholesterol overload which may happen before the onset of the disease (Figure 3). It has to be noted that in this study we focused on HDL composition assessed as apolipoprotein ratio to ApoA-I on HDL particles, which may represent a better marker for conversion to AD and/or pathological changes in AD, rather than individual plasma apolipoprotein levels, which could provide confounding results as many apolipoproteins are shared among several lipoprotein particles (LDL, VLDL).

Interestingly, ApoE/ApoA-I ratio was significantly lower in carriers of the allele APOE ε4 and lowest in the homozygous carrier for the allele APOE ε4. Such ApoE/ApoA-I ratio, which defines HDL composition, was not affected by clinical classification but was solely affected by APOE genotype. As the presence of the allele APOE ε4 is widely considered the biggest risk factor for sporadic AD, such reduced ApoE presence on HDL may reduce HDL functionality, therefore providing an additional explanation for the increased risk associated with APOE genotype. This reduced binding of ApoE ε4 to HDL (with a preferential

binding to chylomicrons and VLDL) is also in accordance with other studies (Poirier et al., 2014). In this regard, several studies which evaluated the effects of ApoE inducers, such as Liver-X-receptor (LXR) agonists, have reported protective effects in transgenic AD mice (Fitz et al., 2010; Lefterov et al., 2007; Riddell et al., 2007), supporting the notion that high levels of ApoE are beneficial in the disease. However, one limitation of this study is that it was not powered to determine the effects of ApoE genotype on disease progression.

When the influence of HDL-cargo on regional brain volumes was assessed, we selected individuals with ongoing brain amyloidosis, in whom regional changes may be more prominent. Higher cholesterol/ApoA-I ratio on HDL resulted to be marginally associated with lower grey matter volume. In parallel, higher cholesterol levels were also associated with greater ventricular volume. While this seems to be counterintuitive at first, as higher HDL cholesterol levels have been associated with protective features, the cholesterol increase here mentioned is per HDL particle (overall plasma HDL levels were unaffected in this study). In this report we suggest that cholesterol overload on HDL is detrimental as it affected brain volumetric parameters, suggesting that HDL quality may be more important than quantity itself. Such a hypothesis would also be in accordance with another report indicating that cholesterol overload on HDL has a negative effect on HDL anti-atherogenic functions (Qi et al., 2015), while an increased cholesterol/ApoA-I ratio on HDL can be a predictor of cardiovascular disease and associated mortality (Rhee et al., 2017). In accordance with these findings, a lower cholesterol/ApoA-I ratio was linked to protective features, as higher levels of small HDL particles (known to have lower cholesterol/ApoA-I ratio) in CSF have been associated with better cognitive performances (Martinez et al., 2022).

Conversely, ApoA-II/ApoA-I, and ApoJ/ApoA-I ratio displayed the opposite effect on grey matter and ventricular volume, with higher levels of both proteins being associated with higher grey matter volume and smaller ventricular volume. In addition, a higher ApoA-II/ApoA-I ratio was also significantly associated with higher hippocampal volume (both left and right). It is, therefore, fitting that ApoA-II and ApoJ on HDL particles displayed protective features, as both proteins have previously been linked to AD for their capacity to bind to Aβ reducing its effects (Bell et al., 2007; Hammad et al., 1997; Yamauchi et al., 2000). Ultimately, we observed a trend between higher cholesterol levels on HDL and higher levels of brain amyloid deposition, further indicating that cholesterol overload on HDL may result in non-functional HDL particles with consequent negative impact.

Taken together, our study suggested that in AD (a) there is a reconfiguration of HDL particles with significant increased cholesterol/ApoA-I ratio (which may take place before the onset of the disease), increased ApoD/ApoA-I and reduced ApoA-II/ApoA-I ratios on HDL which may affect HDL functionality itself; (b) such reconfiguration with the consequent increase of cholesterol/ApoA-I ratio on HDL is associated in individuals with ongoing brain amyloidosis with lower cortical grey matter volume and greater ventricular volume; while (c) other apolipoproteins on HDL, such as ApoA-II and ApoJ, displayed protective features and higher levels of both were associated in individuals with ongoing brain amyloidosis with higher cortical grey matter volume and smaller ventricular volume; and (d) that ApoE/ApoA-I ratio on HDL are solely a function of APOE genotype and reduced levels of ApoE on HDL in APOE ε4 carrier may further alter HDL functionality, reduce its protective features and provide another reason for the increased risk associated with APOE genotype.

Altogether, these data are supporting the notion that the functionality of HDL is related to its protein cargo and it is independent of its absolute levels, and that HDL cargo and HDL functionality may be altered in AD as they are in many other diseases. Further studies will, however, be necessary to better define the extent of HDL functionality in relationship with HDL-cargo in the disease.

## AUTHOR CONTRIBUTIONS

Steve Pedrini: Conceptualization, Methodology, Investigation, Data analysis, Visualization, Writing - original draft, Writing - review/editing. James D. Doecke: Data analysis, Writing - review/editing. Eugene Hone: Investigation, Writing - review/editing. Penghao Wang: Data analysis, Writing - review/editing. Rohith Thota: Conceptualization, Writing - review/editing. Ashley I. Bush: Writing - review/editing. Christopher C. Rowe: Data curation, Writing - review/editing. Vincent Dore: Data curation, Writing - review/editing. Victor L. Villemagne: Data curation, Writing - review/editing. David Ames: Data curation, Writing - review/editing. Stephanie Rainey-Smith: Data curation, Writing - review/editing. Giuseppe Verdile: Conceptualization, Writing - review/editing. Hamid R. Sohrabi: Data curation, Writing - review/editing. Manfred R. Raida: Conceptualization, Methodology, Investigation, Writing - review/editing. Kevin Taddei: Writing - review/editing, Project administration. Sam Gandy: Conceptualization, Writing - review/editing. Colin L. Masters: Data curation, Writing - review/editing. Pratishtha Chatterjee: Conceptualization, Writing - original draft, Writing - review/editing. Ralph N. Martins: Conceptualization, Writing - original draft, Writing - review/editing, Supervision, Project administration.

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All experiments were conducted in compliance with the ARRIVE guidelines.

## CONFLICT OF INTEREST

Ashley Bush is a shareholder in Alterity Ltd, Cogstate Ltd and Mesoblast Ltd. He is a paid consultant for, and has a profit share interest in, Collaborative Medicinal Development Pty Ltd. He has received lecture fees from Biogen and Merck Sharp & Dohme P/L. Dr. Gandy serves as a consultant for Ritrova Therapeutics and as a founder of Recuerdo Pharmaceuticals (inactive). He has served as a consultant in the past for Diagenic, and he has received research support in the past from Warner-Lambert, Pfizer, Baxter, and Avid. He currently receives research support from the NIH. Dr. Victor Villemagne is a Senior Editor for the Journal of Neurochemistry.

## DATA AVAILABILITY STATEMENT

AIBL data have been provided to the Global Alzheimer's Association Interactive Network (GAAIN), and software installed, thereby enabling GAAIN users to interrogate metadata and receive cohort summaries, whereupon users can request further information (and biofluid samples; blood and CSF) if needed by submitting an Expression of Interest (EoI). This mechanism ensures worldwide sharing and utilization of AIBL data (and biofluid samples) and complements the existing framework whereby AIBL imaging scans and demographic data are available on the ADNI LONI (Laboratory of Neuro Imaging, University of Southern California) website for free download and use by researchers worldwide. Up to 3 August 2019, LONI/ GAAIN data applications had been received from over 120 companies, more than 2000 organizations/institutes/departments, and over 3500 individuals, spanning 81 countries. Moreover, hundreds of EoIs from academic and industry-based individuals have been approved resulting in further data and biofluid sample sharing. Data will also be provided to the National Institute on Aging-funded ADOPIC (Alzheimer's Dementia Onset and Progression in International Cohorts) project which will harmonize data from the AIBL, ADNI, Washington University, St. Louis, and University of Washington, Seattle, cohorts to determine factors which influence cognitive decline in AD.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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# PART II

# GFAP, pTau181, NFL AND Aβ42/40 RATIO as POTENTIAL BLOOD BIOMARKERS in ALZHEIMER'S DISEASE

# Chapter 6

## Diagnostic and prognostic plasma biomarkers for preclinical Alzheimer's disease

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FEATURED ARTICLE

# Diagnostic and prognostic plasma biomarkers for preclinical Alzheimer's disease

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## Abstract

**Introduction:** This study involved a parallel comparison of the diagnostic and longitudinal monitoring potential of plasma glial fibrillary acidic protein (GFAP), total tau (t-tau), phosphorylated tau (p-tau181 and p-tau231), and neurofilament light (NFL) in preclinical Alzheimer's disease (AD).

**Methods:** Plasma proteins were measured using Simoa assays in cognitively unimpaired older adults (CU), with either absence (A$\beta$–) or presence (A$\beta$+) of brain amyloidosis.

  

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**Results:** Plasma GFAP, t-tau, p-tau181, and p-tau231 concentrations were higher in Aβ+ CU compared with Aβ− CU cross-sectionally. GFAP had the highest effect size and area under the curve (AUC) in differentiating between Aβ+ and Aβ− CU; however, no statistically significant differences were observed between the AUCs of GFAP, p-tau181, and p-tau231, but all were significantly higher than the AUC of NFL, and the AUC of GFAP was higher than the AUC of t-tau. The combination of a base model (BM), comprising the AD risk factors, age, sex, and apolipoprotein E gene (*APOE*) ε4 status with GFAP was observed to have a higher AUC (>90%) compared with the combination of BM with any of the other proteins investigated in the current study. Longitudinal analyses showed increased GFAP and p-tau181 in Aβ+ CU and increased NFL in Aβ− CU, over a 12-month duration. GFAP, p-tau181, p-tau231, and NFL showed significant correlations with cognition, whereas no significant correlations were observed with hippocampal volume.

**Discussion:** These findings highlight the diagnostic and longitudinal monitoring potential of GFAP and p-tau for preclinical AD.

**KEYWORDS**
Alzheimer's disease, amyloid beta, blood biomarkers, brain amyloid beta, diagnosis, glial fibrillary acidic protein, longitudinal monitoring, neurofilament light, preclinical Alzheimer's disease, p-tau181, p-tau231, single molecule array, tau

## 1 | INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disease that causes cognitive deterioration and ultimately death, is the most common form of dementia and accounts for nearly 60% to 70% of its cases. In 2020, ≈50 million people were living with dementia globally, and there are close to 10 million new cases every year.[1] Given that only symptomatic drugs are available, but yet no cure or disease-modifying treatment for AD, the identification of diagnostic and longitudinal monitoring biomarkers for at-risk populations is paramount to aid in assessing the efficacy of clinical trials.

The existence of a long preclinical phase, that is, prior to the manifestation of clinical symptoms, during which the hallmark proteinopathies (amyloid plaques and neurofibrillary tangles) develop, has provided the opportunity for the investigation of biomarkers that can assist diagnosis and prognosis for such at-risk populations. For instance, positron emission tomography (PET) and cerebrospinal fluid (CSF) analysis can reveal abnormal levels of brain amyloid beta (Aβ) and hyperphosphorylated tau (p-tau), pathologies that begin to accumulate ≈20 years before symptom onset.[2,3] However, routine application of these markers in the clinical setting may be hampered by their limited availability, high costs, and invasiveness, and therefore more accessible diagnostic approaches such as blood-based biomarkers are being investigated intensively.

Several recent studies have reported that plasma glial fibrillary acidic protein (GFAP), total tau (t-tau) and p-tau181 and p-tau231, and neurofilament light (NFL) levels are higher in AD and have suggested that they could serve as potential blood biomarkers for AD, given that they likely reflect AD-related neuropathological processes such as astrogliosis and the disruption of the axonal cytoskeletal structure.[4–9] In the current study, we conducted a parallel investigation of plasma GFAP; t-tau, p-tau181, and p-tau231; and NFL in preclinical AD, by comparing the circulating levels of these proteins between cognitively unimpaired older adults (CU) with absence of brain amyloidosis (Aβ−) and CU who were classified as being within the preclinical stage of AD, characterized by presence of brain amyloidosis (Aβ+). Plasma GFAP, tau (including t-tau, p-tau181, and p-tau231), and NFL levels were measured using an ultra-sensitive, single-molecule array (Simoa) platform, and analyzed cross-sectionally at baseline and at a 12-month follow-up timepoint, to determine if they could differentiate between these groups. We hypothesized that these plasma biomarkers would be higher in the Aβ+ group compared with the Aβ− group at both baseline and the 12-month follow-up. Validating our cross-sectional observations 12 months apart would provide insight into the reliability of these biomarkers in preclinical AD and assess if they have value to assist with the identification of Aβ+ CU for recruitment into clinical trials. We observed higher plasma GFAP, p-tau181, and p-tau231 concentrations in the Aβ+ group compared with the Aβ− group, and GFAP had the highest effect size. No statistically significant differences were observed between GFAP, p-tau181, and p-tau231 in distinguishing between Aβ+ and Aβ− groups; however, GFAP had the highest discriminative accuracy when added to a model comprising the AD risk factors of age, sex, and apolipoprotein E gene (*APOE*) ε4 status compared with the other proteins added to a model comprising the AD risk factors.

In addition, we assessed longitudinal changes in plasma GFAP, t-tau, p-tau181, p-tau231, and NFL in the Aβ− and Aβ+ groups over

a 12-month period, given that understanding longitudinal changes in blood biomarkers over time would provide valuable insight into determining whether the use of these biomarkers as outcome measures may have value for improving the efficacy of designing and interpreting disease-modifying clinical therapeutic trials. We posited that these plasma measures would increase over a 12-month duration in the Aβ+ group. We observed increased GFAP and p-tau181 in the Aβ+ group and increased NFL in the Aβ− group, over this 12-month duration.

We also evaluated the correlations of the plasma markers with cognition and hippocampal volume and observed that GFAP, p-tau181, p-tau231, and NFL showed significant correlations with cognition, whereas no significant correlations were observed with hippocampal volume.

**RESEARCH IN CONTEXT**

1. **Systematic review**: The authors reviewed the literature using PubMed. Although several studies have been conducted on the diagnostic performance of individual plasma biomarkers, a parallel comparison of candidate Alzheimer's disease (AD) plasma biomarkers cross-sectionally and longitudinally within the preclinical stage is lacking.
2. **Interpretation**: Our findings suggest that among all plasma biomarkers included in this study, plasma glial fibrillary acidic protein (GFAP) combined with the AD risk factors (age, sex, and apolipoprotein E gene (*APOE*) ε4 status) had the highest accuracy in differentiating between cognitively unimpaired older adults (CU) with amyloidosis (Aβ+) and without amyloidosis (Aβ−) indicating its potential as a diagnostic marker for preclinical AD. In addition, the increase in GFAP and phosphorylated tau (p-tau181) in CU Aβ+ over 12 months indicates their potential as longitudinal monitoring markers for preclinical AD.
3. **Future directions**: Further studies are required to validate the current observations in independent cohorts, including the establishment of clinical cut-off points for implementation in clinical settings.

**HIGHLIGHT**

- Plasma GFAP, t-tau, p-tau181 and p-tau231 levels are higher in Aβ+ vs Aβ− CU
- No significant difference was observed in plasma NFL levels between Aβ+ and Aβ− CU
- AD risk factors and GFAP combined, had an AUC>90% in differentiating Aβ+ vs Aβ− CU
- GFAP and p-tau181 increased longitudinally over 12 months in Aβ+ CU

## 2 | METHODS

### 2.1 | Cohort

The Kerr Anglican Retirement Village Initiative in Ageing Health (KARVIAH) cohort volunteers ($N$ = 206) were required to meet a set of screening inclusion and exclusion criteria to be eligible for the cohort. Briefly, the inclusion criteria comprised an age range of 65-90 years, good general health, no known significant cerebrovascular disease, fluent in English, adequate/corrected vision and hearing to enable testing, and no objective cognitive impairment as screened by a Montreal Cognitive Assessment (MoCA) score ≥26. MoCA scores lying between 18 and 25 were assessed on a case-by-case basis by the study neuropsychologist following stratification of scores according to age and education.[10] The exclusion criteria comprised the diagnosis of dementia based on the revised criteria from the National Institute on Aging−Alzheimer's Association,[11] the presence of an acute functional psychiatric disorder (including lifetime history of schizophrenia or bipolar disorder), a history of stroke, severe or extremely severe depression (based on the Depression, Anxiety, Stress Scales; DASS), and uncontrolled hypertension (systolic blood pressure [BP] >170 mm Hg or diastolic BP >100 mm Hg). One hundred thirty-four volunteers met the inclusion/exclusion criteria. These 134 participants underwent a 12-month placebo-curcumin intervention (UTN: U1111-1144-1011). One hundred five of these 134 participants underwent neuropsychometric evaluation, blood collection, and cerebral amyloid beta (Aβ) imaging. Within these 105 participants, 100 participants (comprising 50 placebo and 50 curcumin intervention) were considered to have normal global cognition based on their Mini-Mental State Examination (MMSE ≥26) [12] at baseline and were included in the current study.

At baseline, plasma GFAP, t-tau, and NFL concentrations were available in all 100 (n(Aβ−) = 67, n(Aβ+) = 33) participants included in the current study, whereas p-tau181 and p-tau231 were available in 97 (n(Aβ−) = 67, n(Aβ+) = 30) and 96 (n(Aβ−) = 67, n(Aβ+) = 29) participants, respectively, due to sample availability. At the 12-month follow-up timepoint, plasma GFAP, t-tau, and NFL concentrations were available in 95 (n(Aβ−) = 64, n(Aβ+) = 31) participants, whereas p-tau181 and p-tau231 were available in 95 (n(Aβ−) = 64, n(Aβ+) = 31) and 93 (n(Aβ−) = 63, n(Aβ+) = 30) participants, respectively. In addition, participants with a Memory Assessment Clinic - Questionnaire (MAC-Q) score ≥25 were considered as subjective memory complainers (SMC, $n$ = 76; a specific form of subjective cognitive decline, defined by self-reported memory complaints). At baseline, plasma GFAP, t-tau, and NFL concentrations were available in all 76 (n(Aβ−) = 52, n(Aβ+) = 24) SMC participants included in the current study, whereas p-tau181 and p-tau231 were available in 75 (n(Aβ−) = 52, n(Aβ+) = 23) and 73 (n(Aβ−) = 52, n(Aβ+) = 21) SMC participants, and at the 12-month follow-up timepoint, plasma GFAP, t-tau, and NFL concentrations were

available in 74 (n(Aβ−) = 49, n(Aβ+) = 25) SMC participants, whereas p-tau181 and p-tau231 were available in 74 (n(Aβ−) = 49, n(Aβ+) = 25) and 72 (n(Aβ−) = 48, n(Aβ+) = 24) SMC participants, respectively. Details of the participants analyzed within the current study have been reported in Supplementary Figure 1. All participants were based in Sydney, Australia. All volunteers provided written informed consent prior to participation, and the Bellberry and Macquarie University Human Research Ethics Committees provided approval for the study.

## 2.2 | Neuroimaging

Neuroimaging was conducted within 3 months of blood collection at Macquarie Medical Imaging in Sydney. Positron emission tomography (PET) studies were conducted over as a 20-minute static scan (4 × 5 minute frames) that was acquired 50 minutes after an intravenous bolus of $^{18}$F-florbetaben (FBB). Neocortical Aβ load was calculated as the mean standard uptake value ratio (SUVR) of the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions using image processing software, CapAIBL (v2.0).[13, 14] Participants with an FBB PET SUVR ≥1.35 were considered Aβ+, while those with an FBB PET SUVR < 1.35 were considered Aβ−. Available Aβ-PET data for participants at baseline and at 12-month follow-up have been illustrated in Supplementary Figure 1.

In addition, participants passing all standard magnetic resonance imaging (MRI) inclusion/exclusion criteria underwent MRI as described previously using a General Electric (GE) 3 Tesla scanner (Model 750W).[15] Hippocampal volume calculated from the images acquired was normalized with the total intracranial volume comprising the cerebrospinal fluid, gray matter, and white matter. Data for 94 participants at baseline and 81 participants at the 12-month follow-up were available.

## 2.3 | Blood collection, *APOE* genotyping, measurement of plasma GFAP, t-tau, p-tau181, p-tau231, and NFL

A minimum of 10 hours overnight fasted blood was collected from participants using standard processing methods.[16] *APOE* genotype was determined from purified genomic DNA extracted from 0.5 mL whole blood as described previously.[16]

Protein concentrations in ethylenediaminetetraacetic acid (EDTA) plasma were measured employing the ultra-sensitive single-molecule array (Simoa) platform. GFAP, t-tau, and NFL were measured using the Neurology 4-Plex A kit (QTX-102153, Quanterix, Billerica, Massachusetts, United States), which also includesUCH-L1, but this biomarker failed our quality control criteria because of high (>20%) coefficients of variation (CVs). P-tau181 and p-tau231 were measured using the in-house assays developed at the University of Gothenburg, Sweden.[5,8] In addition, p-tau181 was measured using the P-Tau 181 V2 Simoa Advantage Assay (QTX-103714, Quanterix, Billerica, MA, USA) in 19 samples, each at baseline and at the 12-month follow-up timepoint; Supplementary Figure 2 shows the correlation between the two assays. Calibrators and samples were run in duplicate for all assays. Quality control (QC) was achieved by assessing in duplicate the levels of two controls included in the Simoa kits at the beginning of each plate. The analytical lowest limit of quantification was 0.467 pg/mL for GFAP, 0.053 pg/mL for t-tau, 1 pg/mL for p-tau181, 1 pg/mL for p-tau231, and 0.241 pg/mL for NFL. The average %CV was 2.72% for the GFAP assay, 7.48% for the t-tau assay, 8% for the p-tau181 assay, 12% for the p-tau231 assay, and 3.65% for the NFL assay.

## 2.4 | Neuropsychological tests

Study participants underwent a comprehensive battery of neuropsychological testing at baseline and 12 months as described previously, and composite scores were generated for verbal and visual episodic memory and working memory and executive function. In addition, a global composite z-score was constructed using the verbal and visual episodic memory z-scores, working memory and executive function z-scores, and MMSE z-scores as described previously.[17]

## 2.5 | Statistical analyses

Descriptive statistics including means and standard deviations were calculated for Aβ− and Aβ+ groups with comparisons employing Student *t* tests or chi-square tests as appropriate. Linear models were employed to compare continuous variables between Aβ− and Aβ+ groups corrected for covariates age, sex, and *APOE* ε4 carrier status both cross-sectionally and longitudinally (repeated measures). Dependent variables were natural log transformed to better approximate normality and variance homogeneity as required. Spearman correlation coefficient ($r_s$) was employed to investigate correlations between continuous variables. Logistic regression with Aβ−/+ as response was used to evaluate predictive models and receiver-operating characteristic (ROC) curves constructed from the logistic scores. The areas under the curves (AUCs) for different plasma proteins were compared using the DeLong test. To determine the accuracy of each protein in distinguishing between Aβ− and Aβ+ groups, the R package cut-point was used. All analyses and data visualization were carried out using IBM SPSS (v27), GraphPad Prism (v8) or R (v4.0.3).

# 3 | RESULTS

## 3.1 | Cohort characteristics

No significant differences were observed between Aβ− and Aβ+ group characteristics in age, sex, body mass index (BMI), subjective memory complaint (SMC) status, MMSE scores, and hippocampal volumes; however, a significantly higher frequency of *APOE* ε4 allele carriers was observed in the Aβ+ group compared with the Aβ− group ($P < .0001$), as expected (Table 1).

**TABLE 1** Cohort characteristics

| | Aβ− | Aβ+ | p |
|---|---|---|---|
| Age (years, mean ±SD; Aβ− =67, Aβ+ =33) | 77.78±5.56 | 79.00±5.44 | .300 |
| Sex (Male/Female; Aβ− =67, Aβ+ =33) | 19/48 | 13/20 | .266 |
| BMI (mean ±SD; Aβ− =67, Aβ+ =33) | 27.46±4.43 | 27.94±4.85 | .626 |
| *APOE* ε4 carriers (N (%); Aβ− =67, Aβ+ =33) | 5 (7.46) | 16 (48.48) | <**.0001** |
| Subjective memory complainers (N (%); Aβ− =67, Aβ+ =33) | 52 (77.61) | 24 (72.72) | .591 |
| MMSE (mean ±SD; Aβ− =67, Aβ+ =33), baseline | 28.54±1.16 | 28.76±1.12 | .368 |
| MMSE (mean ±SD; Aβ− =64, Aβ+ =32), 12m | 28.87±1.12 | 28.81±1.50 | .818 |
| Hippocampal volume % (mean ±SD; Aβ− =63, Aβ+ =31), baseline | 0.40±0.039 | 0.39±0.038 | .901 |
| Hippocampal volume % (mean ±SD; Aβ− =52, Aβ+ =29), 12m | 0.38±0.04 | 0.38±0.04 | .562 |
| FBB-PET SUVR (mean ±SD; Aβ− =67, Aβ+ =33), baseline | 1.16±0.09 | 1.70±0.24 | - |
| FBB-PET SUVR (mean ±SD; Aβ− =64, Aβ+ =32), 12m | 1.16±0.09 | 1.72±0.24 | - |

Cohort characteristics including age, sex, body mass index (BMI), *APOE* ε4 status, subjective memory complainer status (assessed by the Memory Assessment Clinic - Questionnaire (MAC-Q) score) are presented at baseline. The mini-mental state examination (MMSE) scores, hippocampal volume and brain Aβ load represented by the standard uptake value ratio (SUVR) of ligand $^{18}$F-Florbetaben (FBB) in the neocortical region normalised with that in the cerebellum, have been compared between Aβ- (SUVR<1.35) and Aβ+ (SUVR≥1.35) study participants at baseline and a 12-month follow-up timepoint (12m). Chi-square tests or linear models were employed as appropriate.

## 3.2 | Associations of AD-related risk factors, age, sex, and *APOE* ε4 allele status, with plasma GFAP, t-tau, p-tau181, p-tau231, and NFL measures

Plasma GFAP, p-tau181, p-tau231, and NFL measures were observed to have significant positive correlations with age, at both baseline and the 12-month timepoint ($P < .05$; Supplementary Table 1A). Plasma t-tau was observed to be significantly higher in females compared with males before and after adjusting for covariates, age, and *APOE* ε4 allele status, at both baseline and the 12-month timepoint (Supplementary Table 1B). Plasma p-tau231was observed to be significantly higher in *APOE* ε4 allele carriers compared with non-carriers before and after adjusting for covariates, age and sex, at both baseline and the 12-month timepoint (Supplementary Table 1C). No associations were observed for age, sex, or *APOE* ε4 allele status with protein measures not listed above.

## 3.3 | Cross-sectional comparisons of plasma GFAP, t-tau, p-tau181, p-tau231, and NFL between Aβ− and Aβ+ groups

Plasma GFAP, p-tau181, and p-tau231 were significantly higher in the Aβ+ group compared with the Aβ− group, at both baseline and the 12-month timepoint, before and after adjusting for covariates age, sex, and *APOE* ε4 allele carrier status ($P < .05$). Plasma t-tau was higher in the Aβ+ group compared with the Aβ− group, with a trend toward significance at baseline, and appeared significant at the 12-month timepoint both before and after adjusting for covariates. Plasma NFL, however, was not significantly different between the Aβ− and Aβ+ groups, at baseline and the 12-month timepoint, before and after adjusting for covariates (Table 2A, Supplementary Table 2A, Figure 1). In addition, in these analyses, a large effect size was observed for GFAP, medium to large for p-tau181 and p-tau231, medium for t-tau, and small for NFL.[18] Furthermore, within the SMC subset, similar observations were found for plasma GFAP, t-tau, p-tau181, p-tau231, and NFL between the Aβ+ and Aβ− group, at both baseline and the 12-month timepoint (Table 2B, Supplementary Table 2B).

## 3.4 | Evaluation of plasma GFAP, t-tau, p-tau181, p-tau231, and NFL as predictors of brain Aβ status

At baseline, the diagnostic accuracies between the Aβ− and Aβ+ groups are illustrated using ROC curves in Figure 2. Plasma GFAP was observed to have the highest AUC (79%, 95% confidence interval [CI] 69–89%) in differentiating between the Aβ− and Aβ+ groups when compared with all other proteins considered independently. However, although there was a significant difference in the AUC for GFAP versus t-tau ($P < .05$) and GFAP versus NFL ($P < .005$), there was no statistically significant difference in the AUC for GFAP versus p-tau181 ($P > .05$) or p-tau231 ($P > .05$). In addition, the AUCs of p-tau181 and p-tau231 were also observed to be significantly higher than the AUC of NFL ($P < .05$) (Supplementary Table 3A). Similar observations were also noted at the 12-month timepoint (Supplementary Table 3A).

In addition, at baseline, we generated a base model (BM) incorporating the AD risk factors age, sex, and *APOE* ε4 allele status, and we observed that this base model was significantly outperformed when the plasma protein GFAP ($P = .001$) was added to it, whereas a trend toward significance was observed when p-tau181 ($P = .054$) or p-tau231 ($P = .077$) was added to the BM. However, the addition of t-tau or NFL to the BM did not have a significant additional contribution to the BM AUC in distinguishing Aβ+ from Aβ− (Figure 2, Supplementary

**TABLE 2** Cross-sectional differences in plasma protein measures between Aβ- and Aβ+ at baseline and 12m in all participants and the subjective memory complainer subset

| *Table 2A. All participants* | Aβ- | | Aβ+ | | | | |
|---|---|---|---|---|---|---|---|
| **Baseline** | **Mean** | **SD** | **Mean** | **SD** | **p** | **p[a]** | **Partial $\eta^2$** |
| GFAP (pg/mL; Aβ-=67, Aβ+=33) | 146.96 | 49.48 | 211.39 | 86.04 | **7e-6** | **1.12e-8** | .292 |
| t-tau (pg/mL; Aβ-=67, Aβ+=33) | 1.20 | 0.36 | 1.35 | 0.40 | .065 | .050 | .040 |
| p-tau181 (pg/mL, Aβ-=67, Aβ+=30) | 13.55 | 5.61 | 17.52 | 5.68 | **.002** | **.041** | .045 |
| p-tau231 (pg/mL; Aβ-=67, Aβ+=29) | 11.57 | 6.44 | 19.21 | 7.39 | **2e-6** | **2.7e-4** | .137 |
| NFL (pg/mL; Aβ-=67, Aβ+=33) | 19.39 | 8.90 | 21.05 | 10.35 | .408 | .408 | .007 |
| **12m Follow-up** | **Aβ-** | | **Aβ+** | | | | |
| GFAP (pg/mL; Aβ-=64, Aβ+=31) | 150.84 | 60.35 | 234.87 | 109.29 | **2e-6**† | **6.7e-7** | .241 |
| t-tau (pg/mL; Aβ-=64, Aβ+=31) | 1.22 | 0.39 | 1.45 | 0.41 | **.012** | **.011** | .070 |
| p-tau181 (pg/mL; Aβ-=64, Aβ+=31) | 13.65 | 5.83 | 19.16 | 6.15 | **5.2e-5** | **8.1e-4** | .118 |
| p-tau231 (pg/mL; Aβ-=63, Aβ+=30) | 11.00 | 6.64 | 18.38 | 8.24 | **1.2e-5** | **2.5e-4** | .142 |
| NFL (pg/mL; Aβ-=64, Aβ+=31) | 20.84 | 10.94 | 23.33 | 10.50 | .294 | .492 | .005 |
| *Table 2B: SMCs* | Aβ- | | Aβ+ | | | | |
| **Baseline** | **Mean** | **SD** | **Mean** | **SD** | **p** | **p[a]** | **Partial $\eta^2$** |
| GFAP (pg/mL; Aβ-=52, Aβ+=24) | 147.85 | 47.07 | 229.50 | 93.49 | **6e-6**† | **2.5e-7** | .314 |
| t-tau (pg/mL; Aβ-=52, Aβ+=24) | 1.16 | 0.37 | 1.45 | 0.39 | **.002** | **.016** | .079 |
| p-tau181 (pg/mL; Aβ-=52, Aβ+=23) | 13.35 | 5.41 | 17.92 | 6.06 | **.002** | **.010** | .092 |
| p-tau231 (pg/mL; Aβ-=52, Aβ+=21) | 12.05 | 6.71 | 19.46 | 7.26 | **8.4e-5** | **.001** | .148 |
| NFL (pg/mL; Aβ-=52, Aβ+=24) | 19.47 | 9.13 | 23.08 | 11.00 | .138 | .132 | .032 |
| **12m Follow-up** | **Aβ-** | | **Aβ+** | | | | |
| GFAP (pg/mL; Aβ-=49, Aβ+=25) | 154.10 | 61.00 | 244.68 | 113.35 | **2.7e-5** | **2.9e-5** | .225 |
| t-tau (pg/mL; Aβ-=49, Aβ+=25) | 1.20 | 0.36 | 1.46 | 0.42 | **.007** | **.007** | .101 |
| p-tau181 (pg/mL; Aβ-=49, Aβ+=25) | 13.47 | 5.56 | 17.48 | 6.37 | **8e-5** | **.002** | .129 |
| p-tau231 (pg/mL; Aβ-=48, Aβ+=24) | 11.28 | 6.94 | 18.81 | 8.82 | **1.8e-4** | **.002** | .130 |
| NFL (pg/mL; Aβ-=49, Aβ+=25) | 21.26 | 11.51 | 23.98 | 11.16 | .335 | .547 | .005 |

Plasma protein measures were compared between Aβ- and Aβ+ cognitively normal older adults in (A.) all participants using general linear models. The same analyses were carried out (B.) in a subset of the cohort that only comprised subjective memory complainers (SMCs). † represents p-values obtained from natural log transformed protein concentrations to better approximate normality when required. p[a] represents p-values adjusted for age, sex and *APOE* ε4 status. Partial $\eta^2$ estimates (for analyses adjusted for age, sex and *APOE* ε4 status) represents effect size for comparison Aβ- vs Aβ+ groups. Data are presented in mean±SD. Suggested norms for partial $\eta^2$: small = 0.01; medium = 0.06; large = 0.14.

Table 3B). Similar observations were noted at the 12-month timepoint (Figure 2, Supplementary Table 3B).

Furthermore at baseline, the AUC for BM+GFAP was observed to be significantly higher than the AUCs observed for BM+t-tau ($P$ = .002), BM+p-tau181 ($P$ = .002), BM+p-tau231 ($P$ = .014), and BM+NFL ($P$ = .001). At the 12-month timepoint, the AUC for BM+GFAP was observed to be significantly higher than the AUCs observed for BM+t-tau ($P$ = .049) and BM+NFL ($P$ = .01); however, it became nonsignificant when compared with BM+p-tau181 ($P$ = .10) and BM+p-tau231 ($P$ = .15) (Supplementary Table 3B).

The AUCs from the combination of the three plasma proteins that demonstrated the highest AUCs individually (namely GFAP, p-tau181, and p-tau231, for Aβ−/+ status) at baseline and at the 12-month timepoint were 85% (CI 76-93%) (Figure 2) and 83% (CI: 74-91%) (Figure 2), respectively. At baseline, the AUC of the combination of these three proteins was significantly higher than the AUCs of GFAP, t-tau, p-tau181, and NFL individually, and a trend toward significance was observed for p-tau231. At the 12-month timepoint, the AUC of the combination of these three proteins was only observed to be significantly higher than the AUCs of t-tau, p-tau231, and NFL.

In addition, when these three proteins were combined with the BM, the AUC was further improved to 94% (CI 89-98%) (Figure 2) and 91% (CI 84%-98%) (Figure 2), at baseline and at the 12-month timepoint, respectively (Supplementary Table 3B). At baseline and the 12-month timepoint, the AUC of the combination of these three proteins and the BM was significantly higher than the AUCs of t-tau+BM, p-tau181+BM, p-tau231+BM, and NFL+BM individually; however, no significant difference was observed with GFAP+BM.

Furthermore, at 80% sensitivity, p-tau181 alone was observed to have the highest diagnostic accuracy to detect preclinical AD

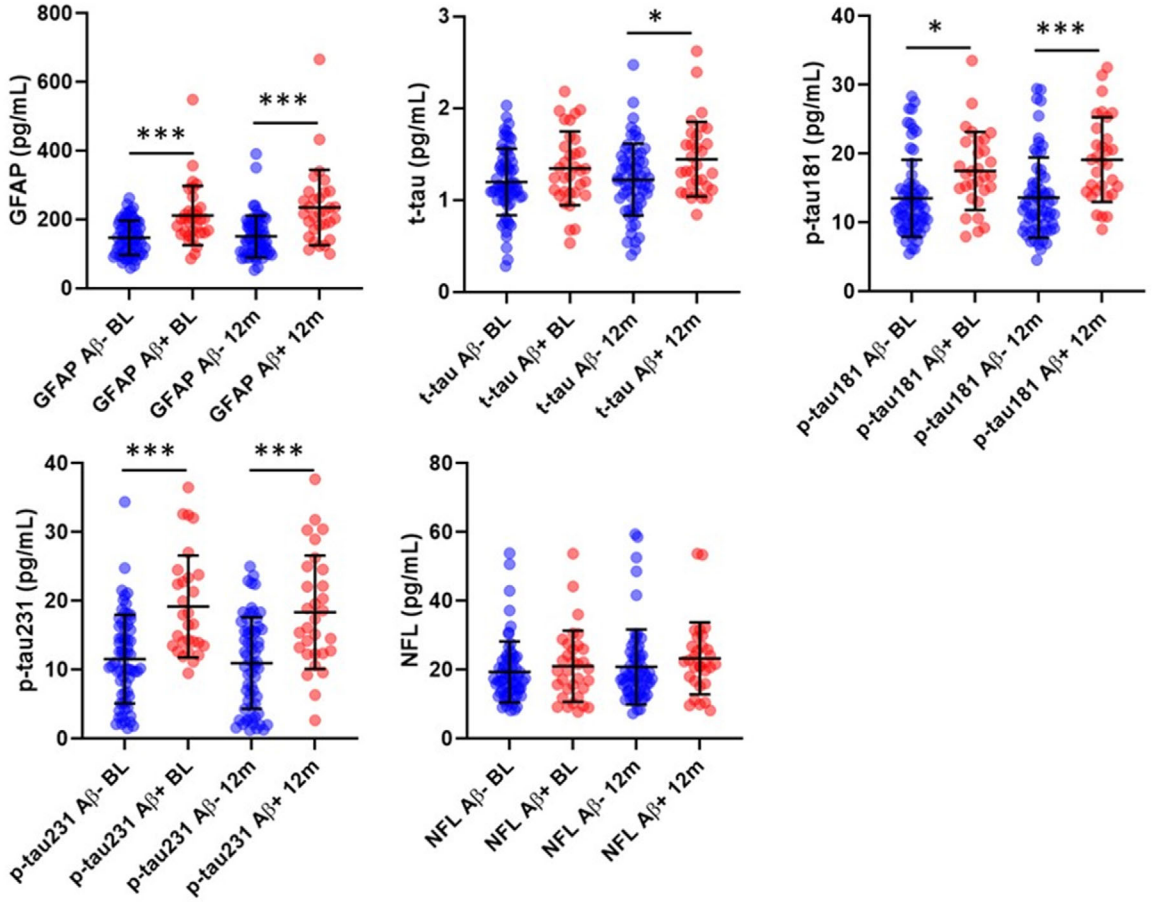


**FIGURE 1** Comparison of plasma GFAP, t-tau, p-tau181, p-tau231, and NFL between Aβ− and Aβ+ cognitively normal older adults at baseline and at 12-month follow-up. Plasma protein measures were compared between cognitively normal Aβ− and Aβ+ older adults using linear models, at baseline (BL) and at the 12-month follow-up timepoint (12 months). The line segment within each jitter plot represents the median of the data and error bars in the graphs represent the interquartile range for the Aβ− and Aβ+ groups. * $P < .05$, ** $P \leq .001$, *** $P \leq .0001$

(BL: accuracy = 68%, specificity = 63%, negative predictive value [NPV] = 88%, positive predictive value [PPV] = 47%; 12 m: accuracy = 69%, specificity = 63%, NPV = 87%, PPV = 50%) when compared with the other proteins independently, whereas the accuracy for GFAP+BM was observed to be the highest (BL: accuracy = 86%, specificity = 88%, NPV = 91%, PPV = 74%; 12 m: accuracy = 85%, specificity = 87%, NPV = 90%, PPV = 74%) compared with all proteins considered individually or in combinations, as shown in Supplementary Table 4A. Sensitivity, specificity, accuracy, Youden's cut point, NPV, and PPV at Youden's index are provided in Supplementary Table 4B.

## 3.5 | Longitudinal changes in plasma GFAP, t-tau, p-tau181, p-tau231, and NFL over a 12-month duration in Aβ− and Aβ+ groups

There was a significant interaction effect of time*Aβ status on GFAP levels in all participants; GFAP increased more over 12 months in the Aβ+ group versus the Aβ- group before adjusting for covariates. However, the interaction term became nonsignificant after adjusting for covariates age, sex, and *APOE* status. No interaction effects between time and Aβ status were observed for t-tau, p-tau181, p-tau231, and NFL changes over 12 months between the Aβ− group and Aβ+ group before and after adjusting for covariates (Table 3A, Supplementary Table 5A).

The main effect of time was significant for plasma GFAP and p-tau181 in all participants and approached significance for plasma NFL, where these protein measures were observed to increase over a 12-month duration, before and after adjusting for covariates. No significant main effect of time was observed for t-tau and p-tau231.

Further investigation of pairwise comparisons for plasma GFAP, p-tau181, and NFL within each Aβ status group in all participants, showed the significant effect of time on GFAP was restricted to the Aβ+ group only, before and after adjusting for covariates. In addition, a significant effect of time on p-tau181 was similarly observed only in the Aβ+ group, but only reached statistical significance after adjusting for covariates. Of interest, a significant effect of time on NFL was observed in the Aβ− group only, before and after adjusting for covariates (Table 3A, Supplementary Table 5A, Figure 3). However, the absolute differences in NFL levels cross-sectionally and in change over time

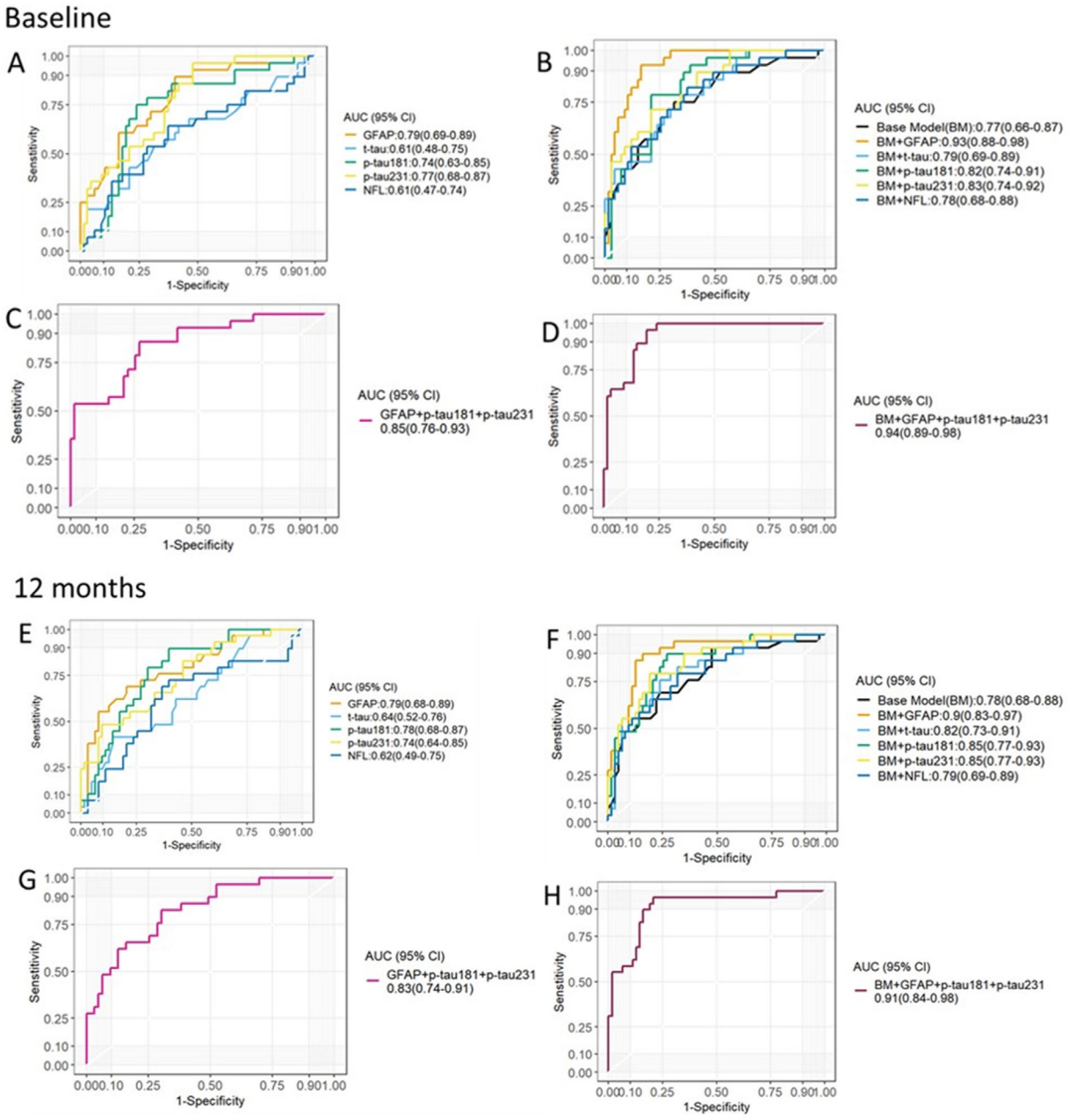


**FIGURE 2** Receiver-operating characteristic curves for the prediction of Aβ− versus Aβ+ participants at baseline and at the 12-month follow-up timepoint. Receiver-operating characteristic (ROC) curves are presented at baseline for (A) GFAP, t-tau, p-tau181, p-tau231, and NFL; (B) base model comprising AD risk factors, age, sex, apolipoprotein E (*APOE*) ε4 allele status (BM), BM+GFAP, BM+t-tau, BM+p-tau181, BM+p-tau231, and BM+NFL; (C) panel of top three performing proteins (GFAP, p-tau181, p-tau231); and (D) BM+ panel of top three performing proteins (GFAP, p-tau181, p-tau231). ROC curves are presented at 12 months for (E) GFAP, t-tau, p-tau181, p-tau231, and NFL; (F) BM, BM+GFAP, BM+t-tau, BM+p-tau181, BM+p-tau231, and BM+NFL; (G) panel of top three performing proteins (GFAP, p-tau181, p-tau231); and (H) BM+ panel of top three performing proteins (GFAP, p-tau181, p-tau231). Data from 95 participants were utilized for analyses at baseline (Aβ−, $n = 67$, Aβ+, $n = 28$) and 92 participants at 12 months (Aβ−, $n = 63$, Aβ+, $n = 29$), given that all required data were available for these participants. Abbreviations: AUC, area under the curve; CI, confidence interval

were similar in Aβ− and Aβ+ in the overall cohort, with considerable overlap between groups; hence NFL may have limited value as a preclinical AD biomarker. The estimates of effect size are presented in Supplementary Table 6, wherein small to medium effect sizes were observed for GFAP and p-tau181 in the Aβ+ and for NFL in the Aβ− groups.[18]

The longitudinal analyses also confirmed our cross-sectional observations with a significant main effect of Aβ−/+ status on plasma GFAP, t-tau, p-tau181, and p-tau231 levels, wherein these proteins were observed to be higher in the Aβ+ group at both timepoints (Table 3A, Supplementary Table 5A). GFAP and p-tau isoforms were significantly different between the Aβ+ and Aβ− group, supporting a potential role

**TABLE 3** Longitudinal changes over a 12-month duration in plasma protein measures between Aβ- and Aβ+ cognitively normal older adults

| *Table 3A. All participants* | Aβ- | | Aβ+ | | Time | | | Time (adjusted for covariates) | | | Time* Aβ status | Time* Aβ status (adjusted for covariates) | Aβ status | Aβ status (adjusted for covariates) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Baseline* | *12m Follow-up* | *Baseline* | *12m Follow-up* | *p* | *Pairwise* | | *p* | *Pairwise* | | | | | |
| | | | | | | $p^{A\beta-}$ | $p^{A\beta+}$ | | $p^{A\beta-}$ | $p^{A\beta+}$ | *p* | *p* | *p* | *p* |
| GFAP (Aβ-=67, Aβ+=31) | 146.96±49.48 | 152.02±60.21 | 211.55±88.77 | 233.87±108.95 | **.001** | .282 | **.002** | **.004** | .169 | **.020** | **.041** | .244 | **5e-6** | **4.5e-8** |
| t-tau (Aβ-=67, Aβ+=31) | 1.20±0.36 | 1.24±0.39 | 1.36±0.41 | 1.37±0.49 | .392 | .214 | .850 | .375 | .283 | .769 | .586 | .752 | .063 | **.043** |
| p-tau181 (Aβ-=67, Aβ+=30) | 13.55±5.61 | 14.08±6.26 | 17.52±5.68 | 18.78±5.51 | **.028** | .237 | .063 | **.022** | .357 | **.047** | .368 | .258 | **5.7e-4** | **.016** |
| p-tau231 (Aβ-=66, Aβ+=29) | 11.59±6.48 | 11.03±6.52 | 19.21±7.39 | 18.67±8.13 | .433 | .471 | .643 | .615 | .289 | .914 | .990 | .530 | **2.6e-7** | **2.3e-5** |
| NFL (Aβ-=67, Aβ+=31) | 19.39±8.90 | 21.23±10.77 | 20.97±10.60 | 22.16±10.76 | **.016** | **.010** | .248 | **.048** | **.003** | .816 | .603 | .167 | .554 | .747 |
| *Table 3B. SMCs* | Aβ- | | Aβ+ | | Time | | | Time (adjusted for covariates) | | | Time* Aβ status | Time* Aβ status (adjusted for covariates) | Aβ status | Aβ status (adjusted for covariates) |
| | *Baseline* | *12m Follow-up* | *Baseline* | *12m Follow-up* | *p* | *Pairwise* | | *p* | *Pairwise* | | | | | |
| | | | | | | $p^{A\beta-}$ | $p^{A\beta+}$ | | $p^{A\beta-}$ | $p^{A\beta+}$ | *p* | *p* | *p* | *p* |
| GFAP (Aβ-=52, Aβ+=23) | 147.85±47.07 | 152.62±55.12 | 229.70±95.59 | 255.00±117.43 | **.009**† | .252† | **.017**† | **.005** | .269 | **.015** | .168† | .154 | **8.4e-7**† | **3.2e-7** |
| t-tau (Aβ-=52, Aβ+=23) | 1.16±0.3.7 | 1.20±0.37 | 1.46±0.39 | 1.45±0.43 | .618 | .213 | .816 | .452 | .429 | .743 | .377 | .896 | **.003** | **.012** |
| p-tau181 (Aβ-=52, Aβ+=23) | 13.35±5.41 | 13.87±6.07 | 17.92±6.06 | 19.39±5.70 | **.044** | .341 | .072 | .060 | .319 | .149 | .326 | .511 | **4.3e-4** | **.005** |
| p-tau231 (Aβ-=51, Aβ+=21) | 12.09±6.78 | 11.31±6.77 | 19.46±7.26 | 19.69±8.95 | .726 | .364 | .866 | .791 | .338 | .773 | .527 | .481 | **1.4e-5** | **1.6e-4** |
| NFL (Aβ-=52, Aβ+=23) | 19.47±9.14 | 21.37±11.23 | 23.34±11.18 | 24.18±11.54 | .074 | **.026** | .508 | .099 | **.024** | .702 | .485 | .405 | .193 | .235 |

Plasma proteins were measured on the Simoa platform and were compared between Aβ- and Aβ+ cognitively normal older adults in (A.) all participants and (B.) in the subjective memory complainer subset separately, using repeated measures analyses at baseline and at the 12-month follow-up timepoint before and after adjustment for covariates age, sex and *APOE ε4* carrier status. Data are presented as Mean±SD. † represents p-values obtained from natural log transformed protein measures to better approximate normality. p<.05 was considered significant and are presented in bold font.

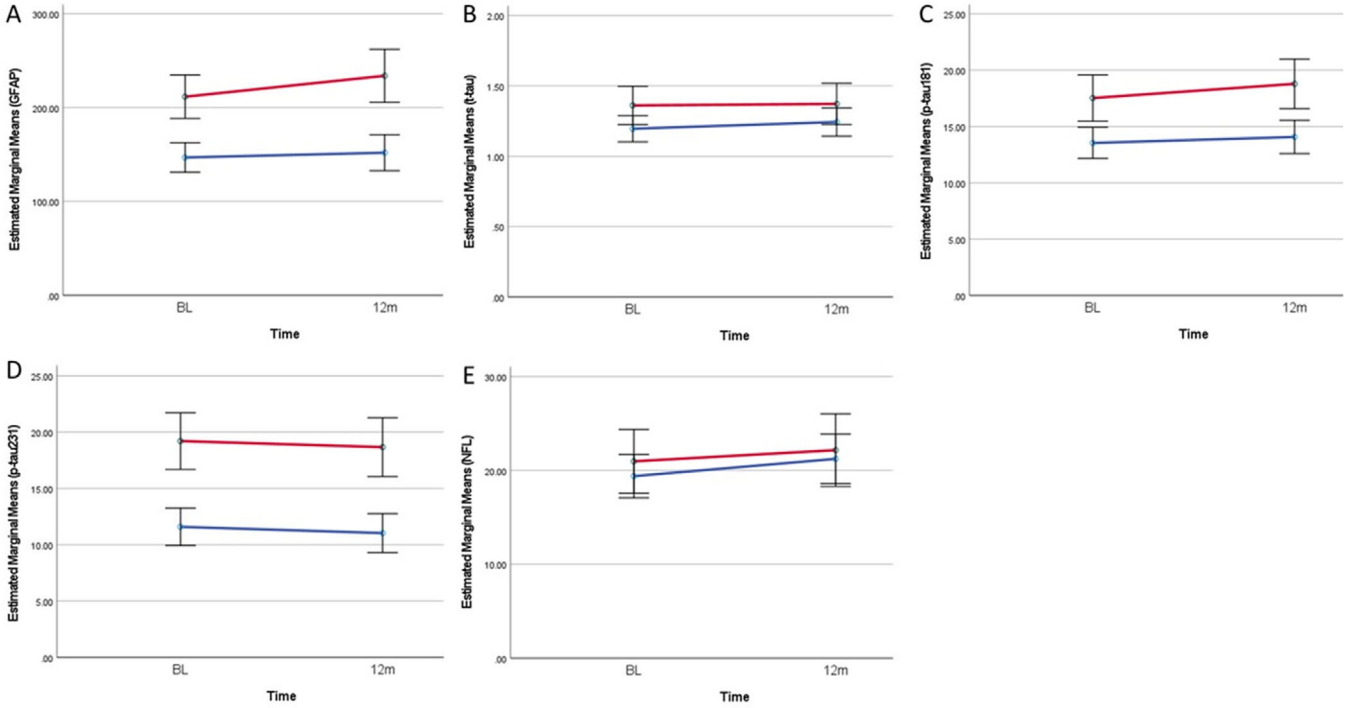


**FIGURE 3** Longitudinal changes in plasma protein measures between Aβ− and Aβ+ cognitively normal older adults over a 12-month duration. The estimated marginal means of the plasma proteins are illustrated for Aβ− (blue) and Aβ+ (red) cognitively normal older adults along time, ie, baseline (BL) to the 12-month follow-up timepoint (12 months). Protein measures for GFAP, t-tau, and NFL were available for 67 Aβ− and 31 Aβ+ at both timepoints, whereas p-tau181 data were available for 67 Aβ− and 30 Aβ+ at both timepoints and for p-tau231, 66 Aβ−, and 29 Aβ+ at both timepoints. Error bars represent ± 2 SE

for these plasma proteins as diagnostic and longitudinal monitoring biomarkers in preclinical AD. Similar observations were also noted within the SMC subset (Table 3B, Supplementary Table 5B, Supplementary Figure 3).

### 3.6 | Association of plasma GFAP, t-tau, p-tau181, p-tau231, and NFL with cognition and hippocampal volume

At baseline, plasma GFAP was observed to be inversely correlated with the working memory and executive function composite score ($r_s = -.257$, $P = .010$) and the global composite score ($r_s = -.200$, $P = .047$). P-tau181 inversely correlated with the global composite score ($r_s = -.209$, $P = .040$), which was also seen for p-tau231 ($r_s = -.278$, $P = .006$), and p-tau231 additionally correlated inversely with the verbal and visual episodic memory composite score ($r_s = -.254$, $P = .013$). As expected, plasma NFL inversely correlated with the verbal and visual episodic memory composite score ($r_s = -.335$, $P = .001$), the working memory and executive function composite score ($r_s = -.347$, $P < .0001$), and the global composite score ($r_s = -.438$, $P < .0001$).[17]

At the 12-month follow-up timepoint, plasma GFAP remained to be inversely correlated with the working memory and executive function composite score ($r_s = -.234$, $P = .021$) and the global composite score ($r_s = -.273$, $P = .007$), whereas a trend toward a significant inverse correlation was observed between p-tau181 and the global composite score ($r_s = -.174$, $P = .086$). As expected, plasma NFL continued to inversely correlate with the verbal and visual episodic memory composite score ($r_s = -.342$, $P = .001$), working memory and executive function composite score ($r_s = -.371$, $P < .001$), and the global composite score ($r_s = -.456$, $P < .001$).

No significant correlation was observed between the plasma proteins and hippocampal volume at baseline, except for a trend toward significance for p-tau231 ($r_s = -.213$, $P = .078$); however, this trend was not observed at the 12-month timepoint.

## 4 | DISCUSSION

In the current study, for the first time to the best of our knowledge, we evaluated plasma GFAP, t-tau, p-tau181, p-tau231, and NFL in parallel between a cognitively unimpaired Aβ+ older adult group (preclinical AD) and a cognitively unimpaired Aβ− older adult group. We found higher plasma GFAP, p-tau181, and p-tau231 in the cognitively unimpaired Aβ+ group. We further validated our cross-sectional findings observed at baseline in a 12-month follow-up timepoint to re-examine the differences in plasma protein levels, and continued to find higher plasma GFAP, p-tau181, and p-tau231 in the preclinical AD group, suggesting that the plasma protein differences observed between these

two groups are consistent and potentially reliable candidate markers for the diagnosis of preclinical AD. Our results held in the subjective memory complainer subset, further supporting the potential utility of GFAP and p-tau isoforms as preclinical AD biomarkers.

We found no significant difference between the AUCs for GFAP, p-tau181, and p-tau231 in differentiating between cognitively unimpaired Aβ+ older adults and cognitively unimpaired Aβ− older adults, although GFAP showed the highest AUC among these three proteins. However, when GFAP was added to the AD risk factors, age, sex, and *APOE ε4* status, the AUC was significantly higher compared with the AUCs of p-tau181 or p-tau231 added to the AD risk factors at baseline. Of interest, although the AUC of the combination of the AD risk factors with the three highest performing proteins (ie, BM+GFAP+p-tau181+p-tau231) was significantly higher than the AUC of t-tau, p-tau181, p-tau231, or NFL combined with the AD risk factors (ie, t-tau+BM/p-tau181+BM/p-tau231+BM/NFL+BM), no significant difference was observed with GFAP+BM at baseline and the 12-month timepoint. Corroborating this observation, GFAP+BM was also observed to have the highest accuracy. Although further studies are required to validate these observations, it may be suggested that plasma GFAP levels may reflect pathological mechanisms additional to those associated with the well-known risk factors for AD within the preclinical stage.

Most interestingly, we also show for the first time that GFAP and p-tau181 increased with time in cognitively unimpaired Aβ+ older adults and NFL increased with time in cognitively unimpaired Aβ− older adults, over a 12-month duration. Similar observations were also noted in the subjective memory complainer subset. Together, these observations suggest that GFAP and p-tau181 may have potential in serving as longitudinal monitoring markers and outcome measures for relatively shorter clinical trials conducted in preclinical AD populations, whereas the longitudinal increase in NFL observed with time in the Aβ− group could possibly indicate that NFL reflects other ongoing neurodegenerative processes that are not directly associated with Aβ pathology.

Among the five proteins investigated in the current study, GFAP, p-tau181, and p-tau231 showed the highest estimates of effect size for the cross-sectional analyses between the Aβ− and Aβ+ groups in all participants (GFAP > p-tau231 > p-tau181). These estimates of effect sizes mostly met the "large" cut-off, which may be indicative of their clinical utility value. Longitudinally, GFAP was observed to have the highest estimates of effect size, followed by p-tau181 in the Aβ+ group, whereas NFL was observed to have the highest estimates of effect size in the Aβ− group. However, these effect sizes fell mostly within the small to moderate range but may still have utility in assessing the efficacy of clinical trials.

In line with our observations of higher GFAP levels observed in the Aβ+ group in this study, GFAP, a marker of astrogliosis,[19] has been reported to be higher in preclinical AD and is associated with brain amyloidosis.[4,7,20–22] Higher GFAP levels have also been reported in AD patients compared with controls.[4,23] Increased GFAP has also been observed around Aβ plaques in the brains of individuals with mild cognitive impairment (MCI) due to AD,[24] and its expression has been observed to correlate with Aβ plaque density in AD.[25] Higher plasma GFAP levels observed in the Aβ+ group within the current study could thus be due to GFAP upregulation associated with astrogliosis in Aβ+ individuals. Astrogliosis has been reported to occur within the early stages of AD pathogenesis, and cultured astrocytes exposed to amyloid isolated from human AD brains have been observed to trigger astrogliosis.[26,27] In addition, studies employing $^{11}$C-deuterium-L-deprenyl PET, further support that reactive astrocytosis is a prodromal feature in the early stages of AD development.[24] Furthermore, similar to our longitudinal findings, Cicognola and colleagues have shown that plasma GFAP increases at a faster rate in Aβ+ MCI compared with Aβ− MCI.[21] In addition, Oeckl and colleagues report that GFAP distinguished between AD and behavior variant frontal temporal dementia, with 89% sensitivity and 79% specificity.[23] However, further studies comparing plasma GFAP levels in AD versus other neuropathologically defined non-AD neurodegenerative diseases are required to confirm the specificity of plasma GFAP alterations for AD, given the existence of mixed pathologies.

In addition, higher plasma p-tau181 levels have been reported in individuals with MCI and AD compared with cognitively unimpaired older adult groups and individuals with other neurodegenerative diseases.[5,28–31] These studies have also showed that p-tau181 levels are higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ− older adults and our findings from the current study are in line with these observations. It has been suggested that the early dysregulation in neuronal tau metabolism is likely to be associated with early Aβ pathology, attributed to the release of soluble p-tau181 in blood.[28,30] Similarly, p-tau231 has relatively recently been reported to be elevated in the blood in individuals with MCI and AD compared with cognitively unimpaired older adult groups and individuals with other neurodegenerative diseases.[8] This study[8] has also reported that p-tau231 levels are higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ− older adults, and our findings at baseline and the 12-month timepoint are in line with these observations. Of interest, p-tau231 has been reported to identify the clinical stages of AD and neuropathology as strongly as p-tau181; however, it increases relatively earlier when compared with p-tau181, with subtle Aβ deposition.[8] It is important to note that plasma p-tau217, like p-tau181 and p-tau231, has also been reported to be higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ− older adults,[32] but has been observed to have a higher discriminative accuracy between AD and non-AD neurodegenerative diseases compared with plasma p-tau181.[33] Of interest, plasma p-tau217 has been reported to correlate with brain amyloidosis in early disease stages.[34] Furthermore, plasma p-tau217 has been observed to increase in *Presenilin 1 (PSEN1)* E280A autosomal dominant AD (ADAD) mutation carriers ≈20 years before symptom onset.[33]

Plasma t-tau is known as a marker of neuronal injury and shows a very marked increase in disorders with acute neuronal injury, such as cardiac arrest.[35] In contrast, although CSF t-tau shows a marked increase in AD,[36] plasma t-tau levels show only a discrete change in AD, and there is no correlation between plasma and CSF t-tau levels in AD-control cohorts.[37] The reason for this discrepancy is not known, but a possible explanation may be that, in contrast to p-tau,

non-phosphorylated tau is also produced in the peripheral nerves or tissue,[38] and it is estimated that only ≈20% of plasma t-tau comes from the CNS,[39] and thus peripherally produced tau will blur possible differences in brain-derived tau in plasma in AD. Although hypothetical, this could explain why the difference in t-tau levels was non-significant between cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ− older adults at baseline, but became significant after 12 months, which may be attributed to a possible increase in preclinical AD pathogenesis severity in 12 months.

Plasma NFL, reflecting neuronal injury, was not significantly higher in cognitively unimpaired Aβ+ older adults compared with cognitively unimpaired Aβ− older adults at baseline or at the 12-month timepoint, suggesting that NFL may not have value as a preclinical AD marker for identifying cognitively unimpaired older adults at risk for AD. These observations are in line with those reported by Mattsson and colleagues, wherein no significant differences in plasma NFL were observed between Aβ− and Aβ+ controls.[40] In contrast, in a *PSEN1* E280A ADAD Colombian kindred, higher plasma NFL levels and a higher annual rate of plasma NFL change have been observed 22 years prior to the estimated age at symptom onset in the mutation carriers compared with non-carriers.[41] Similarly, a higher annual rate of change of serum NFL has been observed 16.2 years before the estimated age at symptom onset in mutation carriers compared with non-carriers from the Dominantly Inherited Alzheimer Network cohort.[42] Elevated blood NFL levels (or a higher annual rate of change of blood NFL levels), reflecting neurodegeneration, observed so early in the ADAD pathogenesis trajectory prior to symptom onset, could be attributed to the aggressive nature of ADAD mutations when compared with sporadic AD.

In the current study, we also noted inverse correlations of plasma GFAP, p-tau181, p-tau231, and NFL with cognitive performance; however, the strength of these associations was at best small to moderate in this cognitively unimpaired cohort. No significant associations were observed for GFAP, p-tau181, p-tau231, and NFL with hippocampal volume. These observations could be attributed to the very early stage within the AD pathogenesis trajectory, the study Aβ+ participants in this study may lie in. However, it is interesting to note that such prominent changes appear in the cognitively unimpaired Aβ+ blood prior to any apparent hippocampal atrophy.

Findings from the current study highlight potential blood biomarkers for the diagnosis and longitudinal monitoring of cognitively unimpaired individuals within the preclinical AD stage. Further studies in larger research cohorts, for example, the Australian Imaging, Biomarker and Lifestyle (AIBL) Study of Aging cohort, are required to validate the current findings. In addition, future studies also need to establish clinical cut-off points for implementation in clinical settings, employing standardized blood collection, processing, and storage protocols. The establishment of clinical cut-off scores will also be assay dependent; for example, the difference in absolute levels observed between the two p-tau181 assays (ie, the in-house assay developed at the University of Gothenburg vs the Quanterix assay) are visible in Supplementary Figure 2, even though a near-perfect correlation was observed between the two assays. In addition, a majority of the studies conducted on the aforementioned proteins are primarily in Caucasian cohorts, and therefore further data from multiple races and ethnic backgrounds need to be investigated. Studies will also need to validate established cut-off points in individuals with other comorbidities in the future.

It is acknowledged that the current study had limitations with regard to its modest sample size. However, the cross-sectional comparisons investigated were consistent, 12 months apart, which is a strength of the study. Although plasma biomarker data available for the maximum number of participants were used in the current study, cross-sectional differences for a direct comparison using the same participants for all biomarkers showing similar observations are presented in Supplementary Table 7. Another limitation to be considered within the current study is that the longitudinal change in plasma GFAP, t-tau, p-tau181, p-tau231, and NFL was investigated over 12 months, which may not have been long enough to observe changes in the other proteins, that is, t-tau and p-tau231. However, given the budget constraints for small scale clinical trials, outcome measures that show changes within 12 months may also be considered suitable. In addition, this cohort underwent a 12-month placebo-curcumin intervention, although all statistical analyses were conducted with and without adjusting for this intervention. Furthermore, longitudinal comparison of plasma protein measures between Aβ− and Aβ+ participants within the placebo group, also had similar observations (Supplementary Table 8). Another limitation within the current study is that Aβ42/Aβ40 ratios and p-tau217 were not included. However, in a previous study we noted that Aβ42/Aβ40 ratios had an AUC <70% in differentiating between cognitively unimpaired Aβ− older adults and cognitively unimpaired Aβ+ older adults, using the same platform in the same cohort[20] and, therefore, more sensitive assays such as immunoprecipitation followed by mass-spectrometry approaches, for example,, that were employed by Nakamura and colleagues may be required.[43] Because we did not have access to the p-tau217 assay, this was not included in the current study.

To conclude, in the current study, we observed higher plasma GFAP, t-tau, p-tau181, and p-tau231 in preclinical AD. Furthermore, plasma GFAP and p-tau181 increased with time in preclinical AD. These observations strongly highlight the diagnostic and longitudinal monitoring potential of plasma GFAP and p-tau isoforms in preclinical AD.

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## CONFLICTS OF INTEREST

All authors report no competing financial interest in relation to the work described in this manuscript. PC received funding from the Macquarie University Seeding Grant, Macquarie University Early Career Researcher collaboration grant, and the Macquarie University Early Career Researcher small grants scheme. TKK was funded by the BrightFocus Foundation (#A2020812F), the International Society for Neurochemistry's Career Development Grant, the Swedish Alzheimer Foundation (Alzheimerfonden; #AF-930627), the Swedish Brain Foundation (Hjärnfonden; #FO2020-0240), the Swedish Dementia Foundation (Demensförbundet), the Swedish Parkinson Foundation (Parkinsonfonden), Gamla Tjänarinnor Foundation, the Aina (Ann) Wallströms and Mary-Ann Sjöbloms Foundation, the Agneta Prytz-Folkes & Gösta Folkes Foundation (#2020-00124), the Gun and Bertil Stohnes Foundation, and the Anna Lisa and Brother Björnsson's Foundation. TKK has a patent on an in vitro assay for measuring phosphorylated tau in a sample; the said assay comprises the use of two antibodies: (i) a capture antibody specific for pS396 on tau and (ii) a detection antibody binding tau on a different epitope that the capture antibody: https://protect-au.mimecast.com/s/5rmBCD1vRkCPylElhBRiat?domain = patents.google.com. EV is a Co-founder of ADx NeuroSciences. EH has received funding from the National Foundation for Medical Research and Innovation Grant (Australia). VLV has received consulting fees from IXICO Hospicom. HRShas received funding from National Institutes of Health (ADOPIC Project; subcontracted by the Melbourne University), Government of Western Australia (COVID-19 and Mental health of older adults), the Australian National Health and Medical Research Council (NHMRC): AUARROW Project and the Australian Alzheimer's Research Foundation (AARF): for research involvement. HRH is the Director of Center for Healthy Ageing at Murdoch University, a member of the Clinical Governance Committee for the Australian Alzheimer's Research Foundation (AARF) and an Australasian Neurosciences Society Council member.HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), and the UK Dementia Research Institute at UCL, payments made to institution. HZ has served at scientific advisory boards as a consultant for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, and CogRx. HZ has given lectures in symposia sponsored by Fujirebio, Alzecure, and Biogen. HZ is chair of the Alzheimer's Association Global Biomarker Standardization Consortium and the Alzheimer's Association Biofluid-Based Biomarker Professional Interest Area. HZ is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. CLM has received consulting fees from Eisai. KB is supported by the Swedish Research Council (#2017-00915), the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615), the Swedish Alzheimer Foundation (#AF-742881), Hjärnfonden, Sweden (#FO2017-0243), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986), the European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), and the National Institute of Health (NIH), USA (grant #1R01AG068398-01). KB has received consulting fees from Abcam, Axon, Biogen, JOMDD/Shimadzu, Lilly, MagQu, Prothena, Roche Diagnostics, Siemens Healthineers. KB is on the advisory board of Julius Clinical and Novartis. KB is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. SP, NJ Ashton, MT, KG, AKS, JS, NJ Armstrong, PRA, KT, VD, and RNM have nothing to disclose.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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# Chapter 7

## Plasma biomarkers in preclinical, prodromal and clinical AD: A cross-sectional and longitudinal study in the AIBL cohort

**Plasma Aβ42/40 ratio, p-tau181, GFAP, and NfL across the Alzheimer's disease continuum: A cross-sectional and longitudinal study in the AIBL cohort.** Chatterjee P#, **Pedrini S**#, Doecke JD, Thota R, Villemagne VL, Doré V, Singh AK, Wang P, Rainey-Smith S, Fowler C, Taddei K, Sohrabi HR, Molloy MP, Ames D, Maruff P, Rowe CC, Masters CL, Martins RN and the AIBL Research Group. Alzheimer's Dement. 2022 (In press). # These authors contributed equally

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FEATURED ARTICLE

# Plasma Aβ42/40 ratio, p-tau181, GFAP, and NfL across the Alzheimer's disease continuum: A cross-sectional and longitudinal study in the AIBL cohort

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## Abstract

**Introduction:** Plasma amyloid beta (Aβ)1-42/Aβ1-40 ratio, phosphorylated-tau181 (p-tau181), glial fibrillary acidic protein (GFAP), and neurofilament light (NfL) are putative blood biomarkers for Alzheimer's disease (AD). However, head-to-head

Pratishtha Chatterjee and Steve Pedrini contributed equally to this work.

cross-sectional and longitudinal comparisons of the aforementioned biomarkers across the AD continuum are lacking.

**Methods:** Plasma Aβ1-42, Aβ1-40, p-tau181, GFAP, and NfL were measured utilizing the Single Molecule Array (Simoa) platform and compared cross-sectionally across the AD continuum, wherein Aβ-PET (positron emission tomography)–negative cognitively unimpaired (CU Aβ–, $n = 81$) and mild cognitive impairment (MCI Aβ–, $n = 26$) participants were compared with Aβ-PET–positive participants across the AD continuum (CU Aβ+, $n = 39$; MCI Aβ+, $n = 33$; AD Aβ+, $n = 46$) from the Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL) cohort. Longitudinal plasma biomarker changes were also assessed in MCI ($n = 27$) and AD ($n = 29$) participants compared with CU ($n = 120$) participants. In addition, associations between baseline plasma biomarker levels and prospective cognitive decline and Aβ-PET load were assessed over a 7 to 10-year duration.

**Results:** Lower plasma Aβ1-42/Aβ1-40 ratio and elevated p-tau181 and GFAP were observed in CU Aβ+, MCI Aβ+, and AD Aβ+, whereas elevated plasma NfL was observed in MCI Aβ+ and AD Aβ+, compared with CU Aβ– and MCI Aβ–. Among the aforementioned plasma biomarkers, for models with and without AD risk factors (age, sex, and apolipoprotein E (*APOE*) ε4 carrier status), p-tau181 performed equivalent to or better than other biomarkers in predicting a brain Aβ–/+ status across the AD continuum. However, for models with and without the AD risk factors, a biomarker panel of Aβ1-42/Aβ1-40, p-tau181, and GFAP performed equivalent to or better than any of the biomarkers alone in predicting brain Aβ–/+ status across the AD continuum. Longitudinally, plasma Aβ1-42/Aβ1-40, p-tau181, and GFAP were altered in MCI compared with CU, and plasma GFAP and NfL were altered in AD compared with CU. In addition, lower plasma Aβ1-42/Aβ1-40 and higher p-tau181, GFAP, and NfL were associated with prospective cognitive decline and lower plasma Aβ1-42/Aβ1-40, and higher p-tau181 and GFAP were associated with increased Aβ-PET load prospectively.

**Discussion:** These findings suggest that plasma biomarkers are altered cross-sectionally and longitudinally, along the AD continuum, and are prospectively associated with cognitive decline and brain Aβ-PET load. In addition, although p-tau181 performed equivalent to or better than other biomarkers in predicting an Aβ–/+ status across the AD continuum, a panel of biomarkers may have superior Aβ–/+ status predictive capability across the AD continuum.

KEYWORDS

Alzheimer's disease, amyloid beta, blood biomarkers, brain amyloid beta, diagnosis, glial fibrillary acidic protein, longitudinal monitoring, neurofilament light, p-tau181, single molecule array

## HIGHLIGHTS

- Area under the curve (AUC) of p-tau181 ≥ AUC of Aβ42/40, GFAP, NfL in predicting PET Aβ–/+ status (Aβ–/+).
- AUC of Aβ42/40+p-tau181+GFAP panel ≥ AUC of Aβ42/40/p-tau181/GFAP/NfL for Aβ–/+.
- Longitudinally, Aβ42/40, p-tau181, and GFAP were altered in MCI versus CU.
- Longitudinally, GFAP and NfL were altered in AD versus CU.

- Aβ42/40, p-tau181, GFAP, and NfL are associated with prospective cognitive decline.
- Aβ42/40, p-tau181, and GFAP are associated with increased PET Aβ load prospectively.

## 1 | INTRODUCTION

Abnormal amyloid beta (Aβ) and tau buildup in the brain measured with positron emission tomography (PET), and Aβ42 and phosphorylated-tau181 (p-tau181) levels in the cerebrospinal fluid (CSF) are the current core biomarkers of Alzheimer's disease (AD). These biomarkers reflect AD neuropathology and begin to manifest two decades before the appearance of clinical symptoms.[1,2] However, the high cost, low throughput, and exposure to radiation associated with PET and the perceived invasiveness and expertise associated with lumbar puncture have all highlighted the need for surrogate markers in the blood.

Plasma Aβ (Aβ1-42/Aβ1-40 ratio), p-tau181, glial fibrillary acidic protein (GFAP) and neurofilament light (NfL) are some of the putative blood-based biomarkers for AD.[3,4] Circulating levels of these biomarkers have been reported to reflect AD-related neuropathological processes such as impaired clearance of brain Aβ, disruption of the axonal cytoskeletal structure, and reactive astrogliosis.[3,5–9] Previous studies have reported lower plasma Aβ1-42 and Aβ1-42/Aβ1-40 ratio[5,10–14] and higher plasma p-tau181 and GFAP in preclinical AD, prodromal AD, and AD dementia.[5,6,12,15–17] In addition, blood-based NfL levels have been observed to be higher in both prodromal AD and AD dementia.[18–20]

However, head-to-head studies of the aforementioned plasma biomarkers across the AD continuum are lacking. Therefore, in the current study, we carried out a head-to-head comparison of plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL alterations between Aβ-PET–negative (Aβ–) and Aβ-PET–positive (Aβ+) individuals across the AD continuum and evaluated the Aβ–/+ status predictive performance of these biomarkers against each other before and after the addition of AD risk factors, as well as evaluated their Aβ–/+ predictive performance as a biomarker panel before and after the addition of AD risk factors. In addition, we investigated the longitudinal changes in plasma biomarkers between the diagnostic groups over 36 months and investigated the association of plasma biomarkers at baseline with prospective cognitive decline and brain Aβ-PET load over a duration of 7 to 10 years.

## 2 | METHODS

### 2.1 | Participants

Participants were from the Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL) cohort. Participant exclusion criteria are described in detail elsewhere.[21] Briefly, exclusion criteria comprised a history of non-AD dementia, schizophrenia, bipolar disorder, significant current (but not past) depression, Parkinson disease, cancer (other than basal cell skin carcinoma) within the last 2 years, symptomatic stroke, uncontrolled diabetes, or current regular alcohol use exceeding two standard drinks per day for women or four per day for men. Participants were classified as individuals with AD based on the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria[22] and mild cognitive impairment (MCI) based on reduced cognitive performance often involving memory, representing a high-risk state for the development of AD.[23,24] Participants were defined as preclinical AD (cognitively unimpaired [CU] Aβ+), prodromal AD (MCI Aβ+), or AD (AD Aβ+) for cross-sectional analyses based on clinical criteria and Aβ+ status. Plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL data were available for 225 participants (81 CU Aβ–, 39 CU Aβ+, 26 MCI Aβ–, 33 MCI Aβ+, and 46 AD Aβ+) at timepoint 1. Follow-up samples were not available for 49 of the 225 participants at timepoint 1. Therefore, plasma biomarker data at the 18- and 36-month follow-up timepoints were available for 80 CU Aβ– (79 CU Aβ– for p-tau181), 40 CU Aβ+, 13 MCI Aβ–, 14 MCI Aβ+, and 29 AD Aβ+ (28 AD Aβ+ for p-tau181) participants. Aβ–/+ status for participants who did not undergo an Aβ-PET scan at any given timepoint was determined from the previous/next immediate timepoint. Participants were defined as CU ($n$ = 120), MCI ($n$ = 27), or AD ($n$ = 29) based on clinical criteria only, for longitudinal analyses, albeit all AD were Aβ+. All participants provided written informed consent before participation. This study was approved by the Human Research Ethics Committees of St. Vincent's Health (HREC/028/06) and Austin Health (HREC/18/Austin/201) in Melbourne and Hollywood Private Hospital (HPH215) and Edith Cowan University (ECU1878 Martins) in Perth, and Macquarie University (520221061636006) in Sydney.

### 2.2 | Measurement of plasma p-tau181, Aβ1-40, Aβ1-42, GFAP, and NfL

Ethylenediaminetetraacetic acid (EDTA) plasma p-tau181, Aβ1-40, Aβ1-42, GFAP, and NfL concentrations were measured utilizing the ultra-sensitive single molecule array (Simoa) platform. Level of p-tau181 was measured using the P-Tau 181 V2 Simoa Advantage Assay (QTX-103714, Quanterix, Billerica, MA), with calibrators and samples run in duplicates. Average Coefficient of Variation CV)% for p-tau181

**RESEARCH IN CONTEXT**

1. **Systematic Review**: The authors reviewed the literature using PubMed. Several studies have been conducted on the diagnostic performance of individual plasma biomarkers; however, head-to-head comparisons of the putative Alzheimer's disease (AD) plasma biomarkers cross-sectionally and longitudinally across the AD continuum are lacking.
2. **Interpretation**: Our findings suggest that among the plasma biomarkers included in this study, phosphorylated tau181 (p-tau181) performed ≥ the other biomarkers in predicting brain amyloid beta (Aβ)−/+ status across the AD continuum. However, a biomarker panel of Aβ1-42/Aβ1-40, p-tau181, and glial fibrillary acidic protein (GFAP) performed ≥ any of the biomarkers alone in predicting brain Aβ−/+ positron emission tomography (PET) status across the AD continuum. Longitudinally, Aβ1-42/Aβ1-40, p-tau181, and GFAP were altered in prodromal AD, and GFAP and neurofilament light (NfL) were altered in AD. Aβ1-42/Aβ1-40, p-tau181, GFAP, and NfL were associated with prospective cognitive decline and Aβ1-42/Aβ1-40, p-tau181, and GFAP were associated with increased Aβ PET load prospectively.
3. **Future Directions**: Further studies need to validate the current observations in independent cohorts including establishment of clinical cutoffs for implementation in clinical settings.

was 5.58%. Aβ1-40, Aβ1-42, GFAP, and NfL were measured using the Neurology 4-Plex E kit (QTX-103670, Quanterix, Billerica, MA), where calibrators were run in duplicates and samples in singlicates. Average CV% of previous batches run in duplicate in our laboratory for Aβ1-40, Aβ1-42, GFAP, and NfL were 1.56%, 2.91%, 3.26%, and 3.20%, respectively. Quality control (QC) was attained by assessing the levels of the positive controls provided in the Simoa kits. The analytical lowest limit of quantification was 0.338 pg/mL for p-tau181, 4.08 pg/mL for Aβ1-40, 1.51 pg/mL for Aβ1-42, 11.6 pg/mL for GFAP, and 1.6 pg/mL for NfL. The average %CV of the two quality controls was 1.7% and 6.6% for p-tau181, 0.2% and 2.19% for Aβ1-40, 1.28% and 1.06% for Aβ1-42, 1.68% and 1.46% for GFAP, and 0.17% and 1.48% for NfL, respectively.

## 2.3 | Neuroimaging

All participants underwent Aβ-PET imaging with either $^{11}$C-Pittsburgh Compound B (PiB), $^{18}$F-NAV4694 (NAV), $^{18}$F-Flutemetamol (FLUTE), or $^{18}$F-Florbetapir (FBP) to determine neocortical Aβ load. PiB, NAV, and FBP PET scan acquisition consisted of 20 min (4 × 5 min) dynamic scans acquired at 50 min after an intravenous bolus injection of 370 MBq (±10%) for PiB or 185 MBq (±10%) for NAV or FBP (±10%). Similarly, the participants who received FLUTE also underwent a 20 min (4 × 5 min) PET acquisition starting at 90 min after injection of 185 MBq (±10%) of FLUTE. All Aβ imaging results were expressed in Centiloids (CL). Aβ-PET scans were spatially normalized using CapAIBL.[25] The standard CL method was applied to determine Aβ burden. A CL value >20 was selected to determine a high Aβ (Aβ+) scan.

## 2.4 | Neuropsychological testing

Participants underwent a comprehensive battery of neuropsychological tests as described previously.[21] For this study, the primary measures used to examine global cognitive abilities were the Mini-Mental State Examination (MMSE; scores range from 0 to 30, indicating severe impairment to no impairment),[26] Clinical Dementia Rating scale (CDR; scores range from 0 to 3, indicating no impairment to severe impairment),[27] CDR-Sum of Boxes (CDR-SOB; scores range from 0 to 18, indicating no impairment to severe impairment), and the Preclinical Alzheimer Cognitive Composite (PACC) constructed using episodic memory, executive function, and orientation as described previously.[28]

## 2.5 | Statistical analyses

Descriptive statistics including means and standard deviations were calculated for each group with comparisons employing Kruskal-Wallis tests for continuous variables with non-parametric distributions, general linear models for continuous variables with parametric distributions, and chi-square tests for categorical variables. Linear models employed to compare plasma biomarkers between groups cross-sectionally were adjusted for covariates age, sex, apolipoprotein E (*APOE*) ε4 carrier status, Aβ-PET tracer, and site. Logistic regression with Aβ−/+ as response was used to evaluate predictive models and receiver-operating characteristic (ROC) curves were constructed from the logistic scores. To determine the diagnostic performance of each protein in distinguishing between groups, the R package cut point was used. The areas under the curves (AUCs) for different plasma proteins were compared using DeLong test. Linear mixed-effects models were used to compare plasma biomarkers longitudinally between diagnostic groups and were adjusted for the covariates age, sex, *APOE* ε4 carrier status, Aβ−/+ status, and PET tracer. Associations between plasma biomarker levels at timepoint 1 with prospective longitudinal cognitive decline were investigated using linear mixed-effects models adjusting for age, sex, *APOE* ε4 carrier status, years of education, and Aβ−/+ status in all participants and in the cognitively unimpaired and cognitively impaired subsets. Associations between plasma biomarker levels at timepoint 1 with subsequent longitudinal Aβ-PET load were investigated using linear mixed-effects models adjusting for age, sex, *APOE* ε4 carrier status, and Aβ−/+ status in all participants and in the cognitively unimpaired and cognitively impaired subsets. The models utilized for the whole sample (all participants) also included cognitive status as an additional covariate. Cognitive data were available for an average period of 6.5 years and Aβ-PET data were available for an average

period of 4.5 years for participants whose plasma samples were available at timepoint 1. Plasma biomarkers were natural log transformed to better approximate normality and variance homogeneity as required for analyses. All analyses and data visualization were carried out using IBM SPSS (v27) or R (v4.0.4). $p < 0.05$ was considered as statistically significant and all statistical tests were two-tailed.

## 3 | RESULTS

### 3.1 | Cohort characteristics

Participant cohort characteristics are presented in Table 1. There was no significant difference in the frequency of males and females, mean age, or mean body mass index (BMI) between CU Aβ−, CU Aβ+, MCI Aβ−, MCI Aβ+, and AD Aβ+ groups; however, the frequency of the *APOE* ε4 carriers was significantly higher in the Aβ+ groups (CU Aβ+, MCI Aβ+, and AD Aβ+) compared with Aβ− groups (CU Aβ− and MCI Aβ−) as expected. Significant differences in cognitive performance between groups were observed, wherein lower MMSE and PACC scores and higher CDR-SOB scores were observed in MCI (Aβ− and Aβ+) and AD Aβ+ compared with CU (Aβ− and Aβ+) as expected. Timepoints 2 (Table S1A) and 3 (Table S1B) had similar cohort characteristics.

### 3.2 | Association of AD risk factors, age, sex, and *APOE* ε4 carrier status, and BMI with plasma biomarkers

Although plasma Aβ1-42/Aβ1-40 ratio was not observed to correlate with age, plasma p-tau181, GFAP, and NfL correlated with age in all participants, and after stratifying participants based on diagnosis, except in the AD group, where only plasma NfL was observed to correlate with age (Table S2A). Plasma GFAP was observed to be significantly higher in females compared with males in all participants and after stratification by diagnosis, following correction for potential confounding variables, except in the AD group (Table S2B). No significant differences in plasma biomarker levels were observed between *APOE* ε4 non-carriers and carriers in all participants and after stratification by diagnosis, following correction for potential confounding variables (Table S2C). Lower BMI, likely to be a consequence of the disease rather than a risk factor, correlated inversely with p-tau181, GFAP, and NfL (Table S2D).

### 3.3 | Cross-sectional comparison of plasma biomarkers between groups

#### 3.3.1 | Aβ1-42/Aβ1-40 ratio

Plasma Aβ1-42/Aβ1-40 ratio was significantly lower in CU Aβ+, MCI Aβ+, and AD Aβ+ compared with CU Aβ− ($p < 0.0001$) and MCI Aβ− ($p < 0.0001$), whereas no significant difference was observed between CU Aβ+, MCI Aβ+, and AD Aβ+ and between CU Aβ− and MCI Aβ− (Figure 1). Similar observations were found after bias correction and bootstrapping with 1000 random samples (Table S3). Absolute value data of Aβ1-42 and Aβ1-40 at timepoint 1 are presented in Table S4.

#### 3.3.2 | p-tau181

Plasma p-tau181 was significantly higher in CU Aβ+, MCI Aβ+, and AD Aβ+ compared with CU Aβ− ($p < 0.0001$) and MCI Aβ− ($p < 0.0001$), whereas no significant difference was observed between CU Aβ+, MCI Aβ+, and AD Aβ+ and between CU Aβ− and MCI Aβ− (Figure 1). Similar observations were found after bias correction and bootstrapping with 1000 random samples, except that higher p-tau181 was also observed in AD Aβ+ compared with MCI Aβ+ (Table S3).

#### 3.3.3 | GFAP

Plasma GFAP was significantly higher in CU Aβ+, MCI Aβ+, and AD Aβ+ compared with CU Aβ− ($p < 0.0001$) and MCI Aβ− ($p < 0.0005$), whereas no significant difference was observed between CU Aβ+ and MCI Aβ+ and between CU Aβ− and MCI Aβ−; however, plasma GFAP was observed to be higher in AD Aβ+ compared with CU Aβ+ ($p < 0.01$) and MCI Aβ+ ($p < 0.001$) (Figure 1). Similar observations were found after bias correction and bootstrapping with 1000 random samples (Table S3).

#### 3.3.4 | NfL

Plasma NfL was significantly higher in MCI Aβ+ compared with CU Aβ− ($p = 0.014$) and MCI Aβ− ($p = 0.031$) and higher in AD Aβ+ compared with CU Aβ− ($p < 0.0001$), CU Aβ+ ($p < 0.005$), MCI Aβ− ($p < 0.001$), and MCI Aβ+ ($p = 0.049$) (Figure 1). Similar observations were found after bias correction and bootstrapping with 1000 random samples, except that no significant difference was observed in NfL levels between AD Aβ+ and MCI Aβ+ ($p = 0.071$, Table S3).

Mean differences and confidence intervals of Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL between CU Aβ−/MCI Aβ− and CU Aβ+/MCI Aβ+/AD Aβ+ are presented in Table S4. These observations were consistent before and after adjusting for covariates age, sex, *APOE* ε4 carrier status, Aβ-PET tracer, and site. Figure S1 shows similar findings at timepoints 2 and 3. Similar observations were noted on adding BMI as a covariate along with other covariates (data not shown).

### 3.4 | Diagnostic performance of plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL

The diagnostic performance parameters of plasma biomarkers including AUCs, specificity, sensitivity, accuracy, negative predictive value,

**TABLE 1** Participant characteristics at timepoint 1

| Timepoint 1 | *Total Sample* | CU Aβ− | CU Aβ+ | MCI Aβ− | MCI Aβ+ | AD Aβ+ | *P* | *p*[a] |
|---|---|---|---|---|---|---|---|---|
| N | 225 | 81 | 39 | 26 | 33 | 46 | - | - |
| Sex, Female % | 50.67 | 53.09 | 51.28 | 46.15 | 39.39 | 56.52 | 0.606 | - |
| Mean age, years (SD) | 74.23 (7.22) | 73.74 (5.96) | 74.9 (6.96) | 71.31 (11.46) | 75.61 (5.66) | 75.17 (7.20) | 0.234 | - |
| Mean body mass index (SD) | 26.19 (4.54) | 26.71 (4.32) | 25.29 (4.65) | 27.28 (5.05) | 25.84 (4.76) | 25.69 (4.32) | 0.339 | - |
| *APOE* ε4 carriage, N (%) | 104 (46.22) | 21 (25.93) | 21 (53.85) | 2 (7.69) | 24 (72.73) | 36 (78.26) | <**0.0001** | - |
| Mean MMSE (SD) | 26.84 (4.15) | 29.04 (1.03) | 28.92 (1.24) | 27.27 (1.89) | 27.58 (1.48) | 20.41 (4.87) | <**0.0001** | - |
| Mean CDR-SOB (SD) | 1.43 (2.66) | 0.025 (0.11) | 0.026 (0.11) | 0.519 (0.264) | 0.606 (0.325) | 6.21 (2.36) | <**0.0001** | - |
| Mean PACC score (SD) | −0.844 (1.53) | 0.175 (0.65) | 0.177 (0.74) | −1.105 (0.80) | −1.446 (0.53) | −3.55 (0.77) | <**0.0001** | - |
| Aβ PET tracer PiB/NAV/FLUTE/FBP, N | 148/4/65/8 | 51/1/28/1 | 22/0/17/0 | 20/1/5/0 | 23/0/8/2 | 32/2/7/5 | **0.021** | - |
| Mean Aβ PET Centiloid (SD) | 41.65 (46.65) | 1.31 (6.70) | 61 (26.85) | 0.30 (7.01) | 77.63 (30.01) | 102.31 (28.55) | <**0.0001** | - |
| Mean hippocampal volume, right, $cm^3$ (SD) | 2.79 (0.43) | 2.97 (0.31) | 2.98 (0.27) | 2.91 (0.30) | 2.7 (0.33) | 2.15 (0.31) | <**0.0001** | - |
| Mean hippocampal volume, left, $cm^3$ (SD) | 2.72 (0.44) | 2.89 (0.31) | 2.89 (0.28) | 2.84 (0.36) | 2.74 (0.30) | 2.04 (0.31) | <**0.0001** | - |
| Mean Aβ1-42/Aβ1-40 ratio (SD) | 0.054 (0.011) | 0.058 (0.010) | 0.047 (0.008) | 0.062 (0.011) | 0.050 (0.008) | 0.049 (0.007) | <**0.0001**† | <**0.0001**† |
| Mean p-tau181 pg/mL (SD) | 3.01 (1.64) | 2.16 (1.14) | 3.67 (2.02) | 1.87 (0.74) | 3.65 (1.39) | 4.12 (1.42) | <**0.0001**† | <**0.0001**† |
| Mean GFAP pg/mL (SD) | 179.60 (85.09) | 135.06 (54.67) | 205.26 (84.76) | 133.07 (72.35) | 196.47 (91.22) | 250.50 (71.37) | <**0.0001**† | <**0.0001**† |
| Mean NFL pg/mL (SD) | 25.66 (14.05) | 22.46 (11.62) | 25.15 (10.56) | 20.49 (10.00) | 28.56 (17.80) | 32.58 (16.66) | <**0.0001**† | <**0.0001**† |

Kruskal-Wallis tests were used for continuous variables with non-parametric distributions and general linear models were used for continuous variables with parametric distributions, whereas chi-square tests were used for categorical variables. Data for composite AIBL PACC scores are presented for 79 CU Aβ−, 39 CU Aβ+, 25 MCI Aβ−, 32 MCI Aβ+, and 35 AD individuals, data for hippocampal volume are presented for 73 CU Aβ−, 35 CU Aβ+, 17 MCI Aβ−, 21 MCI Aβ+, and 31 AD individuals and Centiloid data are presented for 81 CU Aβ−, 39 CU Aβ+, 24 MCI Aβ−, 30 MCI Aβ+, and 40 AD individuals based on data availability. Aβ−/+ status for participants who did not undergo an Aβ PET scan at timepoint 1 was determined from the next immediate timepoint. CU individuals comprised 55 non-subjective memory complainers (non-SMC; Aβ− = 39, Aβ+ = 16) and 65 SMC (Aβ− = 42, Aβ+ = 23). P[a] are adjusted for age, sex, site, *APOE* ε4 carriage, and Aβ PET tracer. $p < 0.05$ was considered significant. †Represents plasma biomarkers natural log transformed to better approximate normality and variance homogeneity. CU: cognitively unimpaired, MCI: mild cognitively impaired, AD: Alzheimer's disease, MMSE: Mini-Mental State Examination, CDR-SOB: Clinical Dementia Rating Sum of Boxes, PACC score: Preclinical Alzheimer Cognitive Composite score, Aβ: amyloid beta, PiB: $^{11}$C-Pittsburgh Compound B, NAV: $^{18}$F-NAV4694, FLUTE: $^{18}$F-Flutemetamol, FBP: $^{18}$F-Florbetapir, PET: positron emission tomography, p-tau181: phosphorylated-tau 181, GFAP: glial fibrillary acidic protein, NfL: neurofilament light chain.

positive predictive value, and Youden's optimal cut point are presented in Table S5.

### 3.4.1 | CU Aβ− versus CU Aβ+

The AUCs of Aβ1-42/Aβ1-40 ratio (AUC = 0.836), p-tau181 (AUC = 0.805), and GFAP (AUC = 0.749) were significantly different, but all had significantly higher AUCs than NfL (AUC = 0.609, $p < 0.01$) in distinguishing between the groups (Table S6A, Figure 2).

### 3.4.2 | CU Aβ− versus MCI Aβ+

P-tau181 had a significantly higher AUC (AUC = 0.858) than GFAP (AUC = 0.716, $p = 0.019$) and NfL (AUC = 0.641, $p < 0.001$), but not

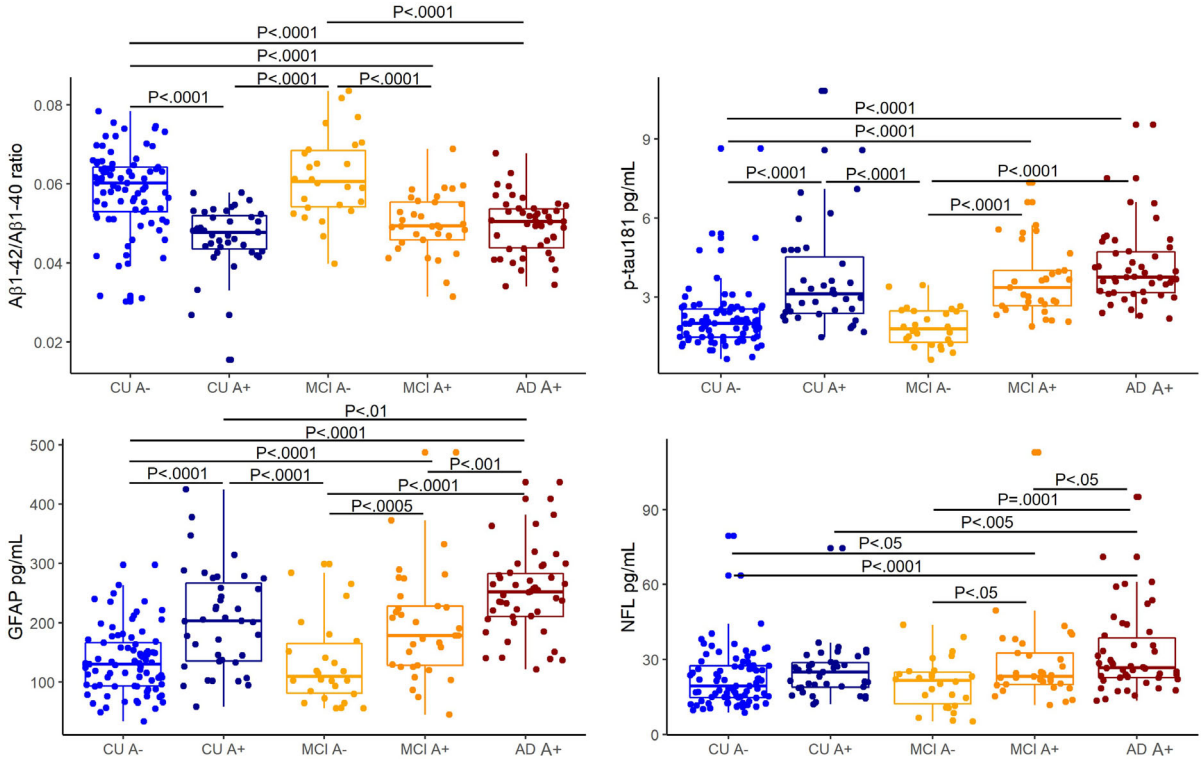


**FIGURE 1** Boxplots comparing plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL between CU Aβ−, CU Aβ+, MCI Aβ−, MCI Aβ+, and AD Aβ+ groups at timepoint 1. Plasma measures were compared between groups using linear models with age, sex, APOE ε4 carrier status, PET tracer, and site as covariates. Data from 81 CU Aβ−, 39 CU Aβ+, 26 MCI Aβ−, 33 MCI Aβ+, and 46 AD Aβ+ participants were utilized for analyses. The line segments within each boxplot represent the median of the data. *p*-values were obtained from natural log-transformed plasma biomarker data to better approximate normality and variance homogeneity. $p < 0.05$ was considered statistically significant.

compared with Aβ1-42/Aβ1-40 ratio (AUC = 0.772) in distinguishing between the groups (Table S6B, Figure 2).

### 3.4.3 | CU Aβ− versus AD Aβ+

P-tau181 (AUC = 0.920) and GFAP (AUC = 0.904) had significantly higher AUCs than Aβ1-42/Aβ1-40 ratio (AUC = 0.784, $p < 0.01$) and NfL (AUC = 0.717, $p < 0.0001$) in distinguishing between the groups (Table S6C, Figure 2).

### 3.4.4 | MCI Aβ− versus MCI Aβ+

P-tau181 (AUC = 0.902) had a significantly higher AUC compared with GFAP (AUC = 0.730, $p < 0.01$) and NfL (AUC = 0.646, $p < 0.0001$), but not compared with Aβ1-42/Aβ1-40 ratio (AUC = 0.825) in distinguishing between the groups (Table S6D, Figure 2).

### 3.4.5 | MCI Aβ− versus AD Aβ+

P-tau181 (AUC = 0.957) had a significantly higher AUC compared with Aβ1-42/Aβ1-40 ratio (AUC = 0.839, $p = 0.036$) and NfL (AUC = 0.741, $p < 0.0001$), but not compared with GFAP (AUC = 0.868) in distinguishing between the groups (Table S6E, Figure 2).

## 3.5 | Diagnostic performance of plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL along with AD risk factors

### 3.5.1 | CU Aβ− versus CU Aβ+

On adding the plasma biomarkers to a base model (BM) incorporating the AD risk factors age, sex, and *APOE ε4* allele carrier status, Aβ1-42/Aβ1-40 ratio+BM (AUC = 0.859), p-tau181+BM (AUC = 0.812), and GFAP+BM (AUC = 0.826) had no significant differences between their AUCs but had significantly higher AUCs compared with the BM (AUC = 0.694, $p < 0.01$) and NfL+BM (AUC = 0.708, $p < 0.01$) in distinguishing between the groups (Table S7A, Figure 2).

### 3.5.2 | CU Aβ− versus MCI Aβ+

Aβ1-42/Aβ1-40 ratio+BM (AUC = 0.884) and p-tau181+BM (AUC = 0.874) had significantly higher AUCs than BM (AUC = 0.809,

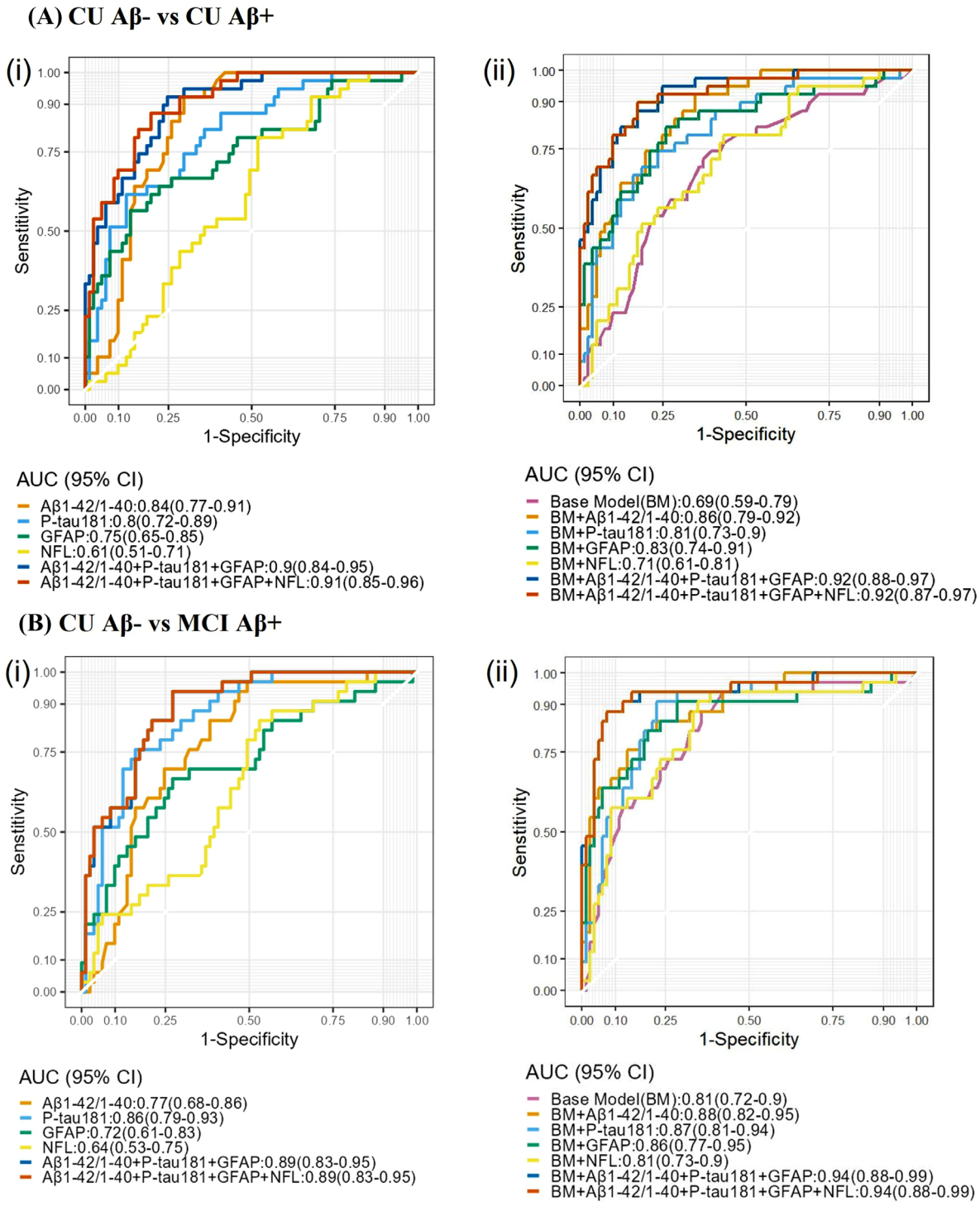


**FIGURE 2** Receiver-operating characteristic (ROC) curves for distinguishing between (A) CU Aβ− and CU Aβ+, (B) CU Aβ− and MCI Aβ+, (C) CU Aβ− and AD Aβ+, (D) MCI Aβ− and MCI Aβ+, and (E) MCI Aβ− and AD Aβ+ participants at timepoint 1. ROC curves are presented for A, B, C, D, and E for (i) Aβ1-42/Aβ1-40, p-tau181, GFAP, and NfL, Aβ1-42/Aβ1-40+ p-tau181+GFAP, and Aβ1-42/Aβ1-40+p-tau181+GFAP+NfL and (ii) base model comprising AD risk factors, age, sex, *APOE* ε4 allele status (BM), BM+Aβ1-42/Aβ1-40, BM+p-tau181, BM+GFAP, BM+NfL, BM+Aβ1-42/Aβ1-40+p-tau181+GFAP, and BM+Aβ1-42/Aβ1-40+ p-tau181+GFAP+NfL. Data from 81 CU Aβ−, 39 CU Aβ+, 26 MCI Aβ−, 33 MCI Aβ+, and 46 AD Aβ+ participants were utilized for analyses. AUC: area under the curve; CI: confidence interval.

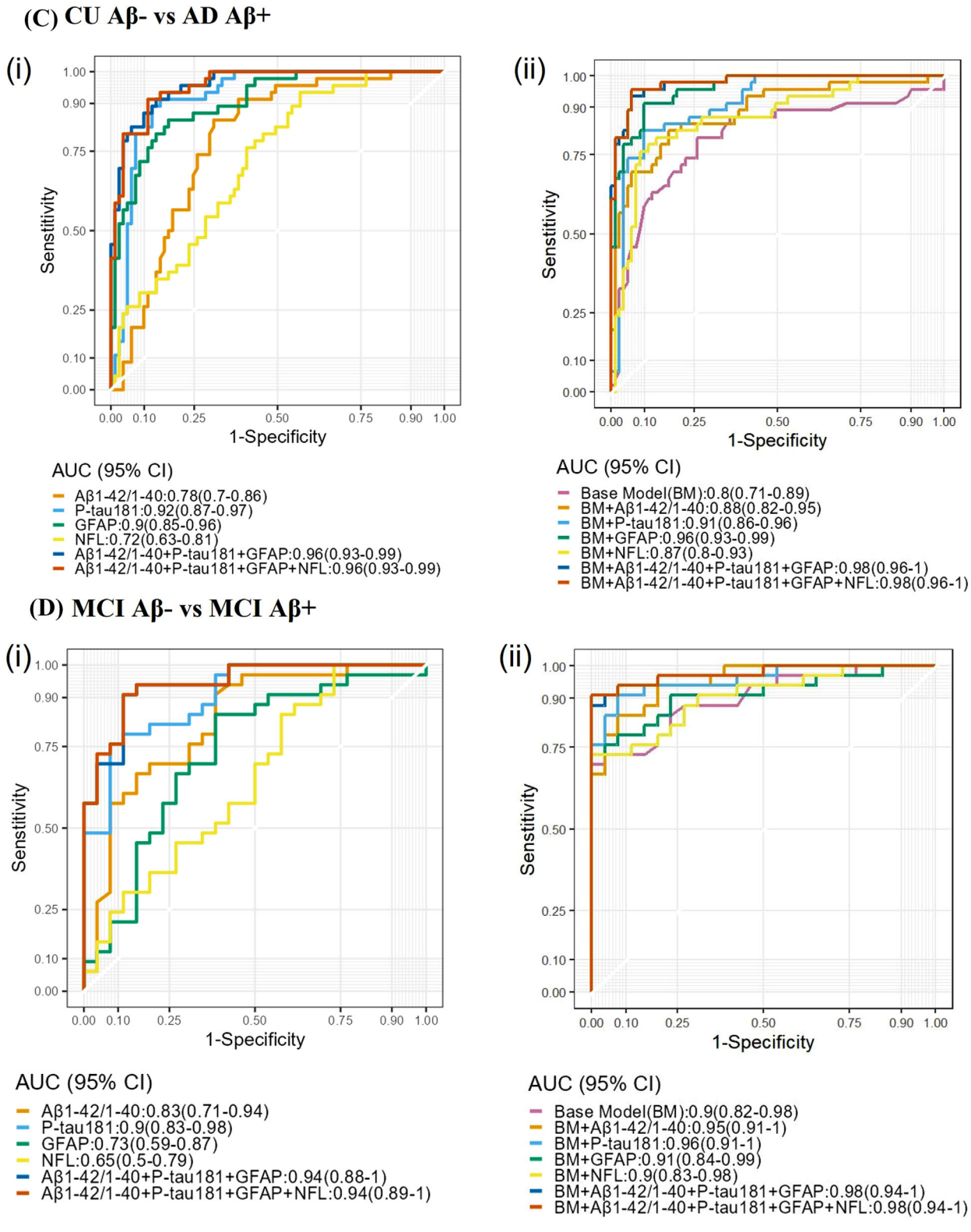


**FIGURE 2** Continued

$p$ = 0.023) and NfL+BM (AUC = 0.814, Aβ1-42/Aβ1-40 ratio+BM: $p < 0.01$; p-tau181+BM: $p = 0.031$) but not compared with GFAP+BM (AUC = 0.861) in distinguishing between the groups (Table S7B, Figure 2).

### 3.5.3 | CU Aβ− versus AD Aβ+

Aβ1-42/Aβ1-40 ratio+BM (AUC = 0.884), p-tau181+BM (AUC = 0.910), GFAP+BM (AUC = 0.959), and NfL+BM (AUC = 0.866)

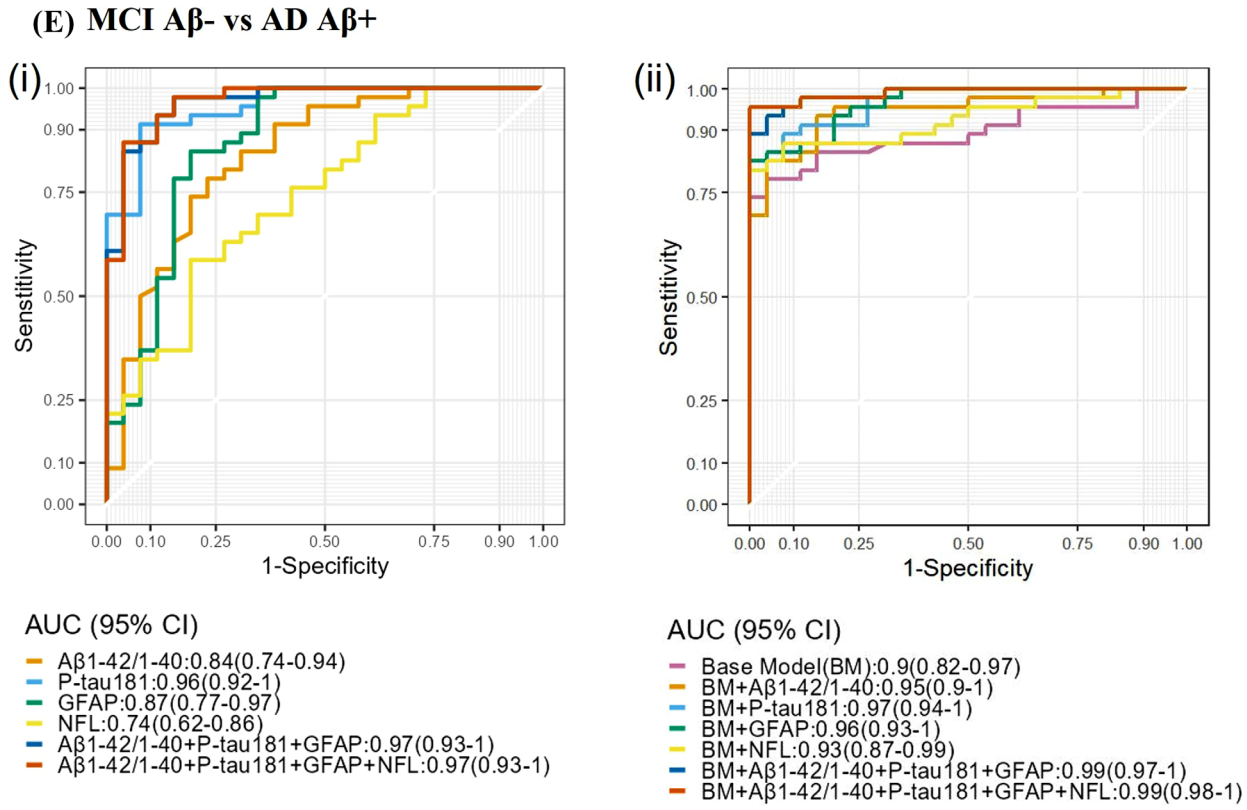


**FIGURE 2** Continued

had significantly higher AUCs than BM (AUC = 0.803, $p = 0.018$), and GFAP+BM had a significantly higher AUC than Aβ1-42/Aβ1-40 ratio+BM ($p < 0.01$) and NfL+BM ($p < 0.01$) in distinguishing between the groups (Table S7C, Figure 2).

### 3.5.4 | MCI Aβ− versus MCI Aβ+

Aβ1-42/Aβ1-40 ratio+BM (AUC = 0.952) had a significantly higher AUC compared with BM (AUC = 0.900, $p = 0.048$), and p-tau181+BM (AUC = 0.958) had significantly higher AUCs compared with BM ($p = 0.018$), GFAP+BM (AUC = 0.911, $p = 0.028$), and NfL+BM (AUC = 0.904, $p = 0.015$) in distinguishing between the groups (Table S7D, Figure 2).

### 3.5.5 | MCI Aβ− versus AD Aβ+

Aβ1-42/Aβ1-40 ratio+BM (AUC = 0.947), p-tau181+BM (AUC = 0.969), and GFAP+BM (AUC = 0.965) had significantly higher AUCs compared with BM (AUC = 0.895, Aβ1-42/Aβ1-40 ratio+BM: $p = 0.032$; p-tau181+BM: $p < 0.01$; GFAP+BM: $p = 0.013$), but not compared with NfL+BM (AUC = 0.926) in distinguishing between the groups (Table S7E, Figure 2).

In addition, we assessed whether combining the BM with the plasma biomarkers significantly improved plasma biomarker diagnostic performance. In distinguishing between CU Aβ− and CU Aβ+, we noted a significantly higher AUC when combining BM with GFAP in a model compared with GFAP alone ($p = 0.049$). In distinguishing between CU Aβ− and MCI Aβ+ groups, CU Aβ− and AD Aβ+ groups, MCI Aβ− and MCI Aβ+ groups, and MCI Aβ− and AD Aβ+ groups, we noted significantly higher AUCs when combining BM with Aβ1-42/Aβ1-40 ratio compared with Aβ1-42/Aβ1-40 ratio alone (CU Aβ− vs MCI Aβ+: $p = 0.019$; CU Aβ− vs AD Aβ+: $p = 0.011$; MCI Aβ− vs MCI Aβ+: $p = 0.014$; MCI Aβ− vs AD Aβ+: $p = 0.017$), BM with GFAP compared with GFAP alone (CU Aβ− vs MCI Aβ+: $p < 0.01$; CU Aβ− vs AD Aβ+: $p < 0.01$; MCI Aβ− vs MCI Aβ+: $p < 0.01$; MCI Aβ− vs AD Aβ+: $p = 0.028$) and BM with NfL compared with NfL alone ($p < 0.01$). No significant difference in diagnostic performance of p-tau181 across the AD continuum was observed before and after the addition of the BM (Table S8).

## 3.6 | Diagnostic performance of a panel of plasma biomarkers comprising Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL

### 3.6.1 | CU Aβ− versus CU Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) had a significantly higher AUC (AUC = 0.898, Aβ1-42/Aβ1-40 ratio: $p = 0.016$; p-tau181: $p < 0.01$; GFAP: $p < 0.001$; NfL:

$p < 0.0001$) than any of these proteins alone in distinguishing between the groups (Table S6A, Figure 2).

#### 3.6.2 | CU Aβ− versus MCI Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) had a significantly higher AUC (AUC = 0.886) compared with the AUC of Aβ1-42/Aβ1-40 ratio ($p < 0.01$), GFAP ($p < 0.001$), and NfL ($p < 0.0001$), but not p-tau181 in distinguishing between the groups (Table S6B, Figure 2).

#### 3.6.3 | CU Aβ− versus AD Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) had a significantly higher AUC (AUC = 0.958) compared with the AUC of Aβ1-42/Aβ1-40 ratio ($p < 0.0001$), GFAP ($p < 0.01$), and NfL ($p < 0.0001$), but not p-tau181, in distinguishing between the groups (Table S6C, Figure 2).

#### 3.6.4 | MCI Aβ− versus MCI Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) had a significantly higher AUC (AUC = 0.941) compared with the AUC of Aβ1-42/Aβ1-40 ratio ($p = 0.011$), GFAP ($p < 0.01$), and NfL ($p < 0.0001$), but not p-tau181, in distinguishing between the groups (Table S6D, Figure 2).

#### MCI Aβ− versus AD Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) had a significantly higher AUC (AUC = 0.967) compared with the AUC of Aβ1-42/Aβ1-40 ratio ($p < 0.01$), GFAP ($p = 0.012$), and NfL ($p < 0.001$), but not p-tau181, in distinguishing between the groups (Table S6E, Figure 2).

### 3.7 | Diagnostic performance of a panel of plasma biomarkers comprising plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL along with AD risk factors

#### 3.7.1 | CU Aβ− versus CU Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) along with BM was observed to have a significantly higher AUC (AUC = 0.924) than Aβ1-42/Aβ1-40 ratio+BM ($p = 0.014$), p-tau181+BM ($p < 0.01$), GFAP+BM ($p < 0.01$), and NfL+BM ($p < 0.0001$) in distinguishing between the groups (Table S7A, Figure 2).

#### 3.7.2 | CU Aβ− versus MCI Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) along with BM was observed to have a significantly higher AUC (AUC = 0.938) than Aβ1-42/Aβ1-40 ratio+BM ($p = 0.026$), p-tau181+BM ($p < 0.01$), GFAP+BM ($p < 0.01$), and NfL+BM ($p < 0.001$) in distinguishing between the groups (Table S7B, Figure 2).

#### 3.7.3 | CU Aβ− versus AD Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) along with BM was observed to have a significantly higher AUC (AUC = 0.978) than Aβ1-42/Aβ1-40 ratio+BM ($p < 0.001$), p-tau181+BM ($p < 0.01$), GFAP+BM ($p = 0.016$), and NfL+BM ($p < 0.001$) in distinguishing between the groups (Table S7C, Figure 2).

#### 3.7.4 | MCI Aβ− versus MCI Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) along with the BM was observed to have a significantly higher AUC (AUC = 0.976) than BM ($p = 0.018$), GFAP+BM ($p = 0.027$), and NfL+BM ($p = 0.016$), but not p-tau181 or Aβ1-42/Aβ1-40 ratio, in distinguishing between the groups (Table S7D, Figure 2).

#### 3.7.5 | MCI Aβ− versus AD Aβ+

A model incorporating Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP (with and without NfL) along with BM was observed to have a significantly higher AUC (AUC = 0.988) than BM ($p < 0.01$), Aβ1-42/Aβ1-40 ratio+BM ($p = 0.025$), NfL+BM ($p = 0.013$), but not GFAP+BM and p-tau181+BM, in distinguishing between the groups (Table S7E, Figure 2).

In addition, whether combining the BM with the plasma biomarker panel significantly improved the diagnostic performance of the plasma biomarker panel was assessed. No significant improvement was observed after combining the BM with the plasma biomarker panel when compared with the plasma biomarker panel in distinguishing CU Aβ− versus CU Aβ+, MCI Aβ− versus MCI Aβ+, and MCI Aβ− versus AD Aβ+ groups. In distinguishing between CU Aβ− and MCI Aβ+, significantly higher AUCs were noted on combining the BM with the plasma biomarker panel compared with the plasma biomarker panel alone ($p = 0.043$) (Table S9).

### 3.8 | Longitudinal changes in plasma biomarkers in MCI and AD compared with CU

Plasma Aβ1-42/Aβ1-40 ratio decreased significantly ($p = 0.024$), and plasma p-tau181 ($p \leq 0.01$) and GFAP ($p < 0.01$) increased

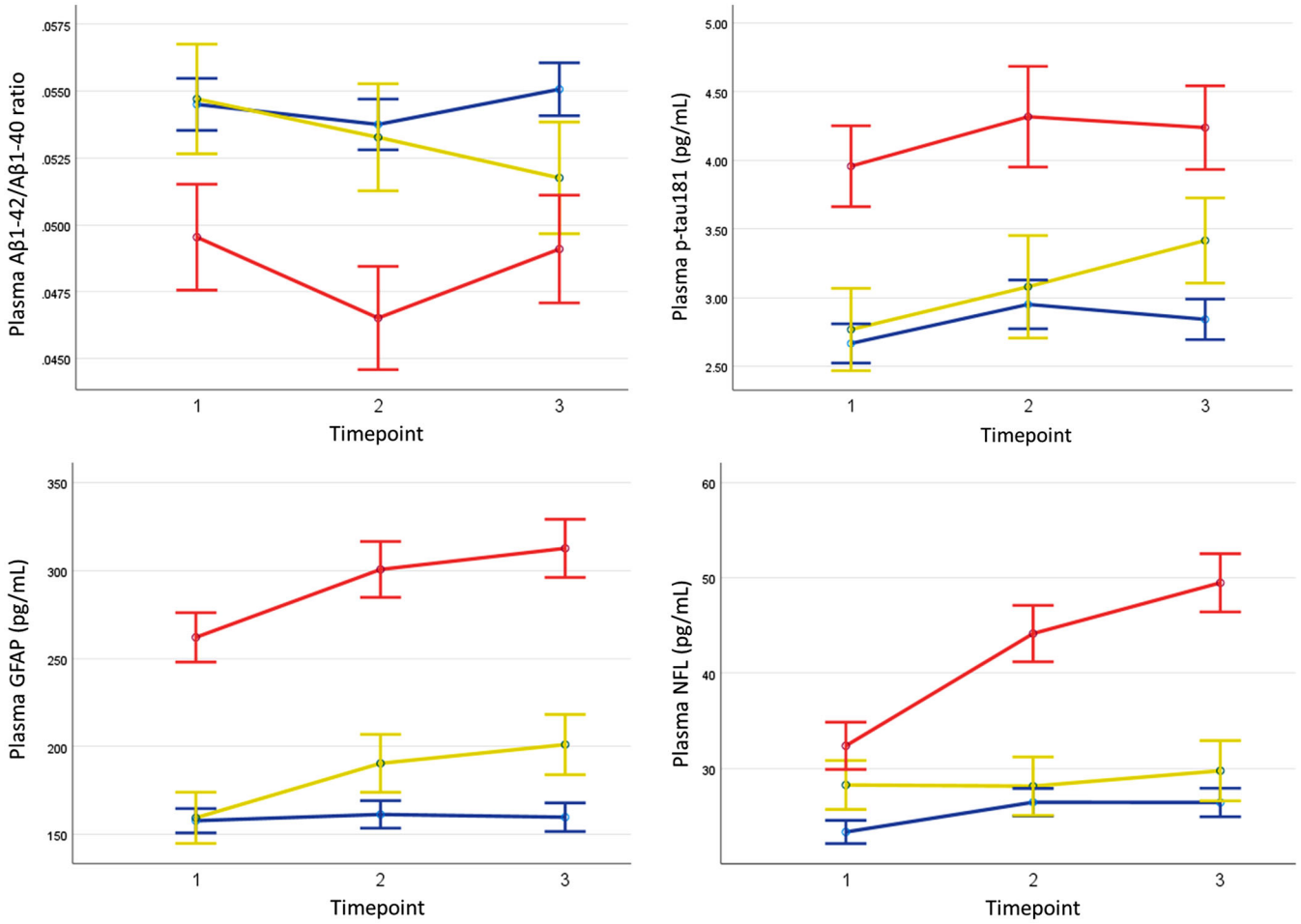


**FIGURE 3** Longitudinal changes in plasma biomarkers over 36 months between CU, MCI, and AD groups. Estimated marginal means of plasma biomarkers Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL for CU (blue), MCI (yellow), and AD (red) participants are presented at three timepoints, 18 months apart. Data for Aβ1-42/Aβ1-40 ratio, GFAP, and NfL are presented in 120 CU, 27 MCI, and 29 AD participants and for p-tau181 are presented in 119 CU, 27 MCI, and 28 AD. Error bars represent ±1 SE.

**TABLE 2** Longitudinal changes in plasma biomarkers over 36 months in MCI and AD individuals compared to CU individuals

| | CU versus MCI | | | | CU versus AD | | | |
|---|---|---|---|---|---|---|---|---|
| | B (SE) | *p* | B (SE) [a] | *p*[a] | B (SE) | *p* | B (SE) [a] | *p*[a] |
| Aβ1-42/Aβ1-40 ratio | −0.020 (0.009) | **0.027** | −0.021 (0.009) | **0.024** | −0.008 (0.009) | 0.36 | −0.008 (0.009) | 0.332 |
| P-tau181 | 0.041 (0.016) | **0.010** | 0.043 (0.016) | **0.008** | −0.009 (0.015) | 0.544 | −0.008 (0.015) | 0.596 |
| GFAP | 0.059 (0.022) | **0.009** | 0.059 (0.023) | **0.009** | 0.042 (0.021) | **0.049** | 0.043 (0.021) | **0.047** |
| NFL | −0.009 (0.020) | 0.630 | −0.009 (0.020) | 0.653 | 0.071 (0.019) | **2e-04** | 0.071 (0.019) | **2e-04** |

Longitudinal changes in plasma proteins were compared between CU and MCI participants and, CU and AD participants, using linear mixed models, before and after ($P^a$) adjustment for the covariates age, sex, *APOE* ε4 carrier status, Aβ−/+ PET-status, and Aβ PET tracer. Data from 120 CU, 27 MCI, and 29 AD participants were utilized for Aβ1-42/Aβ1-40 ratio, GFAP, and NfL and from 119 CU, 27 MCI, and 28 AD participants for p-tau181. CU: cognitively unimpaired, MCI: mild cognitively impaired, AD: Alzheimer's disease. Plasma biomarkers were natural log transformed to better approximate normality and variance homogeneity. $p < 0.05$ was considered significant.

significantly in MCI compared with CU over 36 months before and after correcting for covariates age, sex, *APOE* ε4 carrier status, Aβ-/+ status, and tracer (Table 2). In addition, plasma GFAP ($p = 0.049$) and NfL ($p < 0.001$) increased significantly in AD compared with CU over 36 months before and after correcting for covariates (Figure 3, Figure S2, Table 2).

### 3.9 | Association of baseline plasma biomarker levels with prospective cognitive decline and Aβ-PET load

Analyses were performed to investigate whether plasma biomarker levels from a single timepoint were associated with prospective cognitive decline and cerebral Aβ accumulation. In all participants, lower baseline plasma Aβ1-42/Aβ1-40 ratio was associated with increased future cognitive decline (MMSE: $p = 0.041$; CDR-SOB: $p = 0.049$) and higher baseline p-tau181 (MMSE: $p < 0.0001$; CDR-SOB: $p < 0.0001$; PACC: $p < 0.0001$), GFAP (MMSE: $p < 0.0001$; CDR-SOB: $p < 0.0001$; PACC: $p < 0.001$), and NfL (MMSE: $p < 0.0001$; CDR-SOB: $p < 0.0001$; PACC: $p < 0.0001$) measures were observed to be associated with increased future cognitive decline (Table 3). On stratifying participants based on cognitive status, in cognitively unimpaired participants, baseline plasma Aβ1-42/Aβ1-40 ratio was not observed to be associated with future cognitive decline; however, higher baseline plasma p-tau181 (PACC: $p < 0.001$), GFAP (PACC: $p = 0.020$) and NfL (MMSE: $p = 0.019$; PACC: $p = 0.046$) measures were observed to be associated with increased future cognitive decline (Table 3). In cognitively impaired participants (MCI and AD), lower baseline plasma Aβ1-42/Aβ1-40 ratio was associated significantly with prospective decline in CDR-SOB ($p = 0.020$). Furthermore, higher baseline plasma p-tau181 (MMSE: $p < 0.0001$; CDR-SOB: $p < 0.0001$; PACC: $p < 0.0001$), GFAP (MMSE: $p < 0.001$; CDR-SOB: $p < 0.0001$; PACC: $p < 0.01$), and NfL (MMSE: $p < 0.01$; CDR-SOB: $p < 0.01$; PACC: $p < 0.01$) measures were observed to be associated with increased future cognitive decline (Table 3). In addition, lower baseline plasma Aβ1-42/Aβ1-40 ratio ($p < 0.001$) and higher p-tau181 ($p < 0.0001$) and GFAP ($p < 0.01$) were observed to be associated with increased future Aβ-PET load in all participants; however, upon stratification by cognitive impairment status, the preceding observations remained significant only in cognitively unimpaired participants. Relationships between low and high plasma biomarker levels at baseline (based on the optimal cut point at Youden's index for comparisons between CU Aβ− and AD Aβ+) and the rate of change in cognition and brain Aβ−PET load are presented in Figure S3.

## 4 | DISCUSSION

In the current study we showed that plasma Aβ1-42/Aβ1-40 ratio was lower, and p-tau181 and GFAP levels were higher in Aβ+ individuals across the AD continuum, and that plasma NfL levels were higher in cognitively impaired Aβ+ individuals compared with controls. p-tau181 followed by GFAP showed the highest change in magnitude in Aβ+ compared with Aβ− individuals along the AD continuum. To our knowledge this is the first head-to-head study cross-sectionally investigating plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL along the AD continuum employing Aβ+ defined preclinical AD, prodromal AD, and AD participants in a highly characterized Australian cohort utilizing an ultrasensitive platform. We also showed that Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP had non-significant differences in their discriminative capabilities for preclinical AD based on AUCs, and outperformed NfL. In the cognitively impaired stages, we showed that p-tau181 outperformed NfL and Aβ1-42/Aβ1-40 ratio or GFAP. Furthermore we showed that combining plasma biomarkers (particularly Aβ1-42/Aβ1-40 ratio, p-tau181, or GFAP) with the known AD risk factors, age, sex, and *APOE* ε4 carrier status, most often significantly improved the discriminative performance of the known AD risk factors between CU Aβ+/MCI Aβ+/AD Aβ+ and Aβ− CU individuals. On the other hand, we also showed that although the discriminative performance of Aβ1-42/Aβ1-40 ratio, GFAP, and NfL improved when the AD risk factors were combined with the plasma biomarkers, this was not the case for p-tau181. In our longitudinal analyses, we showed that the plasma Aβ1-42/Aβ1-40 ratio decreased and p-tau181 increased in MCI participants, GFAP increased in MCI and AD participants, and NfL increased in AD participants over 36 months compared with controls. We also showed that baseline plasma Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP, and NfL levels are associated with prospective cognitive decline and baseline plasma Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP are associated with prospective Aβ-PET load.

Our observations of lower plasma Aβ1-42/Aβ1-40 ratio,[10,13,29] and elevated plasma p-tau181[6,15,16,29,30] and GFAP[12,17,31] in preclinical AD, prodromal AD, and AD, corroborate findings from earlier studies; however, in the current study we did not always observe a consistent progressive magnitude decrease in plasma Aβ1-42/Aβ1-40 ratio or increase in plasma p-tau181 levels and GFAP levels across the AD continuum. Further validation studies are required to confirm whether these observations could be attributed to the differences in sample size between groups. Our observations of elevated NfL in prodromal AD and AD but not in Aβ+ defined preclinical AD are also in line with previous studies.[32–34] In addition, abnormal NfL levels have been reported in other neurological diseases, such as multiple sclerosis,[35] Parkinson disease[36,37] and other diseases affecting the central nervous system,[38] thus serving as a putative marker of neurological insults or ongoing neuroaxonal damage but unspecific to AD.

Although head-to-head studies for plasma biomarkers across the AD continuum are largely missing, one study reported that p-tau181 outperformed Aβ1-42/Aβ1-40 ratio, GFAP, and NfL in differentiating between AD and CU; however, unlike the current study, these findings are not from Aβ−/+ status confirmed participants.[3] Autopsy studies demonstrate that diagnosis of AD based on clinical criteria has limited sensitivity and specificity,[39] whereas Aβ-PET and CSF biomarkers have over 90% sensitivity and specificity.[40,41] In the current study, we observed that there was no significant difference in the discriminative performance of p-tau181 and GFAP between AD Aβ+ and CU Aβ−, and that both outperformed Aβ1-42/Aβ1-40 ratio and NfL. Our observations of non-significant differences between the AUCs of p-tau181 and GFAP in CU Aβ− versus CU Aβ+ are in line with our previous observations in an independent cohort, wherein plasma p-tau181 and GFAP had non-significant differences in their discriminative capabilities for preclinical AD and both significantly outperformed plasma NfL.[16] Strikingly, in the current study at timepoint 1, plasma Aβ1-42/Aβ1-40 ratio showed unexpectedly high AUCs in differentiating between CU Aβ− and CU Aβ+ (AUC = 0.84, 95% CI: 0.77-0.91),

**TABLE 3** Association of baseline plasma biomarkers with longitudinal cognitive decline and brain Aβ-PET load

| | Aβ42/40 ratio | P-tau181 | GFAP | NfL |
|---|---|---|---|---|
| **MMSE** | | | | |
| *All participants* | | | | |
| B (SE) | 0.911 (0.442) | −0.927 (0.177) | −0.870 (0.180) | −0.884 (0.199) |
| *P* | **0.041** | **5.52E-07** | **3.29E-06** | **1.66E-05** |
| *CU participants* | | | | |
| B (SE) | 0.094 (0.090) | −0.029 (0.042) | −0.074 (0.041) | −0.111 (0.047) |
| *P* | **0.297** | **0.499** | **0.073** | **0.019** |
| *CI participants* | | | | |
| B (SE) | 2.124 (1.081) | −1.885 (0.373) | −1.371 (0.374) | −1.340 (0.397) |
| *P* | **0.054** | **4.62E-06** | **5.17E-04** | **0.001** |
| **CDR-SOB** | | | | |
| *All participants* | | | | |
| B (SE) | −0.460 (0.232) | 0.531 (0.092) | 0.530 (0.093) | 0.487 (0.103) |
| *P* | **0.049** | **3.18E-08** | **5.07E-08** | **4.76E-06** |
| *CU participants* | | | | |
| B (SE) | −0.027 (0.035) | 0.012(0.016) | 0.011 (0.017) | 0.028 (0.019) |
| *P* | 0.441 | 0.460 | 0.507 | 0.131 |
| *CI participants* | | | | |
| B (SE) | −1.209 (0.509) | 0.932 (0.172) | 0.765 (0.173) | 0.608 (0.186) |
| *P* | **0.020** | **7.63E-07** | **3.37E-05** | **0.002** |
| **PACC** | | | | |
| *All participants* | | | | |
| B (SE) | 0.069 (0.042) | −0.100 (0.018) | −0.070 (0.018) | −0.090 (0.020) |
| *P* | 0.102 | **9.76E-08** | **2.05E-04** | **1.35E-05** |
| *CU participants* | | | | |
| B (SE) | 0.034 (0.038) | −0.064 (0.017) | −0.042 (0.018) | −0.041 (0.020) |
| *P* | 0.374 | **3.37E-04** | **0.020** | **0.046** |
| *CI participants* | | | | |
| B (SE) | 0.213 (0.141) | −0.214 (0.048) | −0.166 (0.048) | −0.156 (0.049) |
| *P* | 0.139 | **6.66E-05** | **0.001** | **0.003** |
| **Aβ-PET** | | | | |
| *All participants* | | | | |
| B (SE) | −6.035 (1.555) | 2.823 (0.675) | 2.075 (0.708) | 1.473 (0.786) |
| *P* | **1.56E-04** | **4.72E-05** | **0.003** | 0.063 |
| *CU participants* | | | | |
| B (SE) | −6.014 (1.521) | 2.844 (0.706) | 2.215 (0.767) | 1.212 (0.866) |
| *P* | **1.28E-04** | **9.71E-05** | **0.005** | 0.165 |
| *CI participants* | | | | |
| B (SE) | −5.646 (4.302) | 2.711 (1.656) | 1.619 (1.569) | 1.467 (1.670) |
| *P* | 0.196 | 0.107 | 0.307 | 0.384 |

Relationships between plasma biomarkers and change in cognition (represented by MMSE, CDR-SOB, and PACC scores) were assessed using linear mixed effects models adjusting for age, sex, *APOE* ε4 carrier status, and years of education. Models for all participants were also adjusted for cognitive status. $p < 0.05$ was considered as statistically significant. Plasma biomarkers were natural log transformed to better approximate normality and variance homogeneity.

not seen previously using the Simoa platform.[10,12,42] Similar analyses between the same CU Aβ− and CU Aβ+ participants at follow-up visit timepoint 2 generated an AUC = 0.78 (95% CI: 0.70-0.87) and timepoint 3 generated AUC = 0.79 (95% CI: 0.70-0.87). It could be posited that this superior performance of plasma Aβ1-42/Aβ1-40 ratio in preclinical AD at timepoint 1 compared to the later timepoints may be reflective of the nature of the early changes of this biomarker in the AD pathogenesis trajectory; however, further confirmatory studies are required.

Combining plasma biomarkers (particularly Aβ1-42/Aβ1-40 ratio, p-tau181, or GFAP) with the known AD risk factors most often significantly improved the discriminative performance of the AD risk factors between CU Aβ+/MCI Aβ+/AD Aβ+ and Aβ− CU individuals. However, combining the AD risk factors with the plasma biomarkers improved the discriminative performance of Aβ1-42/Aβ1-40 ratio, GFAP, and NfL but not p-tau181. Similar to our findings, previous studies have reported improved plasma Aβ1-42/Aβ1-40 ratio or GFAP performance when combined with AD risk factors in differentiating between Aβ−/+ individuals,[5,10,43] whereas plasma p-tau181 combined with AD risk factors did not significantly perform better than p-tau181 alone.[6] This may suggest that p-tau181 levels are largely independent of age, sex, and *APOE* ε4 carrier status in distinguishing CU Aβ+, MCI Aβ+, and AD Aβ+ from Aβ− CU individuals.

Furthermore, our observations within the current study suggest that employing a panel of plasma biomarkers comprising Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP may provide better discriminative performance than individual plasma biomarkers, particularly when combined with the AD risk factors. In line with our observations, Janelidze and colleagues reported a significantly higher AUC when combining p-tau181 with Aβ42/Aβ40 ratio compared with Aβ42/Aβ40 ratio alone in differentiating between Aβ− and Aβ+ individuals.[15] In addition, Verberk and colleagues showed that a panel comprising Aβ1-42/Aβ1-40 ratio, GFAP, age, and *APOE* ε4 carrier status optimally identified Aβ+ individuals, and also reported no significant improvements with the addition of NfL,[5] similar to our findings with regard to NfL. However, further studies investigating an optimal panel of biomarkers along with AD risk factors are required.

To date only a handful of studies have investigated longitudinal changes in the aforementioned plasma biomarkers in clinically classified MCI and AD. In the current study, we observed a longitudinal decrease in plasma Aβ1-42/Aβ1-40 ratio and a longitudinal increase in plasma p-tau181 in MCI participants compared with controls; however, no significant longitudinal changes were observed in plasma Aβ1-42/Aβ1-40 ratio and p-tau181 levels in AD participants compared with controls. These findings are consistent with previous CSF and plasma familial AD studies reporting that alterations in Aβ1-42/Aβ1-40 ratios and p-tau181 levels along the disease trajectory ultimately begin to plateau following the first progressive symptom (e.g., memory, motor, or behavior) onset.[2,44] Furthermore, Rodriguez and colleagues show that the trajectory of p-tau181 is associated with the duration of AD status, wherein increases in plasma p-tau181 in AD patients were observed up to 8 to 4 years prior to death, which later plateaued.[45] Given that we do not have data on the duration of AD status for participants in the current study, further studies are required to investigate the trajectory of p-tau181 levels in AD participants from disease onset to death. A previous study reported significant longitudinal increases in GFAP in MCI Aβ+ and MCI who progressed to dementia compared with MCI Aβ− and stable MCI, respectively.[43] Within the current study, we show that GFAP longitudinally increased in MCI and AD compared with controls, and although NfL did not significantly increase longitudinally in MCI, a significant longitudinal increase was observed in AD compared with controls. These findings suggest a sequence in the progression of biomarkers reflecting the underlying pathological process.

In the current study we showed that plasma biomarker levels are associated with prospective cognitive decline. Our observations of the association of baseline plasma biomarker levels with prospective cognitive decline are in line with previous studies, wherein lower baseline plasma Aβ42/40 ratio or Aβ42 levels have been reported to be associated with faster cognitive decline[46,47] and higher baseline plasma p-tau181,[48,49] GFAP[31] and NfL[19,33,48,50] levels have been reported to be associated with faster cognitive decline. Furthermore, observations from the current study extend results from previous findings, wherein the majority of the aforementioned studies report associations in sample sets comprising a mix of CU and CI individuals, and not independently.

Baseline plasma Aβ1-42/Aβ1-40 ratio, p-tau181, and GFAP were also observed to be associated with future brain Aβ accumulation, in line with previous reports. Schindler and colleagues reported a 15-fold greater risk of conversion to Aβ+ in Aβ− cognitively normal individuals with plasma Aβ42/Aβ40 ratio < 0.1218 compared with individuals with plasma Aβ42/Aβ40 ratio > 0.1218.[51] In addition, Shen and colleagues reported that individuals with abnormal baseline plasma p-tau181 levels had a higher risk of progression to pathological brain amyloid load.[52] Furthermore, Pareira and colleagues have reported that plasma GFAP levels predicted Aβ accumulation before and after adjusting for age, sex, baseline Aβ status, presence of cognitive impairment, and tau PET load.[31]

The strengths of the current study include Aβ+ defined classification, the availability of serial plasma measurements to assess longitudinal changes in plasma biomarkers, and the availability of longitudinal data on cognition and brain Aβ-PET load. It is acknowledged that this study also has its limitations. Aβ+ defined classification was not used to assess longitudinal changes in plasma biomarkers as only a modest Aβ-PET sample size with follow-up timepoints was available; however, analyses were adjusted for Aβ−/+ status at baseline. Preliminary, longitudinal changes in plasma biomarkers in groups classified using both clinical and Aβ−/+ status are; however, presented in Table S10, albeit further validation studies are required. In addition, analyses could not include tau-PET−/+ status to assess early or late preclinical AD stages, given that these data were not available for the analyzed sample set. Furthermore, the measurement of Aβ42/Aβ40 using the Simoa platform has been reported to perform inferiorly to immunoprecipitation followed by mass-spectrometry methods or the Elecsys immunoassay with respect to its predictive performance for Aβ−/+ status.[42]

To conclude, results from the current study suggest that plasma biomarkers are altered cross-sectionally and longitudinally, sequentially along the AD continuum, and are associated with prospective cognitive decline and increase in brain A$\beta$-PET load. These findings provide further evidence of the diagnostic and prognostic potential of plasma biomarkers. Findings from the current study have significance and potential implications for (1) clinical trials (e.g., identifying preclinical and prodromal AD participants for clinical trials, and demonstrating superiority of some biomarkers/combinations for this distinction earlier in the AD continuum, compared to NfL) and (2) clinical translation (e.g., earlier, and simpler precision diagnosis of AD). Studies comparing differences in the putative plasma biomarkers between AD and other non-AD neurodegenerative diseases and non-neurodegenerative psychiatric disorders in clinical settings are required. Further in-depth head-to-head comparisons between the putative plasma and CSF AD biomarkers are required; however, Tables S11-S12 and Figure S4 show comparisons and associations of plasma versus CSF A$\beta$42 and p-tau181 pilot data. Future validation studies are required with an emphasis on more ethnically diverse populations.

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## AUTHOR CONTRIBUTIONS

Pratishtha Chatterjee and Ralph N. Martins conceptualised the study. Steve Pedrini measured plasma protein concentrations using the Simoa platform. Pratishtha Chatterjee carried out the statistical analyses, data visualization and interpretation, and James D. Doecke, Abhay K. Singh, and Penghao Wang validated the statistical analyses. Victor L. Villemagne, Vincent Doré, and Christopher C. Rowe provided input on neuroimaging data. Pratishtha Chatterjee wrote the manuscript. All authors critically reviewed the manuscript.

## CONFLICT OF INTERESTS

V.V. is and has been a consultant or paid speaker at sponsored conference sessions for Eli Lilly, Life Molecular Imaging, ACE Barcelona, and IXICO. S.R.S. has received grant support from the National Health and Medical Research Council, Alzheimer's Association (USA) Research Grant, Alzheimer's Drug Discovery Foundation, and the BrightFocus Foundation and honorarium for lectures from the Mature Adults Learning Association Inc. K.T., H.R.S., and R.N.M. are Directors of SMarT Minds Western Australia. H.R.S. has been partially supported by the Australian Alzheimer's Research Foundation, Western Australia. H.R.S. has received reimbursements from Alector and Alnylam Pharmaceuticals. P.M. is a full-time employee of Cogstate Ltd. C.C.R. has received research grants from NHMRC, Enigma Australia, Biogen, Eisai, and Abbvie. He is on the scientific advisory board for Cerveau Technologies and has consulted for Prothena, Eisai, Roche, and Biogen Australia. The other authors did not report any conflict of interest. Author disclosures are available in the supporting information.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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# Chapter 8

## Conclusions

Alzheimer's disease is the leading the leading cause of dementia in the elderly accounting for more than 50% of all dementia cases. Currently there are no cures for it and the few medical treatments approved are aimed at managing symptoms and delaying disease progression. In order to be effective, however, timed medical treatments rely on a quick and definitive diagnosis. It is known that amyloid deposition in the brain happens decades before the onset of clinical symptoms, but the lack of an early diagnosis hampers the possibilities for such early treatments. Unfortunately, current techniques are either (a) expansive or (b) invasive and cannot be used in community-wide screening. Cerebral PET scans for the detection of amyloid deposits in the brain parenchyma are one of the means by which AD is diagnosed, but they are expensive and due to the small amount or radiation used, cannot be repeated too frequently. On the other hand, biomarker analysis in the cerebrospinal fluid (CSF), relies on lumbar punctures, which is an invasive technique that can be performed only by trained personnel. The danger associated with such technique may also induce individuals to stay away from such procedure. Regardless, the absence of a community-wide screening fails to detect the numerous individuals who are in the early stages of the disease or individuals in which brain amyloid deposition has already started but clinical symptoms are yet to appear. All these problems could be solved by unveiling a cheap and non-invasive set of blood biomarkers that can reliably indicate (a) which healthy individuals are more at risk for AD, or (b) which individuals are in the early stages of the disease. In both cases, thanks to this quick, cheap and non-invasive diagnosis, early therapies could be commenced in a timely fashion to either delay the onset of the disease and/or slow its progression. Either way, this would improve the quality of life for affected individuals and would delay the placement of affected individuals in aged-care facilities, strongly reducing the associated costs for families and for government bodies.

The current thesis had access to samples from the AIBL cohort and from the KARVIAH cohort. For both cohort there was access to brain amyloid data assessed by PET scan at several time points and for the AIBL cohort there was also access to regional brain volumetric data assessed by MRI and access to cognitive test scores for the AIBL cohort.

## 8.1 Conclusions 1 (Part 1)

The first part focused on the role of HDL in AD with particular emphasis on the role of HDL maturation and its associated HDL-protein cargo (which comprises all proteins which are associated to HDL and are included/excluded from HDL particles during HDL maturation process). To date, altered HDL levels have been associated to AD, albeit with some contradictory results, but data assessing the implications of HDL-maturation process and HDL-cargo are limited. Such controversies could be in accordance with recent evidence indicating that HDL-cargo may play a prominent role in defying HDL beneficial effects, therefore suggesting that quality over quantity may be a better indicator of HDL protective activity and protective features.

To determine whether HDL particles undergo an altered maturation process in the disease, we have assessed the size of HDL particles (Small, Intermediate and Large) in healthy controls (HC), mild cognitive impaired (MCI) and Alzheimer's patients (AD). Our results indicated that with regards to HDL-profile particles during HDL maturation in AD there are lower levels of the smaller HDL particles compared with the MCI or controls. Small HDL have been associated with HDL protective features such as being protective against atherosclerosis and vascular related oxidative stress [890].Our data have also indicated that higher levels of small HDL are positively associated with a higher MMSE score in MCI/AD, strongly enforcing the notion of a protective role of small HDL in AD.

We have subsequently assessed the composition of HDL-cargo (ApoA-I, ApoA-II, ApoC-I, ApoC-III, ApoD, ApoE, ApoH, ApoJ and CRP) and HDL-cholesterol in stable healthy controls (HC), healthy controls converting to AD within 36 months (HC-Conv) and Alzheimer's patients (AD). Our results indicated that HDL-cargo is altered in AD compared to controls displaying increased relative (to ApoA-I) levels of ApoD and reduced relative (to ApoA-I) levels of ApoA-II. Additionally, our results also indicated that in AD there is an overload of HDL-associated cholesterol, which has already been linked to HDL with reduced anti-atherogenic functions. Such cholesterol overload on HDL appears to begin a few years before the onset of clinical signs of the disease. These early finding could suggest therefore that HDL-associated cholesterol represent an early change in the disease and could be used in a wider blood-based biomarker panel. Additionally, in amyloid positive individuals, cholesterol overload on HDL (relative to ApoA-I) has also been associated to reduced grey matter volume and greater ventricular volume. On the other hand, ApoA-II and ApoJ levels (relative to ApoA-I) have been associated to greater grey matter volume and smaller ventricular volume. Altogether, these data indicated that HDL subclasses and HDL-cargo proteins are altered either before or during AD, and these changes are also associated with brain volumetric AD-related parameters, indicating a strong involvement of HDL in the disease and the possibility that some of the HDL-related parameters could be used for the creation of a broad blood-based biomarker panel for the early diagnosis of the disease.

## 8.2 Conclusions 2 (Part 2)

The second part focused on determining the involvement of specific AD biomarkers (Aβ1-40, Aβ1-42, p-tau181, p-tau231, t-tau, GFAP, NFL) in several clinical cohorts. For this purpose, a cohort with pre-AD individuals (KARVIAH, comprising controls with and without brain amyloidosis) and a cohort with controls, MCI and AD participants (AIBL) were used in these

studies. Overall, higher plasma GFAP, p-tau181 and p-tau231 were observed in the pre-clinical AD group (brain Aβ+ group), just like higher plasma levels of GFAP and p-tau181 were found in Aβ+ participants (CU, MCI and AD) from the AIBL cohort (p-tau231 was not assessed in this study), compared to Aβ- participants. In addition, A decreased Aβ1-42/Aβ1-40 ratio was also consistently observed in Aβ+ participants, suggesting that these biomarkers could potentially be used to discriminate individuals with ongoing brain amyloidosis compared to individuals without brain amyloid deposition. In both cohorts, longitudinal analysis corroborated data obtained at the initial timepoint, suggesting that these specific biomarkers are reliable candidates for the diagnosis of individuals with ongoing brain deposition. On the other hand, NFL levels, also increased in AD, appears to be a marker of generic neurodegeneration and not necessarily associated with brain amyloidosis, limiting their value as potential predictor of AD. However, in the KARVIAH cohort, when analysing the AUCs we found non-significant differences for GFAP, p-tau181 and p-tau231 in differentiating between cognitively unimpaired Aβ+ and cognitively unimpaired Aβ-. Similarly, in the AIBL cohort, Aβ1-42/Aβ1-40 ratio, p-tau181 and GFAP had non-significant differences in predicting performances for preclinical AD. However, in both cohorts, the addition of known AD risk factors (age, sex and *APOE* ε4 status) to the original biomarkers significantly improved the predictive performance for preclinical AD. However, in the AIBL cohort while the predictive performance of Aβ1-42/Aβ1-40 ratio, GFAP and NFL improved with the addition of known AD risk factors, this was not the case for p-tau181. Interestingly, in the KARVIAH cohort we observed a longitudinal increase of GFAP and p-tau181 cognitively unimpaired Aβ+ and in the AIBL cohort a longitudinal increase of GFAP increased in MCI and AD. In the same cohort we also observed a longitudinal decrease in plasma Aβ1-42/Aβ1-40 ratio and a longitudinal increase in plasma p-tau181 in MCI participants. Additionally, NFL did not significantly increase longitudinally in MCI, but it

showed a significant longitudinal increase in AD. Finally, we also reported that Aβ1-42/Aβ1-40 ratio, p-tau181, GFAP levels were associated with cognitive decline Aβ PET load.

These two studies indicate that several plasma biomarkers that are linked to AD are altered cross-sectionally and longitudinally and are associated with increased brain Aβ deposition and cognitive decline. These findings provide further evidence that a selected set of AD-related biomarkers may have diagnostic and prognostic potential. However, further studies are necessary not only to unveil additional plasma biomarkers for a more specific diagnostic panel, but also to determine the relationship between these biomarkers in ethnically different populations.

# Appendix

# Appendix A – Contribution to Chapter 6

To Whom It May Concern,

I, Steve Pedrini, contributed by assessing the levels of GFAP, t-tau and NFL (along with p-Tau181 in a small subset of samples) using SIMOA kits and participated in editing the final version of the manuscript entitled **Diagnostic and prognostic plasma biomarkers for preclinical Alzheimer's disease.**

[redacted]

I, Prof. Ralph Martins, as the supervisor and corresponding author of the manuscript, endorse that this level of contribution by the Candidate indicated above is appropriate.

[redacted]

# Appendix B – Contribution to Chapter 7

To Whom It May Concern,

I, Steve Pedrini, contributed by assessing the levels of GFAP, NFL, Aβ1-40, Aβ1-42 and p-Tau181 using SIMOA kits and participated in editing the final version of the manuscript entitled **Plasma biomarkers in preclinical, prodromal and clinical AD: A cross-sectional and longitudinal study in the AIBL cohort**.

I, Prof. Ralph Martins, as the supervisor and corresponding author of the manuscript, endorse that this level of contribution by the Candidate indicated above is appropriate.

# Appendix C –Chapter 2 – PDF

Appendix C is not available in this version of the thesis

# Appendix D –Chapter 3 - PDF

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# Appendix E –Chapter 4 – PDF

Appendix E is not available in this version of the thesis

# Appendix F –Chapter 5 – PDF

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ORIGINAL ARTICLE

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# Plasma high-density lipoprotein cargo is altered in Alzheimer's disease and is associated with regional brain volume

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## Abstract

Cholesterol levels have been repeatedly linked to Alzheimer's Disease (AD), suggesting that high levels could be detrimental, but this effect is likely attributed to Low-Density Lipoprotein (LDL) cholesterol. On the other hand, High-Density Lipoproteins (HDL) cholesterol levels have been associated with reduced brain amyloidosis and improved cognitive function. However, recent findings have suggested that HDL-functionality, which depends upon the HDL-cargo proteins associated with HDL, rather than HDL levels, appears to be the key factor, suggesting a quality over quantity status. In this

Abbreviations: AD, Alzheimer's Disease; Aβ, amyloid-β; BBB, blood-brain barrier; CAA, cerebral amyloid angiopathy; CRP, C-reactive protein; CSF, cerebrospinal fluid; GM, grey matter; HC, healthy control; HC-Conv, healthy control converter; HDL, high-density lipoprotein; HL, hippocampus left; HR, hippocampus right; LD, lipid droplets; LDL, low-density lipoprotein; LTP, long-term potentiation; MMSE, mini-mental state examination; SAA, serum amyloid A; SUV, standardized uptake values; SUVR, standardized uptake value ratio; VLDL, very low-density lipoproteins; WM, white matter.

# Appendix G –Chapter 6 – PDF

Appendix G is not available in this version of the thesis

# Appendix H –Chapter 7 – PDF

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FEATURED ARTICLE

## Plasma Aβ42/40 ratio, p-tau181, GFAP, and NfL across the Alzheimer's disease continuum: A cross-sectional and longitudinal study in the AIBL cohort

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**Abstract**

**Introduction:** Plasma amyloid beta (Aβ1-42/Aβ1-40 ratio, phosphorylated-tau181 (p-tau181), glial fibrillary acidic protein (GFAP), and neurofilament light (NfL) are putative blood biomarkers for Alzheimer's disease (AD). However, head-to-head

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