University College London

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Blood and cerebrospinal fluid biomarkers for Alzheimer's disease: from clinical to preclinical cohorts

A thesis submitted for the Degree of Doctor of Philosophy

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I, Ashvini Keshavan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Dementia is a major contributor to global morbidity, mortality and costs associated with health and social care. Alzheimer's disease (AD) is a common pathology culminating in dementia, but it has a preclinical phase of one to two decades, with early brain deposition of amyloid and tau, followed by synaptic and neuronal degeneration. Early detection during the preclinical phase of AD might enable disease-modifying therapies to be applied during a window of opportunity in which they would be more likely to work. Currently the main biomarkers of AD pathology are neuroimaging markers, which can be costly, or cerebrospinal fluid markers, which require invasive sampling. Blood biomarkers would be relatively less invasive and could be a more cost-effective means for risk stratification, early detection, monitoring progression and measuring response to treatment.

The work described here used sensitive assay technology including the Simoa digital immunoassay platform, in large and well-characterised cohorts, to examine candidate blood biomarkers linked to the core AD pathologies of amyloid, tau and neurodegeneration, as specified by the National Institute on Aging and Alzheimer's Association 2018 research framework. Firstly, experiments on samples from a cognitive clinic cohort established the stability of the blood biomarkers Aβ40, Aβ42, total tau and neurofilament light chain (NFL – a marker of neurodegeneration) to multiple freeze-thaw cycles, and the optimal blood fraction to use for quantifying each of these biomarkers in onward studies.

Secondly, an unique large preclinical cohort with life course data (Insight 46, the neuroscience sub-study of 502 individuals from the MRC National Survey of Health and Development; the 1946 British birth cohort) was used to examine the cross-sectional relationships between these blood biomarkers, neuroimaging biomarkers (^{18}F-florbetapir amyloid PET, whole brain and hippocampal volumes, white matter hyperintensity volume and cortical thickness in an AD signature region) and cognitive performance (PACC: preclinical Alzheimer's composite and its constituents). Through a collaboration with the University of Gothenburg, a novel liquid chromatography-mass spectrometry (LC-MS) method for quantification of plasma amyloid-β species was compared with the commercial Simoa assays in Insight 46. This was the first direct method comparison study of plasma amyloid-β species for the detection of preclinical cerebral amyloid deposition. It showed that the LC-MS method, when combined with age, sex and *APOE* ε-4 carrier status, was able to distinguish PET amyloid status with an optimal (Youden's cut point) sensitivity of 85.7% and specificity of 72.7%. The Simoa biomarkers of plasma total tau and serum NFL were confirmed to be potentially useful prognostic markers, as lower AD signature cortical thickness was associated with higher plasma total tau and serum NFL, lower whole brain volume was associated with higher plasma total tau, and higher ventricular volume was associated with higher serum NFL. Lower PACC scores were associated with higher serum NFL and lower scores for a paired associative memory test in particular were associated with higher plasma total tau and serum NFL.

Thirdly, through a collaboration with Harvard University and the University of California San Diego, a new N-terminal tau biomarker was developed in CSF

and plasma that showed good accuracy in distinguishing individuals with symptomatic CSF-defined AD pathology from healthy controls.

Taken together, this work has demonstrated the impact of pre-analytical factors on measurements of AD blood biomarkers, validated these biomarkers as indicators of the core pathologies of AD and helped to develop a new tau blood biomarker in AD.

Impact statement

The work presented in this thesis has several potential impacts on research and application to clinical contexts.

The examination of pre-analytical variables - both modifiable, such as freeze-thaw cycling and blood fraction choice, and participant-specific, such as sex, renal function and body mass index – has provided important information on potential confounds for variation in blood biomarkers, which must be controlled or accounted for in future studies, especially those examining longitudinal changes.

The study of plasma amyloid-β peptides highlights the differences between assay methods and their ability to predict cerebral PET amyloid status. It demonstrates the potential of the liquid-chromatography-mass spectrometry assay to exceed the predictive ability of *APOE* genotype and the commercially available Simoa assays as pre-screeners to amyloid PET scan in recruitment to therapeutic trials. The studies of plasma total tau and serum NFL provide evidence in a large cohort of cognitively normal individuals of the cross-sectional associations of these biomarkers with neuroimaging biomarkers of neurodegeneration and subtle cognitive performance indicators. This sets the stage for studies, both in Insight 46 and other cohorts, that will contribute to evidence for the ability of these blood biomarkers in the preclinical phase of AD to predict longitudinal brain atrophy and cognitive decline.

The development of the novel plasma N-terminal tau assay raises an exciting prospect of a minimally invasive tau biomarker that may enable pre-screening

for asymptomatic tau pathology, if validated in preclinical cohorts such as Insight 46.

All of the work detailed in this thesis has either been published in peer-reviewed journals or presented at national and international AD conferences, enabling wide dissemination of the findings. It has strengthened existing international collaborations and fostered new ones. The most direct application of this work is likely to be its contribution toward potential screening tests for AD pathology in asymptomatic individuals. Clinical trials may benefit from utilising blood biomarkers for screening and for monitoring, and if any of the trialled therapeutic agents achieves disease-modifying effects, the blood biomarkers described here may translate from research into clinical practice.

Acknowledgements

I thank all the study participants across the cohorts, whose previous and ongoing contributions have made this work possible.

The large team of people contributing to this research, either through collaboration, administration or education, is detailed in the “Statement of Contribution” (Chapter 11, page 323), and I am thankful to all of them.

I am deeply grateful to my supervisors Jonathan Schott, Henrik Zetterberg and Amanda Heslegrave, who have not only guided my learning with their sound advice, but enthusiastically supported my every endeavour and fostered a collegiate culture of which I am proud to be a part.

For their love and unwavering support, I am thankful to Kapil and my family.

Glossary of abbreviations

a.a.	Amino acid residue
Aβ1-42	Amyloid-β 1-42, as measured by mass spectrometry techniques
Aβ1-40	Amyloid-β 1-40, as measured by mass spectrometry techniques
Aβ42	Amyloid-β x-42, as measured by Simoa techniques
Aβ40	Amyloid-β x-40, as measured by Simoa techniques
Aβo	Amyloid-β oligomers
AC	Affinity chromatography
AD	Alzheimer's disease
ADAS-Cog	Alzheimer's disease assessment scale – cognitive
ADC	Amsterdam Dementia Cohort
ADNI	Alzheimer's Disease Neuroimaging Initiative
AGES-RS	Age Gene/Environmental Susceptibility – Reykjavik Study
AIBL	Australian Imaging Biomarker and Lifestyle study
AIC	Akaike Information Criterion
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ART	Alzheimer Research Trust
AT(N)	Amyloid, tau and neurodegeneration biomarker classification system
AUC	Area under the receiver operating characteristics curve
BIOFINDER	Swedish study of Biomarkers for Identifying Neurodegeneration Early and Reliably
BLSA	Baltimore Longitudinal Study of Aging
BMI	Body mass index
BMP	Bone morphogenetic protein
BNP	Brain natriuretic peptide

BSHRI	Banner Sun Health Research Institute
CA1	Cornu ammonis 1 region of the hippocampus
CDR	Clinical Dementia Rating scale
CEA	Carcinoembryonic antigen
CERAD	Consortium to Establish a Registry for Alzheimer's Disease
CJD	Creutzfeldt-Jakob disease
CNS	Central nervous system
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CT	Computed tomography
CTh	Alzheimer’s disease signature region of interest cortical thickness
CV	Coefficient of variation
DELCODE	German Center for Neurodegenerative Diseases longitudinal study on cognition and dementia
DIAN	Dominantly Inherited Alzheimer’s Network
DLB	Dementia with Lewy Bodies
DRC	Dementia Research Centre at University College London
DSS	Digit symbol substitution
DWI	Diffusion weighted imaging
ECL	Electrochemiluminescence
EDTA	Ethylenediamenetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMIF	European Medical Information Framework
ES	Erlangen Score for classification of probability of Alzheimer’s pathology based on cerebrospinal fluid profile

ESTHER	German (Saarland) epidemiological study on chances of prevention, early detection and optimized treatment of chronic diseases in the elderly population
EYO	Estimated year of onset
FAD	Familial Alzheimer's Disease
FCN2	Ficolin-2
FDG-PET	18-F fluorodeoxyglucose positron emission tomography
FHS	Framingham Heart Study
FLAIR	Fluid attenuated inversion recovery magnetic resonance imaging
fMRI	Functional magnetic resonance imaging
FNAME-12	12-item face-name associative memory examination
FTLD	Frontotemporal lobar degeneration
FTD	Frontotemporal dementia
GDF	Growth differentiation factor
GE-067-005	Study for investigation of ^{18}F-flutemetamol in prediction of conversion of amnestic mild cognitive impairment to probable Alzheimer's disease dementia
HABS	Harvard Aging Brain Study
HR	Hazard ratio
HV	Hippocampal volume
IGFBP2	Insulin-like growth factor binding protein 2
IL-17	Interleukin-17
IMR	Immunomagnetic reduction
Insight 46	Neuroscience sub-study of the MRC National Survey of Health and Development (the 1946 British Birth Cohort)
INSIGHT-preAD	Investigation of Alzheimer's predictors in Subjective Memory Complainers
IP	Immunoprecipitation
KARVIAH	Kerr Anglican Retirement Village Initiative in Ageing Health
KBASE	Korean Brain Aging Study for the Early Diagnosis and Prediction of Alzheimer's disease

LATE-NC	Limbic-predominant age-related transactive response DNA binding protein 43 encephalopathy neuropathologic change
LC	Liquid chromatography
LMD	Logical memory delayed
LonDOWNS	London Down Syndrome Consortium
MALDI-TOF	Matrix-assisted laser desorption ionization–time of flight
MAPT	Microtubule associated protein tau
MaR	Matrix reasoning
MC	Mutation carrier (e.g. of an autosomal dominant Alzheimer's disease mutation)
MCSA	Mayo Clinic Study of Aging
MCI	Mild cognitive impairment
MIP-1δ	Macrophage inflammatory protein-1δ
MMSE	Mini Mental State Examination
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MSD	Mesoscale Discovery
NC	Non-carrier of a mutation (e.g. in autosomal dominant Alzheimer's disease)
NCGG	Japanese National Center for Geriatrics and Gerontology
NFL	Neurofilament light chain
NFT	Neurofibrillary tangle
Ng	Neurogranin
NIA-AA	National Institute on Aging and Alzheimer's Association
NICE	National Institute for Health and Care Excellence
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and the Alzheimer's Disease and Related Disorders Association
NSHD	MRC National Survey of Health and Development (the 1946 British Birth Cohort)

NTUH	National Taiwan University Hospital
OR	Odds ratio
PACC	Preclinical Alzheimer's cognitive composite
PDGF-BB	Platelet derived growth factor BB
PDGFRβ	Platelet derived growth factor receptor-β
Penn	University of Pennsylvania
PET	Positron emission tomography
PI	Principal Investigator
PiB	11-C Pittsburgh-B compound amyloid tracer
PPY	Pancreatic polypeptide or prohormone
PSEN1	Presenilin 1 gene
PSEN2	Presenilin 2 gene
PSP	Progressive supranuclear palsy
p-tau	Phosphorylated tau (position not specified)
p-tau-181	Tau phosphorylated at position 181
PVC	Partial volume correction
ROC	Receiver operating characteristics
sAD	Sporadic Alzheimer's disease
SCD	Subjective cognitive decline
SCIENCe	Dutch Subjective Cognitive Impairment cohort
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Simoa	Single molecular array
SOP	Standard operating protocol
SPECT	Single positron emission computed tomography
SST	Serum separator tube

sTREM2	Soluble triggering receptor expressed on myeloid cells 2
SUVR	Standardised uptake value ratio
SWI	Susceptibility weighted imaging
T1	MRI spin-lattice or longitudinal relaxation time
T2	MRI spin-spin or transverse relaxation time
TDP-43	Transactive response DNA binding protein 43
TGFβ	Transforming growth factor-β
TIV	Total intracranial volume
TREM2	Transmembrane receptor expressed on myeloid cells 2
t-tau	“Total” tau, as quantified usually by mid-region-directed assays
UCSD	University of California, San Diego
UCL	University College London
UKDRI	United Kingdom Dementia Research Insititute
VaD	Vascular dementia
VCAM	Vascular cell adhesion molecule
VIF	Variance Inflation Factor
VV	Ventricular volume
WashU	Knight Alzheimer’s Disease Research Center at Washington University School of Medicine
WB	Western blot
WBV	Whole brain volume
WMHV	White matter hyperintensity volume

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1 Introduction

1.1 Publication statement

The contents of this chapter from section 1.6 onward have been published previously in an abridged format [1] but were updated extensively for inclusion here. A copyright licence was obtained to reproduce the published work (Springer Nature licence: 4576580755724). All other figures, tables or data have been reproduced under a Creative Commons Licence (http://creativecommons.org/licenses/by/4.0/), or licences obtained from individual publishers as specified.

1.2 Dementia as an important global problem

Dementia is defined in the International Classification of Diseases (ICD-10, [2]) as a “syndrome due to disease of the brain, usually of a chronic or progressive nature, in which there is disturbance of multiple higher cortical functions, including memory, thinking, orientation, comprehension, calculation, learning capacity, language, and judgement”, without clouding of consciousness.

The World Alzheimer Report 2015 [3] detailed that the global prevalence of dementia was estimated to be 46.8 million people over the age of 60 years, which ranges from 4.6% of this population sector in Europe, to 8.7% in North Africa and the Middle East. Projections for the number of people who will be living with dementia are 74.7 million in 2030 and 131.5 million in 2050, with greater increases in prevalence expected in low- and middle-income countries (Figure 1.1, page 38).

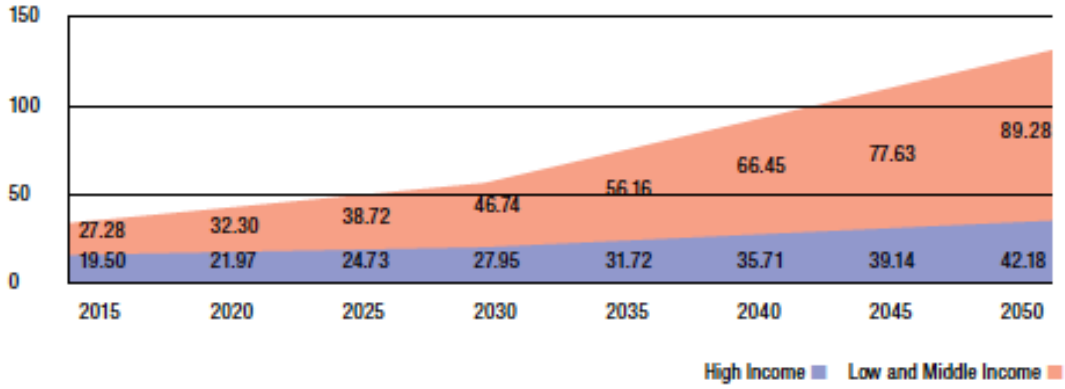


Figure 1.1: Estimates of growth in numbers of people living with dementia (millions) from 2015 to 2050.
Reproduced from the World Alzheimer Report 2015 [3], Alzheimer's Disease International, as per its permissions policy.

Despite the potential for reducing dementia incidence in developed countries with time, which would continue a trend that has been observed in the last 10-20 years from studies in the United Kingdom, France, the Netherlands and the Unites States of America [4], this increase in dementia prevalence is expected due to a combination of increasing dementia incidence with age (Figure 1.2), and global population ageing.

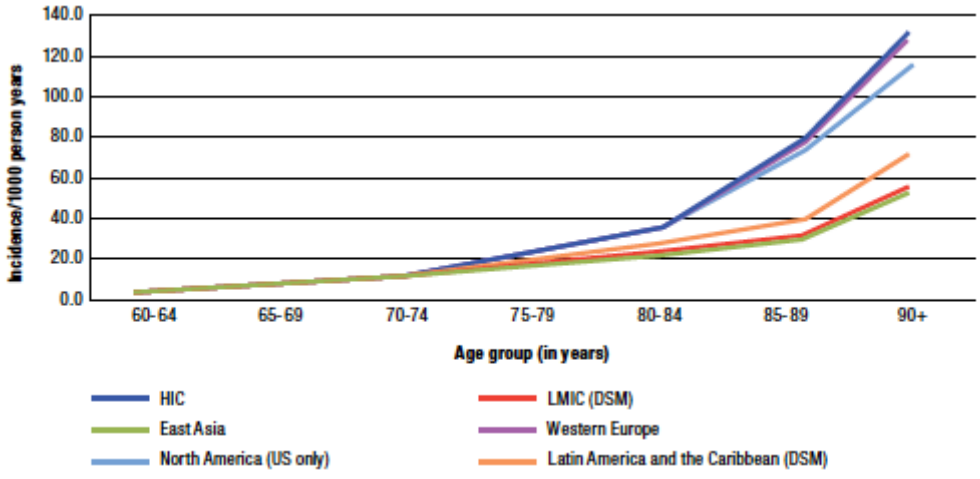


Figure 1.2: Estimated age-specific annual incidence of dementia by world region, where meta-analysis data are available.
Reproduced from the World Alzheimer Report 2015 [3], Alzheimer's Disease International, as per its permissions policy.
DSM, diagnosed by the Diagnostic and Statistical Manual-IV criteria; HIC, High-income countries; LMIC, low- to middle-income countries.

The increasing financial burden of disease is also notable. From 2010 to 2015, the total global cost of dementia per year was estimated to rise from 604 to 818 billion US dollars, with *per capita* costs increasing by 7% in low-income countries and up to 14% in high-income countries over this period [3].

1.3 **Heterogeneity of dementia syndromes**

Dementia is often considered as a unified entity for the purpose of epidemiology, particularly on the global scale, where data on incidence and prevalence of specific diagnoses are not always available. Even in the UK, only about 540 000 of the 850 000 individuals living with dementia (that is, about 66%) have a diagnosis given by a general practitioner or a memory service, as recorded in the National Health Service digital records of the four nations over 2017/2018 [5]. However, specific diagnosis of the underlying cause is important, as the varied clinical syndromes that come under the umbrella term of dementia have distinct evolving clinical features and prognoses, and are linked to distinct pathophysiological processes. Receipt of an accurate diagnosis of dementia sub-type also allows patients to access information and support that is more relevant to their individual conditions.

A brief non-exhaustive list of some of the primary neurodegenerative dementias is included below:

- *Alzheimer's disease (AD)*

  AD was first described in 1907 by Alois Alzheimer [6] (translated in [7]) as a case report of a 51 year old woman whose first clinical features included jealousy and profound episodic and topographical memory deficits, followed by disorientation and delusions, but with initially normal

motor function. The term AD has since been extended to include familial and sporadic forms with both typical (amnestic) and atypical presentations. Sporadic AD is the commonest dementia diagnosis in the UK (accounting for about two thirds of dementia diagnoses: see Figure 1.3, page 42). The current neuropathological criteria are detailed in section 1.4 (page 43) and clinical criteria in section 1.6 (page 52).

- *Vascular dementia (VaD)*

  VaD is characterized by brain infarction due to vascular disease, including hypertensive cerebrovascular disease. The infarcts are usually small but cumulative in their effect, which can include focal neurological symptoms and signs, early gait and/or urinary dysfunction, and subcortical features such as global slowing of thinking and motor function, as detailed in the consensus research criteria [8]. The term VaD has been superseded in more recent literature by vascular cognitive impairment and dementia (VCI and VCID) [9] but is retained here in view of the use of VaD in the most recent UK prevalence estimates.

- *Frontotemporal dementia (FTD)*

  FTD involves early progressive changes of behaviour and social deterioration, which may be followed by memory and/or language impairment and may be associated in some cases with extrapyramidal features or motor neuron disease. The current diagnostic criteria for the behavioural variant (bvFTD) were published by Rascovsky *et al.* [10] and for the language variants of semantic (svPPA) and non-fluent variant (nfvPPA) primary progressive aphasia by Gorno-Tempini *et al*. [11]. Core

proteins involved in the pathophysiology of FTD spectrum conditions include transactive response deoxyribonucleic acid binding protein 43 (TDP-43), tau and fused in sarcoma (FUS).

- *Dementia with Lewy Bodies (DLB) and Parkinson's disease dementia (PDD)*

  The core clinical features of DLB are cognitive fluctuations, visual hallucinations and rapid eye movement sleep behaviour disorder as detailed by McKeith and colleagues [12]. PDD is a closely related condition, in which the motor symptoms of Parkinson's disease precede cognitive changes by at least a year. Both conditions share alpha-synuclein-containing Lewy bodies as a key neuropathological feature.

- Sporadic Creutzfeldt-Jakob disease (CJD)

  CJD is a prion disease characterised by rapid progression, usually over weeks and months, of neuropsychiatric symptoms, myoclonus, visual or cerebellar signs, pyramidal/extrapyramidal signs or akinetic mutism [13]. Sporadic and inherited versions of this condition involve propagation through the brain of aggregates of the prion protein (PrP).

The relative prevalence in the UK of the commonest dementia syndromes is illustrated in Figure 1.3, page 42.

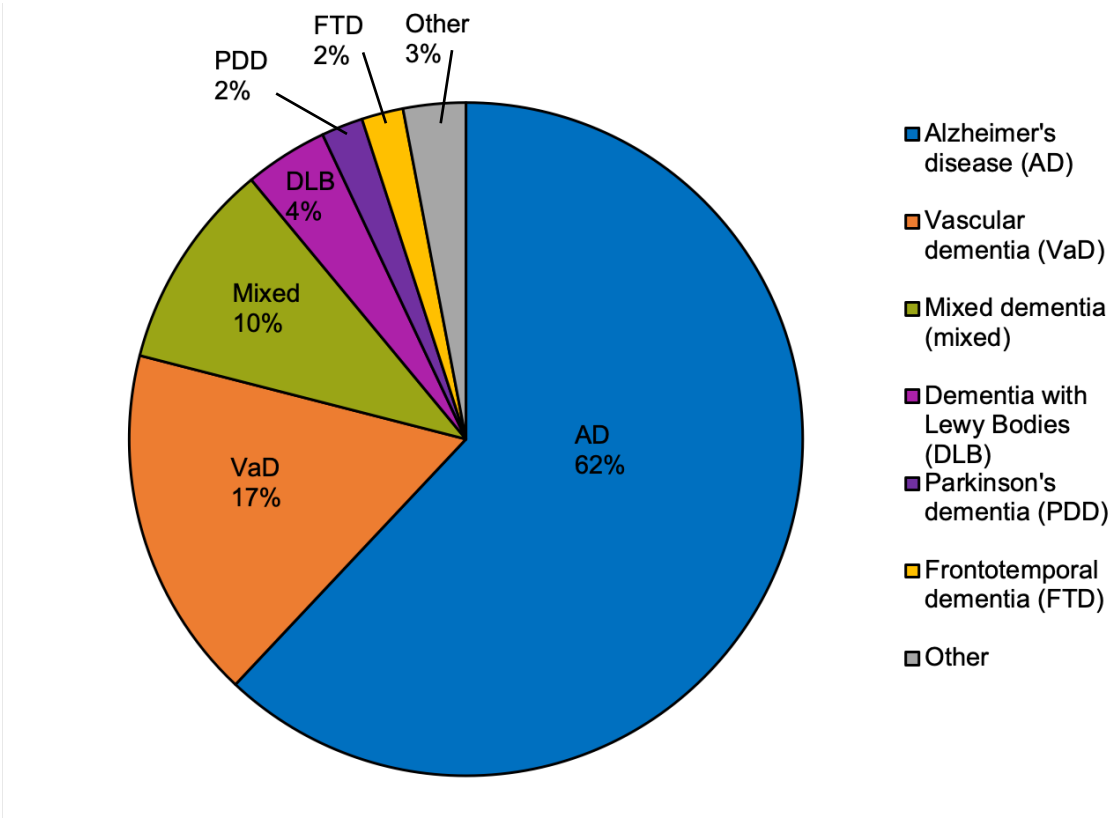


Figure 1.3: Dementia diagnoses in the UK.
Data from the Alzheimer's Society 2014 report [14].

1.4 **Alzheimer's disease: pathology underlying a common dementia**

While most dementia syndromes are clinically defined, the brain diseases underlying them have been extensively described in *post mortem* studies of the human brain. Alois Alzheimer's initial case report included a neuropathological examination revealing extracellular "minute military foci caused by the deposition of a special substance in the cortex", intracellular neurofibrils and tangles, and disappearance of many neurons [6, 7]. The National Institute on Aging–Alzheimer's Association (NIA-AA) 2012 guidelines for diagnosis of AD neuropathologic change [15] continue to incorporate these three core features, which are now known to be:

- extracellular senile plaques (of which a principal component is the protein amyloid-β: Aβ),
- neurofibrillary pathology (neuropil threads and neurofibrillary tangles, of which a main component is hyperphosphorylated tau protein) and
- neurodegeneration, which is associated with neuritic plaques

These core features form the basis of the NIA-AA 2012 neuropathological staging criteria (the "ABC" scoring system). In addition, the histopathology of AD includes astrogliosis, microglial activation and cerebral amyloid angiopathy.

1.4.1 Aβ and senile plaques

Aβ peptides are hydrophobic proteolytic cleavage products derived from the amyloid precursor protein (APP), which is encoded on human chromosome 21q21. APP is a 115-130 kDa integral membrane protein (Figure 1.4A, page 45) that is conserved across mammalian species [16] and expressed at medium levels in cortical, hippocampal , caudate and cerebellar neurons, and lower

levels in hippocampal and caudate glia, and glandular cells of the gastrointestinal and reproductive tracts [17, 18].

APP has three main alternative splicing isoforms in human neurons (APP_{695}, APP_{751} and APP_{770}). Canonical pathways of APP processing in cells involve cleavage by either α-secretase (initiating a non-amyloidogenic pathway) or β-secretase (initiating an amyloidogenic pathway), as shown in Figure 1.4B (page 45). Non-canonical pathways initiated by other enzymes (such as δ-secretase, η-secretase, meprin-β and caspases) may also occur, but the physiological relevance of these pathways and their products, apart from Aβ, is less well-known [19]. After the initial cleavage at the extracellular or transmembrane domain, all amyloidogenic pathways require the action of the γ-secretase complex at the transmembrane domain (Figure 1.4C, page 45). γ-secretase is a membrane-associated complex consisting of presenilin 1 or 2, presenilin enhancer 2 (PEN2), nicastrin and anterior pharynx defective (APH-1). It performs sequential cleavages, generating the APP intracellular domain (AICD) and the Aβ peptides. Extracellular Aβ monomers may aggregate into dimers, oligomers, protofibrils, fibrils and eventually network-like structures, forming plaques. While the major cleavage products in the amyloidogenic pathway include Aβ40, one of the minor cleavage products, Aβ42, forms the principal Aβ constituent of senile plaques [20]. Aβ42 has been demonstrated to be more aggregation-prone than Aβ40 *in vitro* and is able to seed the aggregation of other Aβ peptides [21]. Conversely, Aβ40 is the principal Aβ constituent of cerebrospinal fluid (CSF) [22], with CSF Aβ40 concentrations measured at around 100 times the CSF Aβ42 concentrations within the same individuals [23].

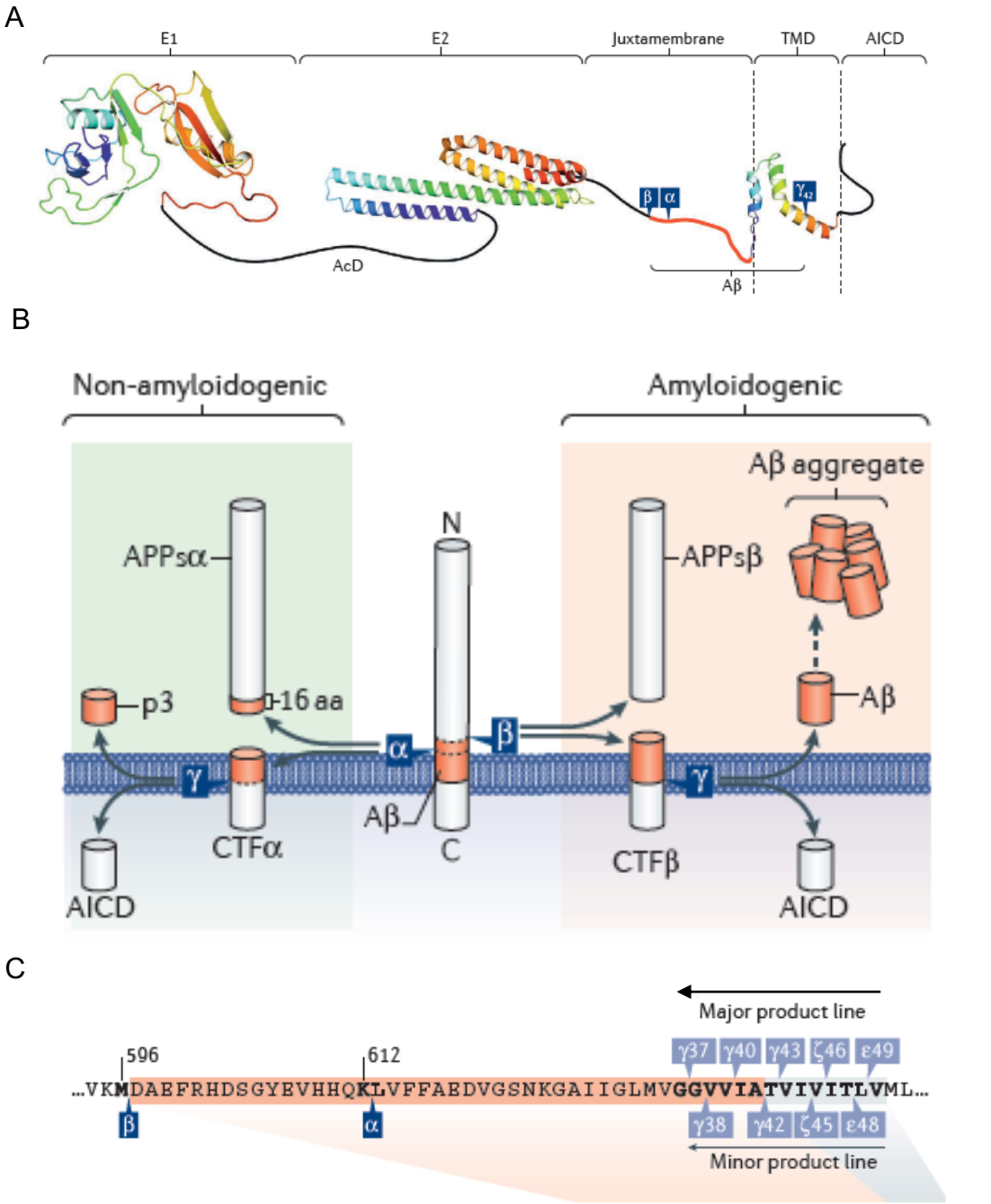


Figure 1.4: Amyloid precursor protein (APP) structure and processing.
A: Structure of APP_{695}, showing the extracellular domains (E1 and E2), bridged by the acidic domain (AcD); the juxtamembrane domain in which α- and β-secretase-mediated cleavage occurs; the transmembrane domain (TMD) in which γ-secretase-mediated cleavage occurs to yield the Aβ peptides, and the amyloid precursor protein intracellular domain (AICD).
B: Canonical pathways of APP processing. The non-amyloidogenic pathway is initiated by α-secretase and yields APPsα, p3 and AICD after γ-cleavage. The amyloidogenic pathway Is initiated by β-secretase and yields APPsβ, Aβ and AICD after γ-cleavage.
C: Amino acid sequence of the Aβ sequence-containing region of APP, with the target sites of α-, β- and sequential γ-cleavage indicated. The location of the initial γ-cleavage site (the ε-cleavage site) determines the peptides generated by sequential cleavage, as γ-secretase catalyses cleavage at every third amino acid. The major Aβ products including Aβ40 are generated after cleavage at ε49, and the minor products including Aβ42 are generated after cleavage at ε48.
Reproduced from Müller *et al*. [19] with permission (Springer Nature licence: 4577190672861).

The NIA-AA 2012 criteria build upon methods for identifying senile plaques by Aβ immunohistochemistry (Figure 1.5A), described by Thal and colleagues in 2002 [24] as a hierarchical sequence of Aβ deposition in the entire brain, with five phases depicted in Figure 1.5B. These phases have been consolidated in the current staging criteria to form four “A” scores (Table 1.1, page 47).

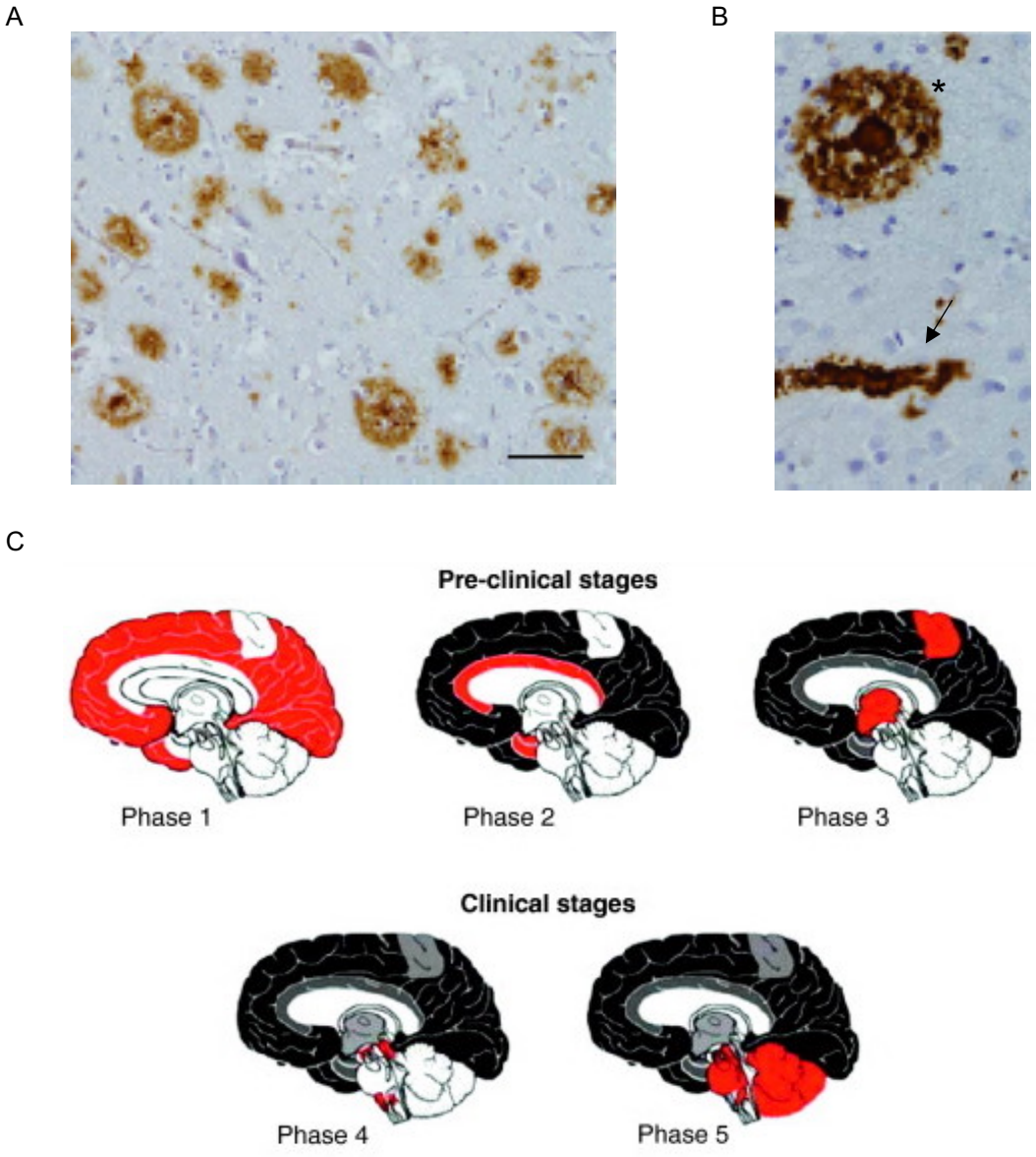


Figure 1.5: Aβ pathology in AD.
Aβ immunohistochemistry shows senile plaques in *post mortem* brain tissue from the frontal cortex of a patient with sporadic AD (A and B*); capillary deposition of Aβ also indicates cerebral amyloid angiopathy in B (arrow). The bar represents 50 µm in A and 25 µm in B. Reproduced from Lane *et al.* [25] with permission.
C: Phases of Aβ deposition, as depicted in Thal *et al*. [26] with newly involved areas for each phase marked in red; reproduced with permission.

Table 1.1: Staging of Aβ-containing plaques in the NIA-AA 2012 criteria for diagnosis of AD neuropathologic change, with the corresponding Thal phases.

Aβ plaque score [15]	Thal phase [24]	Brain region(s) involved newly at each phase
A0	-	None
A1	1	Neocortex
	2	Allocortex
A2	3	Diencephalic nuclei, striatum, cholinergic nuclei of basal forebrain
A3	4	Brainstem nuclei
	5	Cerebellum

1.4.2 Tau pathology

The microtubule-associated protein tau is encoded by the *MAPT* gene on human chromosome 17q21. It has a very wide tissue expression in central and peripheral neurons, muscle, kidney and breast, and is expressed at lower levels in many epithelial and glandular cells [17, 27]. Tau in neurons has several physiological roles including assembly and stabilization of microtubules, axonal transport and potentially maintaining the integrity of genomic DNA. Tau undergoes extensive splicing that is regulated both developmentally and regionally in the human brain; all six isoforms are expressed in adult neurons (Figure 1.6).

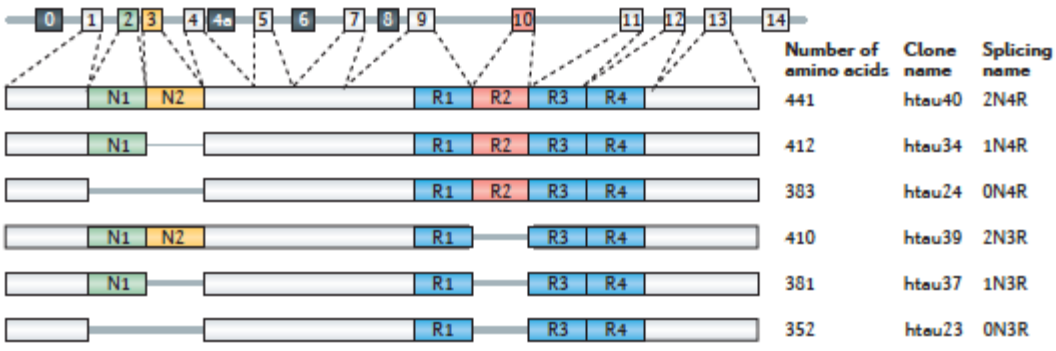
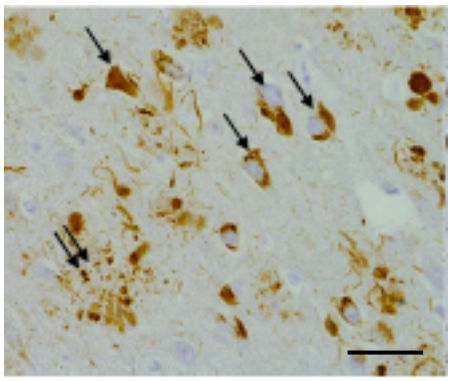


Figure 1.6: Human tau isoforms.
Reproduced from Wang and Mandelkow [28] with permission (Springer licence: 4577680685488).

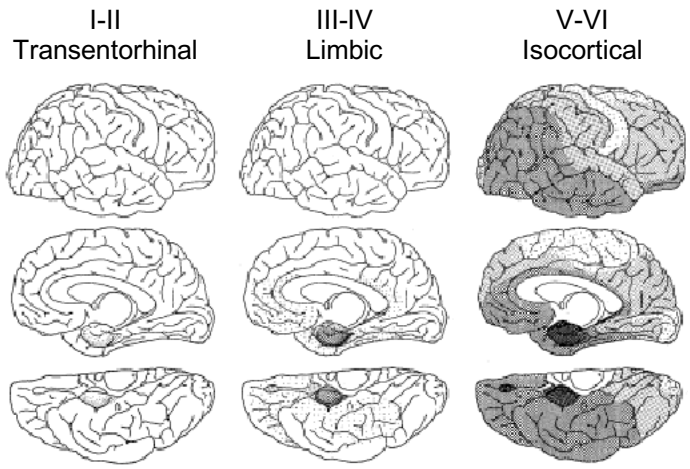
In addition, tau may undergo extensive post-translational modifications, including phosphorylation (at up to 85 potential sites), glycosylation, and others

(reviewed in [28]), which depend on whether tau is in its native unfolded state or folded and aggregated into paired helical filaments (PHF). Hyperphosphorylated PHF comprised of both 4R and 3R tau are principal components of the intraneuronal neurofibrillary tangles (NFT - Figure 1.7A) and neuropil threads of AD. The Braak staging of regional tau pathology distribution [29] forms the “B” score of the NIA-AA 2012 criteria (Figure 1.7B and C).

A



B



C

Braak stage	Neurofibrillary tangle “B” score	Brain region(s) involved newly at each stage
-	0	None
I	1	Transentorhinal
II		Hippocampus CA1
III	2	Entorhinal and subiculum
IV		Amygdala, claustrum and thalamus
V	3	Isocortex (neocortex)
VI		Striatum

Figure 1.7: Tau pathology in AD
A: AT8 immunohistochemistry demonstrating phosphorylated tau in neurofibrillary tangles (single arrows). A neuritic plaque (double arrow) is also shown. The bar represents 50 μm. Reproduced from Lane *et al*. [25] with permission.
B: The six Braak stages of neurofibrillary pathology in AD. Reproduced from Braak and Braak [29] with permission (Springer licence: 4577750089579).
C: Correspondence of Braak stages with the “B” score according to the NIA-AA 2012 criteria [15].

1.4.3 Neurodegeneration and neuritic plaques

Neuritic plaques (Figure 1.7A, double arrow – page 48) display dystrophic neurites and are considered to be the Aβ plaque type that is most closely associated with neuronal injury. Their presence is associated with glial activation and local synapse loss. The "C" score of the NIA-AA 2012 criteria is derived from the scoring system for neocortical neuritic plaque density formalised by the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [30]. Integrating the A, B and C scores gives four possible levels of AD neuropathic change (Table 1.2).

Table 1.2: Levels of AD neuropathic change according to the NIA-AA 2012 criteria. Intermediate or high AD neuropathic change is considered a sufficient explanation for a clinically manifest dementia syndrome.
Reproduced with minor adaptation from [15], with permission (Elsevier licence: 4578730932021).

A: Aβ/amyloid plaque score	C: Neuritic plaque score (CERAD)	B: Neurofibrillary tangle score		
		B0 or B1	B2	B3
A0	C0	Not	Not	Not
A1	C0 or C1 (none to sparse)	Low	Low	Low
	C2 or C3 (moderate to frequent)	Low	Intermediate	Intermediate
A2	Any C	Low	Intermediate	Intermediate
A3	C0 or C1 (none to sparse)	Low	Intermediate	Intermediate
	C2 or C3 (moderate to frequent)	Low	Intermediate	High

1.5 **Pathophysiology of AD**

First proposed in the early 1990's, the amyloid cascade hypothesis [31-33] continues to dominate views of the pathophysiology of AD. However, the hypothesis has evolved as anti-amyloid treatment trials have thus far failed to meet their primary endpoints in altering the course of AD dementia, and as knowledge has advanced through the study of model systems including animal and human cellular models [34]. The sequence of events remains a source of

considerable debate, but Aβ retains a unifying role in both familial AD, in which all pathogenic mutations lead to enhanced production of Aβ, and in sporadic AD, in which clearance of Aβ is likely to be impaired. Aggregation of Aβ occurs when there is an imbalance between production and clearance; it is likely that the toxic forms of Aβ are oligomers rather than plaques. These Aβ oligomers (Aβo) may have synergistic toxic effects with tau already present at neuronal synapses, and through downstream activation of kinases may be involved in pathological tau phosphorylation, but may also be involved in tau-independent mechanisms of excitotoxicity through increasing post-synaptic calcium concentrations (reviewed by Spires-Jones and Hyman [35]).

From observations of gliosis around Aβ plaques, non-neuronal cells have also long been known to be involved in AD, but much is as yet unknown about the extent to which inflammatory responses by CNS macrophages and microglia occur downstream of neural excitotoxicity or compound it. Astrocytes are likely to be involved in Aβ plaque breakdown, which may itself release toxic Aβo. Astrocytes may also have a role in regulating the intramural periarterial drainage of solutes such as Aβ from brain interstitial fluid. Figure 1.8 (page 51) shows a schematic view of some of the mechanisms by which Aβ, tau and different cell types may bring about the synaptopathy and neuronal loss that characterise AD.

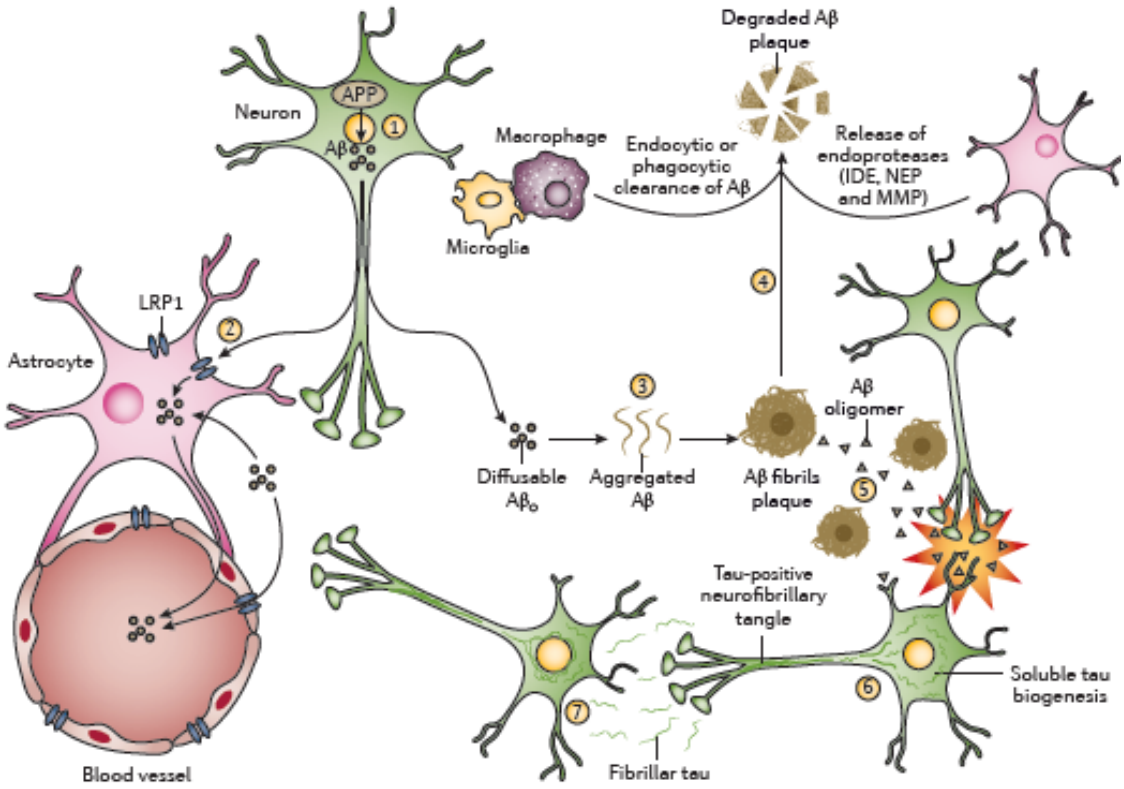


Figure 1.8: Pathways leading to plaque and neurofibrillary tangle formation on the basis of current theories of AD pathogenesis.
Reproduced from Masters *et al*. [36] with permission (Springer licence: 4600290147338).
1. Cleavage of Aβ from APP and release into brain interstitial fluid.
2. Uptake of Aβ into astrocytes via low-density lipoprotein receptor related protein LRP1, with potential influence on intramural periarterial drainage of solutes from brain interstitial fluid.
3. Aggregation of diffusible Aβ oligomers (Aβo) into plaques.
4. Degradation of Aβ plaques by endocytic or phagocytic clearance in microglia and macrophages, and by astrocytic release of endoproteases such as insulin-degrading enzyme (IDE), neprolysin (NEP) and matrix metalloproteinase (MMP).
5. Direct synaptotoxicity of Aβo.
6. Aβo induction of tau phosphorylation and aggregation.
7. Release of fibrillar tau and uptake by healthy neighbouring neurons.

1.6 Clinical criteria for AD dementia and Mild Cognitive Impairment

1.6.1 AD dementia

The first clinical diagnostic criteria for AD dementia formalised by the National Institute of Neurological and Communicative Disorders and the Alzheimer's Disease and Related Disorders Association (NINCDS-ARDRA) in 1984 [37] are compared and contrasted with the updated National Institute on Aging and Alzheimer's Association (NIA-AA) criteria of 2011 [38] in Table 1.3, page 53. The 2011 criteria distinguished the AD dementia syndrome from AD-related mild cognitive impairment (MCI – see section 1.6.2 below) and formalised the diagnosis of non-amnestic presentations of AD dementia, thus updating the 1984 criteria to acknowledge the clinical heterogeneity of AD dementia. The 2011 criteria also detailed investigations that increase the certainty of the AD pathophysiological process being the cause of a dementia syndrome. These included the presence of a genetic mutation associated with familial AD, or a biomarker profile suggestive of AD pathology (see sections 1.7 to 1.12).

1.6.2 MCI

The NIA-AA 2011 working group also produced a set of diagnostic criteria for MCI [39], defining it as a combination of

- cognitive concern from a patient, informant or clinician with
- objective evidence of one or more cognitive deficits on either bedside or neuropsychological evaluation, but
- without impairing independence in work/daily function.

The latter is the key point that distinguishes MCI from the dementia syndrome.

Table 1.3: Comparison of the 1984 and 2011 clinical diagnostic criteria for AD dementia.

NINCDS-ADRDA criteria 1984 [37]	NIA-AA criteria 2011 [38]
Probable AD dementia[a] (all necessary)	Probable AD dementia[b] (all necessary)
• Dementia on clinical examination, confirmed by neuropsychological testing • Deficits in two or more areas of cognition • Progressive worsening of memory and other cognitive functions • No disturbance of consciousness • Onset age 40-90 years (most often >65 years) • Absence of another systemic or neurological disease accounting for the above	• Meets criteria for dementia: cognitive or behavioural symptoms that interfere with ability to function at work/usual activities[c] represent a decline from previous levels of function, are not explained by delirium or major psychiatric disorder and are confirmed on patient and informant history, examination and either bedside examination or neuropsychological testing • A minimum of two domains must be involved, to include: amnesia (verbal or topographical), impaired reasoning/judgement, visuospatial impairment, language dysfunction or changes in personality • Presents either as Amnestic AD or non-amnestic AD including language, visuospatial or dysexecutive presentations • Is excluded if there is evidence of another cause for a dementia syndrome (such as substantial cerebrovascular disease, DLB or FTD), concurrent neurological disease or medical comorbidity that could have a substantial effect on cognition
	Probable AD dementia (increased certainty)
	• Documented cognitive decline increases certainty that there is evolving neuropathology but not that the process is that of AD • Presence of a causative AD genetic mutation (in *APP, PSEN1 or PSEN2*) increases the certainty that the condition is caused by AD pathology
Possible AD dementia	Possible AD dementia
• Dementia syndrome with variation in onset, presentation or clinical course • May occur in the presence of a second systemic or brain disorder sufficient to produce dementia which is not considered to be the cause of the dementia • May be diagnosed when a single gradually progressive severe cognitive deficit is identified without another identifiable cause	• Atypical course or insufficient information on progressive decline OR • Aetiologically mixed presentation
Definite	Pathophysiologically proven AD dementia
Clinical criteria for probable AD dementia AND histopathologic evidence from biopsy or autopsy	Clinical criteria for probable AD dementia AND consensus histopathological criteria for AD are both fulfilled

[a] Supportive clinical and other features, including the use of investigations such as CT scan and spinal fluid (mostly to rule out differential diagnoses), are also detailed, but do not form part of the core criteria for probable AD dementia.
[b] Biomarkers that increase the certainty of the AD pathophysiological process are acknowledged but not advocated for routine diagnostic purposes.
[c] Deficits in cognition that do not interfere significantly with work/usual daily activities are categorised as mild cognitive impairment (see section 1.6.2).

1.6.3 Limitations of clinical definitions

As detailed in both the 1984 and 2011 criteria, a definite diagnosis of AD dementia requires neuropathological evidence, which can usually be obtained only at *post-mortem* examination. In life, these diagnostic criteria are therefore limited to the clinical domain, and rely heavily on patient/informant report of symptoms, as well as on excluding a multitude of other possible causes for the symptoms, which include major psychiatric, other neurological or systemic illnesses. A review of thirteen studies of clinical-pathological correlation [40] showed that the 1984 clinical criteria for "probable AD dementia" achieved an average sensitivity of 80% but an average specificity of only 70% for neuropathologically confirmed AD. Those with mixed AD and Lewy body pathology or mixed AD and vascular pathology were more commonly misjudged by clinicians as having AD only [41] and dementia symptom severity correlated better with neurofibrillary tangle density [42, 43] and synaptic loss [44] rather than senile plaque density. More recently, the term "limbic-predominant age-related TDP-43 encephalopathy neuropathologic change (LATE-NC)" has been coined to describe *post mortem* findings of TDP-43 pathology in the amygdala, hippocampus and middle frontal gyrus, which may be either asymptomatic (and found in community-based autopsy series of those above 80 years, with increasing prevalence with age) or associated with predominantly amnestic symptoms that may mimic typical AD dementia [45]. Current clinical criteria are unable to distinguish between individuals who go on to have neuropathologically-defined AD and LATE-NC.

The difficulty in ascertaining the underlying pathology based on clinical symptoms alone was acknowledged in the 2011 clinical diagnostic criteria, and

the concept of “probable AD dementia with increased certainty” was introduced. In the context of AD research (but not in a routine clinical setting) the 2011 criteria stated that biomarkers should be used to make a link to possible underlying AD pathology, particularly in the context of recruitment to clinical trials.

1.7 **Biomarkers – definitions and characteristics**

A biological marker, or biomarker, has been defined by the WHO as “any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” [46] and more broadly by the National Institutes of Health Biomarkers Definition Working Group in 2001 as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [47].

Ideal biomarkers have different characteristics based on the information they aim to give. For example, an ideal diagnostic biomarker would reliably reflect *in vivo* pathology with high sensitivity and specificity. A screening biomarker would combine high sensitivity with at least moderate specificity and low cost. Conversely, a biomarker of progression may be downstream of the initial pathology but reliably track change over time. Biomarkers related to treatment might confirm the engagement of the molecular target of the treatment, or indicate reversal of effects of disease. For all biomarkers, reliability, cost, and ease of acquisition and processing are important considerations.

1.8 The need for biomarkers of AD pathology and the concept of preclinical AD

To date, no disease-modifying therapy for AD has demonstrated proven efficacy or translated into clinical application. This may at least in part be related to the imprecision of clinical diagnosis in identifying AD pathology (see section 1.6.3) but may also relate to the timing of application of potential therapeutic agents. Neuropathological studies indicate that senile plaque pathology is necessary but not sufficient for developing symptoms of AD-related cognitive impairment, as asymptomatic individuals can have widespread plaques, and in symptomatic individuals, symptom severity correlates with the presence and distribution of tau pathology and neurodegeneration [42, 43]. These findings imply that there is a potential window of opportunity, in which asymptomatic people with the neuropathological signature of AD (if it were able to be identified) might be treated, before the pathological burden reaches a level that brings about symptoms and irreversible damage to brain function. One of the major applications of biomarkers in AD would therefore be for early detection and recruitment to treatment trials, either as a screening tool (identifying at-risk individuals) or as an *in vivo* confirmation of AD pathology. Given the heterogeneity of clinical presentations of AD, other potential roles for biomarkers would be in classifying sub-types, denoting proximity to symptomatic onset and stratifying individuals by prognosis.

As previously mentioned, one of the key changes to the AD diagnostic criteria from 1984 to 2011 was incorporation of biomarkers into *in vivo* diagnosis (see Table 1.3) and this extends to the research criteria for diagnosing the preclinical phase of AD [48], which require evidence of Aβ pathology from biomarkers.

The currently available biomarkers of AD broadly can be categorised as:

- genetic biomarkers (see section 1.9, page 57), derived by a range of genetic analysis methods;
- neuroimaging biomarkers, derived from non-invasive methods of imaging the brain (see section 1.10, page 61) and
- fluid biomarkers, derived from bodily fluids such as cerebrospinal fluid (CSF – see sections 1.11, page 67, and section 1.14, page 88), blood (see section 1.15, page 95), urine or saliva.

1.9 **Genetic biomarkers**

1.9.1 Monogenetic forms of Alzheimer's disease

Less than 1% of all AD is estimated to be due to highly penetrant autosomal dominant mutations in three key genes: *PSEN1* on chromosome 14q24.2 [49], *PSEN2* on chromosome 1q42.13 [50] and *APP* on chromosome 21q21.3 [51]. Their protein products, presenilin 1, presenilin 2 and APP, are all involved in the canonical pathway of APP processing that results in the production of Aβ peptides (see section 1.4.1, page 43) and the known autosomal dominant AD mutations in these genes result in relative overproduction of more aggregation-prone and toxic forms of Aβ peptides via the amyloidogenic pathway. Taken together, it is estimated that these mutations underlie 5-10% of early onset AD dementia (below age 65). In addition, individuals with Down syndrome, in whom there are three copies of *APP* due to triplication of chromosome 21, have Aβ peptide overproduction, plaque deposition and an increased risk of early onset AD dementia. While testing for Down syndrome often occurs as part of prenatal

screening, with confirmatory karyotyping after birth, testing for the autosomal dominant AD mutations is usually offered in one of three clinical contexts:

- Symptomatic individuals with early onset AD in whom there is an autosomal dominant family history of similar symptoms: here genetic testing is offered on a clinical diagnostic basis;
- Asymptomatic individuals from families with known autosomal dominant AD mutations, who choose to have the test after genetic counselling, or
- Pre-implantation genetic testing in the context of *in vitro* fertilisation to reduce the risk of an individual from a family with a known autosomal dominant AD mutation passing this on to a child.

Despite the relative rarity of these conditions, studies of cohorts of individuals with monogenetic forms of AD continue to provide a wealth of information regarding molecular mechanisms and possible other categories of biomarkers that are relevant to sporadic AD.

1.9.2 Sporadic Alzheimer's disease

Up to about 80% of the risk of even sporadic AD is estimated to be attributable to genetic factors [52], with polymorphisms in the involved genes each making a relatively small contribution to risk, and none of them being necessary or sufficient for a diagnosis of AD. The first gene discovered to influence sporadic AD risk was *APOE* [53], which encodes apolipoprotein E (ApoE). ApoE is expressed in liver, brain and other tissues, where it is involved in lipid trafficking and metabolism. In AD it may be involved in multiple Aβ-dependent and Aβ-independent pathways (reviewed by Verghese *et al*. [54]). Single nucleotide polymorphisms (SNPs) in the coding region of the gene (rs7412 and rs429358)

result in three possible allelic forms: ε2, ε3 and ε4. The ε4 allele confers a dose-dependent increase in AD risk: the odds ratio (OR) for AD in the presence of the ε3/ε4 genotype is 3.2 and for ε4/ε4 is 14.9 relative to ε3/ε3. Conversely, the ε2 allele confers reduced risk, with an OR of 0.6 for ε2/ε3 relative to ε3/ε3 [55].

The next level of genetic contribution to AD risk was identified by genome-wide association studies (GWAS); a meta-analysis of over 74 000 individuals of European origin was able to identify 19 loci (in addition to *APOE)* at which SNPs reached genome-wide significance [56]. Further AD-relevant loci with much rarer SNPs have been identified; among these is *TREM2* (Triggering Receptor Expressed On Myeloid Cells 2) [57, 58], which has a similar OR to *APOE* but due to the rarity of the relevant SNPs is likely to contribute a much smaller fraction of the population attributable risk. The functional annotations of the closest genes to these loci encompass diverse pathways that may impact Aβ generation and clearance and tau toxicity, including cytoskeletal function and axonal transport, endosomal vesicle cycling, synaptic function, immune response and lipid metabolism (reviewed in [59]). Figure 1.9 shows risk of AD and the allele frequencies of identified mutations in familial AD and SNPs conferring risk in sporadic AD.

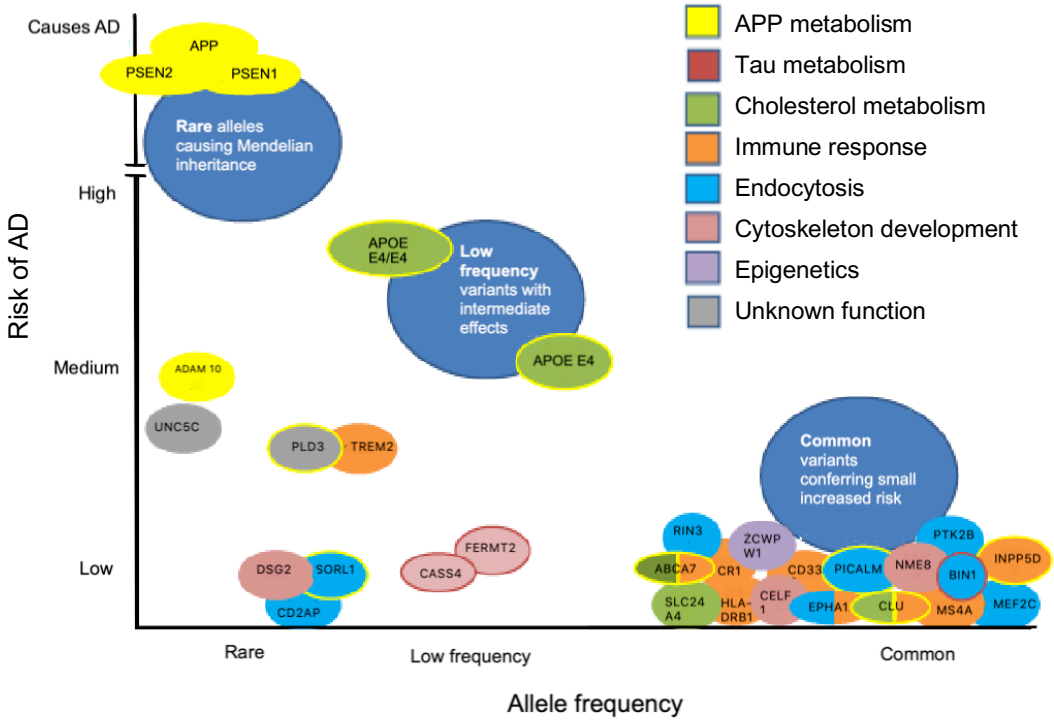


Figure 1.9: Genes identified as causal for familial Alzheimer's disease or as conferring risk for sporadic Alzheimer's disease.
The internal colour corresponds to their understood function. Where there are two internal colours, the gene has been implicated in more than one pathway. Genes circled in yellow are also thought to influence amyloid precursor protein metabolism; genes circled in red are thought to also influence tau metabolism. Reproduced from Lane et al. [25] with minimal modifications for clarity, after Karch and Goate [60] (permission obtained from both publishers; John Wiley and Sons licence 4600180842980 and Elsevier licence 4600181189483).

More recently, the concept of polygenic risk has been extended by incorporating common SNPs that achieve a much lower significance threshold of $p < 0.5$, which significantly improves prediction of AD risk over models including age, sex, *APOE* and the GWAS-identified loci [61].

Current UK National Institute for Health and Care Excellence (NICE) guidelines for dementia assessment do not advocate the use of genetic testing in sporadic AD [62], as not even *APOE* is fully predictive of disease. However, biomarker research and clinical trials in AD routinely utilise *APOE* as a risk stratifier.

1.10 **Neuroimaging biomarkers**

1.10.1 Structural imaging

Computed tomography (CT) and magnetic resonance imaging (MRI) are the two main modalities used in clinical practice. MRI has superior spatial resolution and does not use ionising radiation. Therefore, MRI supersedes CT except when the former is contra-indicated due to presence of MRI-incompatible implants, or in patients with claustrophobia or those with advanced cognitive impairment, in whom MRI is likely to be poorly tolerated.

The main MRI sequences and their uses in clinical practice include:

- T1 weighted: assessment of brain volume/pattern of atrophy – useful in particular in differential diagnosis of AD, in offering predictive value for typical AD (hippocampal atrophy) and in confirming atypical AD diagnoses (for example, posterior cortical atrophy in the context of a biparietal AD syndrome),
- T2 weighted and fluid attenuated inversion recovery (FLAIR): assessment of cerebrovascular disease burden,
- Diffusion weighted (DWI): assessment of acute infarcts and exclusion of changes suggestive of prion disease, and
- Susceptibility weighted (SWI)/T2*: assessment of microhaemorrhages that may result from hypertensive disease or amyloid angiopathy, and superficial siderosis that may suggest amyloid angiopathy.

The main use of MRI in the AD research setting is as a surrogate marker for neurodegeneration (one of the three hallmarks of AD pathology). MRI has been validated against *post mortem* examination, with earlier studies showing that hippocampal volumes on *post mortem* MRI correlated with neuronal counts in

AD and healthy controls [63] and hippocampal volumes on *ante mortem* MRI correlated with Braak stage on *post mortem* examination in mixed pathology cohorts, but the strongest correlation was driven by patients with AD pathology [64]. Longitudinal changes in ventricular volume have also been shown to predict global cortical NFT burden [65].

MRI has also been used to predict clinical progression to AD dementia. In cognitively normal individuals who progressed to MCI, and in individuals with MCI who progressed to AD, annualised rates of change in hippocampal, entorhinal, whole brain and ventricular volumes were greater than in non-progressors [66]. An example of serial T1 coronal MRI showing progressive hippocampal volume decline in a patient with MCI who progressed to neuropathologically-confirmed AD is shown in Figure 1.10 (page 62).

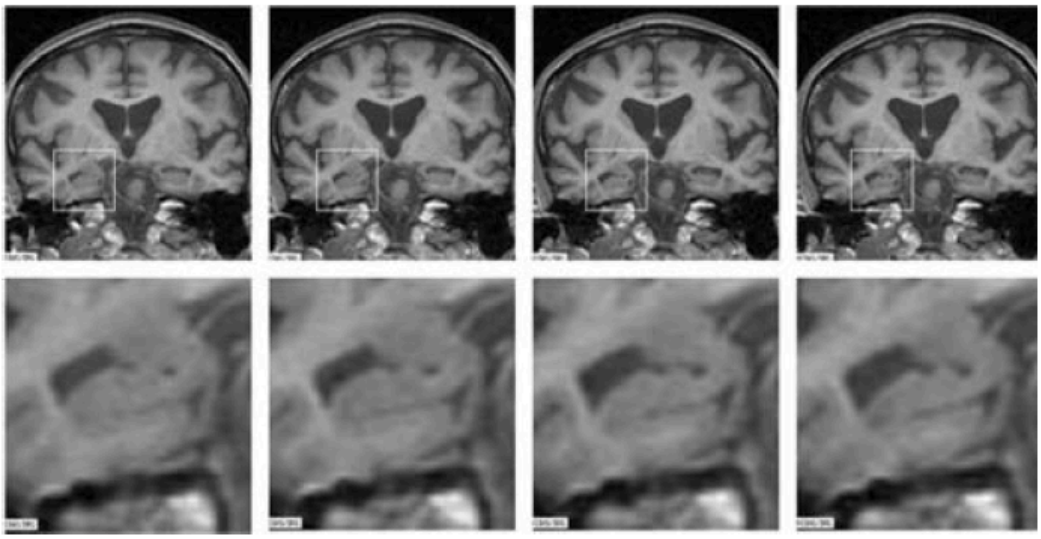


Figure 1.10: Progressive hippocampal atrophy in AD-related MCI.
Serial T1 weighted coronal 1.5T MRI images were taken at 0, 0.75, 2 and 3 years from presentation in a 73-year-old patient with a 2-year history of progressive memory problems. Reproduced from Archer *et al.* [67] according to the publisher's permissions policy.

1.10.2 Functional imaging

1.10.2.1 Fluorodeoxyglucose positron emission tomography (FDG-PET) and single positron emission computed tomography (SPECT)

FDG-PET and SPECT are imaging modalities employing intravenously injected radioisotopes (18-F fluorodeoxyglucose and 99mTc-hexamethylpropyleneamine oxime or HMPAO respectively). The former is considered a more direct measure of brain glucose metabolism while the latter measures perfusion. FDG-PET is generally considered to have greater accuracy than SPECT in contributing to AD diagnosis, as PET has superior sensitivity and specificity in distinguishing AD dementia from other neurodegenerative dementias [68, 69]. FDG-PET findings of temporo-parietal hypometabolism are more accurate than clinical diagnosis alone at predicting *post mortem* AD diagnosis [70, 71].

In the research setting, the locations of earliest regions of FDG-PET hypometabolism in AD include the posterior cingulate [72] and in cognitively normal individuals with high AD risk (such as *APOE* ε4 homozygotes) there is further early involvement in the parietal, temporal, and prefrontal regions [73]. In healthy controls who eventually convert to MCI or AD these regions are also involved, forming part of the same network that shows earlier hyperactivations in functional MRI studies (see section 1.10.2.2 below).

1.10.2.2 Functional MRI

Functional MRI (fMRI) gathers data on blood oxygen level dependent (BOLD) changes in MR signal, either during the resting state or when an individual is performing a cognitive task. There are currently no clinically validated applications of fMRI in AD diagnosis and it is a particularly difficult modality to

employ in severely symptomatic individuals, due to its susceptibility to motion artefacts.

However, fMRI has yielded a wealth of information in research studies on the networks involved in early AD, including in asymptomatic individuals. In particular, the default mode network (consisting of medial temporal lobe, precuneus, posterior cingulate, lateral parietal, lateral temporal, and medial prefrontal regions) has been shown to have increased connectivity in the resting state in asymptomatic individuals at risk for AD due to family history and *APOE* ε4 carrier status [74]. The same network shows diminishing of normal deactivations in older adults with cerebral amyloid deposition who are asymptomatic or mildly symptomatic, compared to those without significant amyloid deposition [75].

1.10.3 Molecular imaging

1.10.3.1 Amyloid PET

The first radioligand with specific binding to Aβ was the 11-C Pittsburgh-B compound (PiB), developed in 2004, with cortical PiB binding showing inverse correlation with FDG-PET signal in the same regions [76]. PiB binding has shown good correlation with Thal phase [77] and CERAD scores [78] in patients scanned within 2-3 years of death. However, the very low half-life of 20 minutes for PiB makes it impractical to use in settings where the production facility is not very closely geographically associated with the scanner. This led to the development of the 18-F amyloid PET tracers, of which currently there are three licensed for use in research in Europe and the USA: florbetaben (Neuraceq^{TM}), florbetapir (Amyvid^{TM}), and flutemetamol (Vizamyl^{TM}). A pair of axial florbetapir

PET images, from a healthy control and a patient with AD dementia, is shown in Figure 1.11.

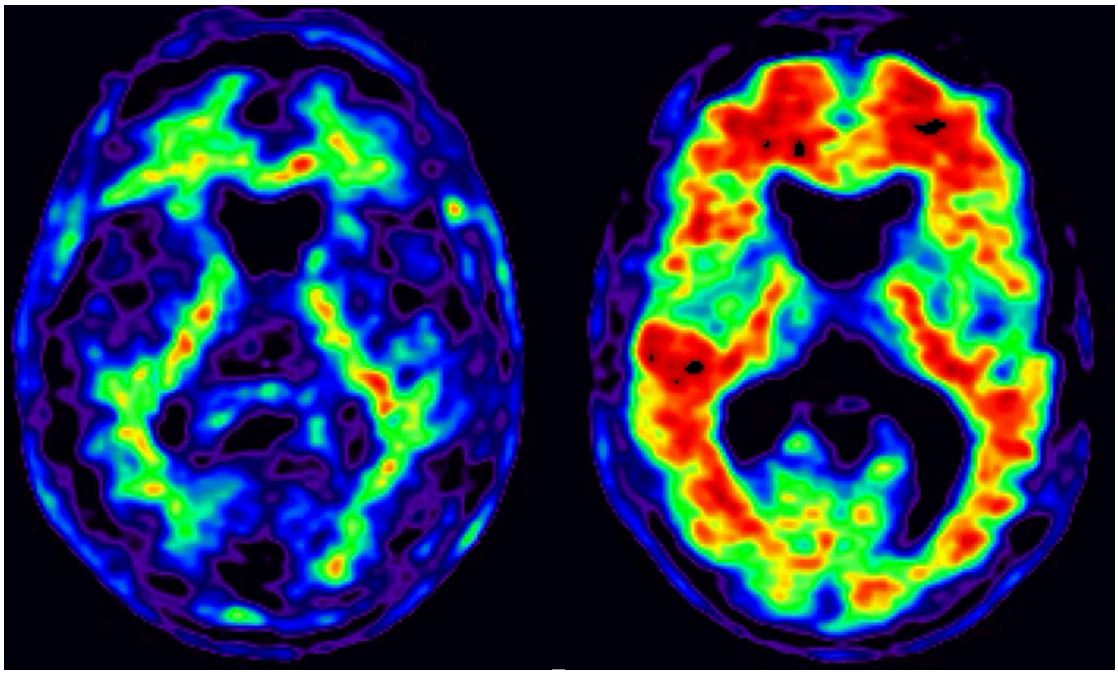


Figure 1.11: ^{18}F-florbetapir PET axial images from a healthy control (left) and a patient with Alzheimer's disease (right).
Warm colours indicate areas of high tracer uptake. Image credit: Dr David Cash and Prof Jonathan Schott.

The three 18-F tracers have shown similar diagnostic accuracy in separating clinically defined AD from controls (about 90% sensitivity and 85% specificity in a meta-analysis [79]). Neuropathological validation of these tracers with lower intervals of 1-2 years between scan and *post mortem* has been undertaken [80-83] and it is possible to use amyloid PET to perform the *in vivo* equivalent of Thal staging [84]. In order to facilitate comparison between results of studies performed using different tracers, a centiloid scale for reporting tracer uptake has been developed [85].

While the specific binding of these tracers to parenchymal fibrillar amyloid has been demonstrated on autoradiography studies (for example, florbetapir binding of both diffuse and neuritic plaques in AD [86]), increased tracer uptake has

also been seen in cases of cerebral amyloid angiopathy (CAA). The diagnostic utility of amyloid PET in distinguishing CAA versus either healthy controls or individuals with deep cerebral haemorrhages was shown to be variable across a meta-analysis of seven studies, with average sensitivity 79% and specificity 78%. This may partly be related to tracer differences, as 11-C PiB and its 18-F analogue flutemetamol have been demonstrated to bind Aβ in atherosclerotic plaques [87] but autoradiography studies of florbetapir and florbetaben have not commented on whether these tracers bind vascular Aβ. Instead, in studies where the latter two tracers have been shown to differentiate *in vivo* CAA from healthy controls or deep cerebral haemorrhages, it may be that the tracers are binding parenchymal Aβ (effectively, comorbid AD pathology) that occurs in a proportion of CAA patients.

In the clinical setting, amyloid PET imaging has been shown to significantly alter clinicians' diagnostic confidence for AD and acetylcholinesterase prescription for symptomatic treatment of AD (increasing both in scan-positive individuals and decreasing both in scan-negative individuals) in multicentre studies in Europe [88] and the USA [89].

1.10.3.2 Tau PET

Post mortem neurofibrillary tangle load (i.e. tau pathology) correlates better with dementia symptom severity in AD than does senile plaque load (i.e. amyloid pathology), as shown in large single-centre neuropathological series [42, 43]. Braak stage was also correlated with worse performance in neuropsychological assessments performed within 1-2 years prior to death in participants in the Alzheimer's Disease Neuro-imaging Initiative [90]. Therefore, tau PET tracers

are being developed in an effort to allow for *in vivo* Braak staging. None has reached clinical application so far; however, tau imaging is being used in the context of therapeutic trials.

Off-target binding of tau tracers has been a significant problem. When using the ligand 18-F flortaucipir, which is the best characterised ligand so far, amyloid-negative semantic variant primary progressive aphasia (svPPA) patients, who would be expected to have TDP-43 type C pathology rather than AD-related tau pathology, have demonstrated binding in the dominant anterior temporal lobe [91] and binding has also been shown in the choroid plexus causing "spill over" to regional tau PET uptake values for hippocampi, which should therefore be interpreted with caution [92]. Despite this, flortaucipir has proved useful as it does not show appreciable binding for tauopathies such as FTLD-MAPT or TDP-43 type B pathology [93], for which other ligands are being sought. Moreover, longitudinal studies of mixed cohorts of healthy controls, MCI and AD dementia has shown that the rate of increase of flortaucipir uptake in regions of interest including the entorhinal cortex, temporal neocortex, posterior cingulate and retrosplenium is greater in amyloid-positive compared to amyloid-negative individuals even among the cognitively unimpaired, and greater in the cognitively impaired than the unimpaired, among those who are amyloid-positive [94, 95]. Similar longitudinal data on a second generation of 18-F tau tracers, which are purportedly less affected by off-target binding, are awaited.

1.11 **Established fluid biomarkers**

1.11.1 CSF as a source of AD biomarkers

Cerebrospinal fluid (CSF) is produced by the choroid plexus epithelium and ventricular ependyma at a rate of 0.4 ml/minute/gram of tissue and its steady

state volume in adults is about 150 ml, so it turns over on average four times daily [96]. CSF circulates around the brain and spinal cord, and is reabsorbed into the cerebral venous sinuses via the arachnoid granulations. As CSF is in communication with the extracellular fluid that bathes the brain parenchyma, molecules secreted into the extracellular fluid by neurons and glia enter it. CSF can be obtained safely by lumbar puncture, which is usually undertaken at the L3/4 or L4/5 space, with the individual either in the lateral decubitus or seated position, in lumbar forward flexion. Consensus guidelines have been published for lumbar puncture in the investigation of suspected neurological disease [97], in which the contraindications and techniques to reduce complications (including the use of atraumatic needles, which are proven to reduce the rate of post-lumbar puncture headache [98]) are discussed. The main types of CSF biomarker used currently in AD diagnosis are Aβ peptides and tau proteins.

1.11.2 Terminology

The terminology adopted here and throughout the thesis for the main established CSF biomarkers of AD pathology is as follows.

"Aβ1-42" refers to the specific peptide starting at the first amino acid (a.a.) residue and terminating at the 42nd a.a. residue of the Aβ sequence. "Aβ42" refers to all Aβ peptides terminating at the 42nd a.a. residue, regardless of the starting a.a. residue.

"t-tau" refers to tau proteins that can be quantified by conventional mid-region tau assays, including the commonly used INNOTEST® assay.

"p-tau" refers to all phosphorylated tau proteins. "p-tau-181" refers to tau phosphorylated at the threonine at a.a. residue 181, which is the epitope recognised by most commonly used INNOTEST® assay [99].

1.11.3 CSF measures of amyloid-β peptides

Soon after it was established that Aβ42 was the main Aβ peptide constituent of senile plaques in both sporadic AD and familial AD with *APP* mutations [20], the first enzyme-linked immunosorbent assay (ELISA) specific to Aβ42 was developed, showing marked reduction of Aβ42 in CSF from patients with clinically diagnosed AD dementia compared to controls [100]. Since then, this finding has been confirmed using numerous other immunoassay methods (ELISA, Luminex xMAP technology, electrochemiluminescence immunoassay, urea-based gel electrophoresis combined with Western blot), and using selected reaction monitoring mass spectrometry (SRM MS), with a meta-analysis of 131 studies examining CSF from 9949 patients with AD and 6841 controls showing that CSF Aβ42 in AD is 0.56 times that in controls [101]. Low Aβ42 has also been confirmed in *pre mortem* CSF from neuropathologically defined AD compared to controls [102-104]. Internationally certified reference materials for measurement of Aβ1-42 based on three human CSF pools (reviewed in [105]) and two International Standards Organisation-certified reference measurement procedures based on liquid chromatography-mass spectrometry (LC-MS: [106, 107]) have been produced. This allows for commercially developed kits and different measurement techniques to be calibrated to the same standards, as CSF Aβ42 measurement is now established in clinical practice.

Later studies have shown that the CSF Aβ42/40 ratio may be a more accurate biomarker for AD pathology than CSF Aβ42 alone [108-111], not only because the ratio may control for inter-individual variation in overall Aβ peptide production, but also because it may partly control for pre-analytical sources of variation such as adsorption or degradation of Aβ peptides with storage [112, 113]. The superiority of the CSF Aβ42/40 ratio compared to CSF Aβ42 alone has been shown in relation to discriminating both *post mortem* amyloid load [110] and *in vivo* amyloid PET [111, 114] as gold standards.

The prevailing theory for the cause of reduced Aβ42 in AD CSF is that it results from the increased tendency of Aβ42 to aggregate in parenchymal plaques. However, another possible contribution to the observed difference in AD vs control levels is that Aβ42 also exists in aggregated forms in AD CSF. These Aβ oligomers (Aβo) may have epitopes partly masked from most assays. Some support for this theory comes from observations that when AD CSF samples are tested under denaturing conditions, the measured Aβ42 concentration is higher than when tested under non-denaturing standard conditions [115, 116]. These findings, in combination with a growing body of evidence that Aβo rather than fibrils or plaques are the neurotoxic species in AD, have prompted the development of CSF Aβo assays (reviewed in [117]). These assays are heterogeneous in their methods and in the oligomeric species they may detect, and the findings of the ten reviewed studies are also somewhat mixed. Eight of these studies reported significant increases in Aβ oligomer levels in AD dementia compared to control CSF, and some studies reported increased levels in AD-MCI compared to control CSF. Aβo assays are now commercially available but are not as yet incorporated into clinical routine.

1.11.4 CSF measures of tau

1.11.4.1 T-tau

A meta-analysis of 151 studies reported CSF data on 11341 patients with AD and 7086 controls, showing agreement between all the studies for elevation of CSF t-tau in AD, with a combined result of 2.54 for the ratio of CSF t-tau in AD to controls [101].

Tau within neurons comprises a spectrum of isoforms and both truncated and full-length forms [118]. However, tau released actively from neurons and present in CSF is predominantly C-terminally truncated [118, 119]. Most studies of CSF tau have employed mid-region-directed assays for t-tau, which would be unable to differentiate between passively released and actively secreted forms, so CSF t-tau has been interpreted as a general neuronal injury marker. However, in the context of many chronic slowly progressive neurodegenerative conditions where individuals do not have a significant reduction in CSF Aβ42, such as behavioural variant FTD and svPPA, CSF t-tau is not usually elevated [120]. There is increasing evidence that tau may be both passively released into CSF with acute neuronal damage/death (as in the case of acute stroke [121], traumatic brain injury [122, 123] and rapidly progressive degenerative conditions like CJD [124]) and actively secreted into CSF at a rate that correlates with age and the presence of cerebral amyloid deposition [118].

Reports of correlation between *ante mortem* CSF t-tau levels and *post mortem* Braak stage in AD are mixed [104, 125], but this may be unsurprising as Braak staging is obtained by immunostaining for hyperphosphorylated intracellular tau.

However, correlation between CSF t-tau and CERAD score has been shown [104] and gives some validation of CSF t-tau as a marker of neurodegeneration in AD.

1.11.4.2 P-tau

89 studies included in a meta-analysis of CSF p-tau, comprising 7498 patients with AD and 5126 controls, showed AD to control p-tau ratios were consistently above one, with an average of 1.88 [101].

P-tau is generally considered to be more AD-specific than t-tau, as p-tau is not elevated to the same extent in conditions with rapid neuronal damage like acute stroke [121] or CJD . For example, the p-tau-231/t-tau ratio is lower in CJD than in AD, and an assay for tau not phosphorylated at threonine 181 has conversely shown good ability to discriminate between CJD and AD [126]. A recent study has also demonstrated that patients with all FTD phenotypes apart from lvPPA (which is usually considered to be due to AD rather than FTLD pathology) had lower CSF p-tau-181/t-tau ratios than controls [127].

Most AD studies have examined CSF p-tau-181 but another commonly assayed moiety is p-tau-231, which has been shown in one study to have superior discriminant ability over p-tau-181 for AD vs control [128]. P-tau-231 levels in brain homogenates correlate with those in *ante mortem* CSF (taken by lumbar puncture 0-3 years prior to autopsy) and CSF p-tau-231 levels correlate with neurofibrillary tangle load in *post mortem* AD brain immunohistochemistry [129] while p-tau-181 does not display this correlation [125, 130].

1.12 **Current clinical applications of established neuroimaging and fluid biomarkers of AD**

The current UK NICE guidelines for dementia assessment indicate that structural imaging (MRI, or CT if MRI is contraindicated) should be offered to "rule out reversible causes and assist with subtype diagnosis, unless dementia is well established and the subtype is clear" [62]. If cognitive impairment remains unexplained despite clinical and structural imaging assessment, the guidance suggests the use of either FDG-PET, SPECT or CSF testing; the latter is directed particularly at identifying molecular pathologies of AD in CSF whereas FDG-PET or SPECT may also be useful in confirming regional hypometabolism or hypoperfusion respectively, in other dementias.

The appropriate use criteria for CSF proposed by the Alzheimer's Association [131] are broader than those supported by NICE (see Table 1.4, page 74). Key points of difference include the Alzheimer's Association recommendations for application of CSF testing for AD biomarkers even in those with typical probable AD, and in those with subjective cognitive decline (SCD) who are considered to be at elevated risk of AD (as established, for example, by *APOE* genotyping).

Table 1.4: Clinical indications for appropriate use of lumbar puncture and CSF testing in the diagnosis of AD, as detailed by the Alzheimer's Association appropriate use criteria. Reproduced with minor adaptations from Shaw *et al.* [131], with permission.
REM, rapid eye movement; SCD, subjective cognitive decline

	Clinical indication
Appropriate	Meeting core clinical criteria for probable AD with typical age of onset
	Symptoms suggesting possible AD
	MCI or dementia with onset age below 65 years
	Persistent, progressing or unexplained MCI
	SCD but considered to be at high risk for AD (persistent decline in memory rather than other cognitive domains; onset in the last 5 years; age at onset >60 years; worries associated with SCD; feeling of worse performance than others of the same age group; confirmation of cognitive decline by an informant; carriage of *APOE* ε4)
	Dominant symptom of change in behaviour, where AD diagnosis is being considered
Inappropriate	Determination of disease severity in patients who have already received a diagnosis of AD
	SCD not considered to be at high risk of AD
	Symptoms of REM sleep behaviour disorder
	Carriers of autosomal dominant AD mutations, with or without symptoms
	In lieu of genotyping for suspected carriers of autosomal dominant AD mutations
	Carriers of *APOE* ε4 with no cognitive impairment
	Cognitively unimpaired on objective testing and no SCD but considered as high risk due to family history of AD
	Cognitively unimpaired on objective testing, no SCD, no expressed concern about developing AD and no condition suggesting high risk

There are also agreed appropriate use criteria for amyloid PET imaging in the clinical setting in the USA, which include patients presenting with dementia with atypical clinical features, or those whose initial workup in the context of persistent MCI (including structural imaging) gives uncertain results [132]. Although NICE guidance in the UK does not yet make recommendations on amyloid PET, the consensus UK Royal College guideline on its use [133] is

based upon the US guideline, and the 18-F tracers are clinically licensed in the UK.

1.13 **Research applications of AD biomarkers**

1.13.1 IWG-2 criteria 2014

The International Working Group (IWG) for Research Criteria for the diagnosis of AD first published a set of criteria in 2007 but updated these in 2014 (the IWG-2 criteria [134]), mostly to take into account the advances in AD biomarkers that had occurred in that time. The clinical aspects of the IWG-2 2014 research criteria are consistent with those of the NIA-AA 2011 clinical criteria, but the former require biomarker evidence of AD pathology for a research diagnosis to be made, and include preclinical AD as a research diagnosis in which there is biomarker evidence of AD pathology without a clinical syndrome. Table 1.5 summarises the IWG-2 2014 research diagnostic criteria for typical, atypical and preclinical AD.

Table 1.5: Summary of the IWG-2 research diagnostic criteria.
A+B is required for a diagnosis in each case, with the clinical AD diagnoses further requiring fulfilment of exclusion criteria; paraphrased from [134].
* Preclinical AD is further categorised as “pre-symptomatic AD” (in those who carry an autosomal dominant AD mutation) or “asymptomatic at risk of AD” (in those who have either of the other two biomarkers).

	Typical AD	Atypical AD	Preclinical AD
A: Clinical	Early progressive episodic memory impairment for at least 6 months with objective evidence of the same on tests validated as measures of hippocampal memory deficits	Presence of a phenotype consistent with a known atypical variant of AD such as posterior (including occipitotemporal or biparietal), logopenic, frontal or Down’s syndrome variants	Asympomatic
B: Biomarker	Decreased CSF Aβ1-42 AND increased t-tau or p-tau, OR Increased amyloid PET tracer retention, OR Presence of an autosomal dominant AD mutation (in *PSEN1*, *PSEN2*, or *APP*)*		
Exclusion	History, clinical or biomarker features (e.g. blood tests or structural brain imaging) indicating a more likely clinical diagnosis		

1.13.2 Hypothetical model of AD biomarkers

The use of biomarkers in AD research has been structured around a hypothetical model first proposed by Jack and colleagues in 2010 [135], in which AD biomarkers change in a structured order as clinical disease stage progresses, commencing with accumulation of parenchymal Aβ, followed by accumulation of tau pathology, brain structural changes, sub-clinical cognitive change and then symptoms (see Figure 1.12 below).

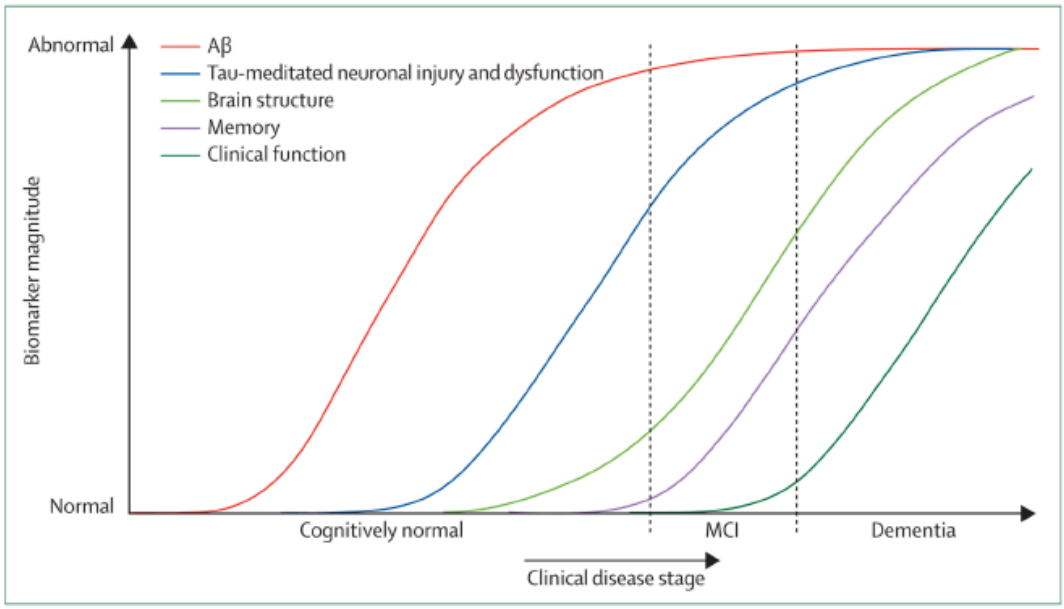


Figure 1.12: A hypothetical model of temporally ordered AD biomarker changes relative to clinical disease stage.
Reproduced from Jack *et al.* [135], with permission (Elsevier licence: 4587550799255).

1.13.3 Evidence for temporal ordering of AD biomarkers

Longitudinal cohort studies in both familial and sporadic AD have provided some evidence for the hypothetical model described above, but have also indicated potentially important refinements to this model and fostered debate on the actual temporal order.

The global Dominantly Inherited Alzheimer's Network (DIAN) study (https://clinicaltrials.gov/ct2/show/NCT00869817) was established in 2009 to

follow individuals from families carrying mutations in familial AD genes including *PSEN1*, *PSEN2* and *APP* over ten years, with the primary aim of AD biomarker identification. Although familial AD accounts for less than 1% of all AD, its penetrance is nearly 100% provided that individuals live long enough, and the estimated age at onset (EAO) is predictable to a great extent from parental age at onset and mutation type, as shown in a meta-analysis of 387 autosomal dominant AD pedigrees including data from DIAN and two other large kindreds from Colombia and Germany [136]. Thus, EAO provides a common anchoring timepoint against which biomarker changes in pre-symptomatic individuals may be assessed according to the estimated years to onset (EYO), which is the difference between an individual's age and the EAO. The rate of change of Aβ markers may be an earlier biomarker than the absolute value of a given measure – hence McDade *et al*. [137] showed that the rate of change of Aβ accumulation (assessed either by CSF Aβ42 or by PiB PET SUVR) was significantly different between mutation carriers (MC) and non-carriers (NC) as early as -25 EYO, but on cross-sectional estimates CSF Aβ42 diverged significantly between MC and NC only from -10 EYO. Longitudinal change estimates also showed CSF Aβ42 change progressively reduced and plateaued around 0 EYO, while it continued to increase for PiB SUVR. Conversely, absolute values of CSF t-tau and p-tau showed better divergence between MC and NC than rates of change, which showed no difference for t-tau at any EYO, and a plateau for p-tau at -4 EYO. Markers of neurodegeneration such as FDG PET uptake in the precuneus and hippocampal volume ascertained by MRI showed consistent findings whether using longitudinal or cross-sectional estimates, but the former diverged much earlier (-14 EYO versus -1 EYO).

A large body of evidence on biomarker changes in sporadic AD has been derived from cohort studies employing multimodal biomarkers, such as the Alzheimer's Disease Neuroimaging Initiative (ADNI: http://adni.loni.usc.edu/about/), which was established in 2004 and has had four successive phases since. Broadly the findings from ADNI have supported the assumptions of the hypothetical model, but in sporadic AD the choice of anchoring timepoint is not as clear as in familial AD, so revisions of the hypothetical model have advocated use of time from a defined clinical state (e.g. incident MCI or dementia diagnosis) as the horizontal axis [138]. Models of longitudinal CSF measurements in ADNI have shown CSF Aβ1-42 changes to occur from about 9 years before crossing the threshold for abnormality in AD, and CSF p-tau-181 changes from about 2 years before [139], but supported the idea that there may be two distinct sub-populations with differing rates of change in these biomarkers, dependent upon their baseline values (see Figure 1.13 A and B below). Conversely, the rate of change of CSF t-tau showed a unimodal distribution (see Figure 1.13C below).

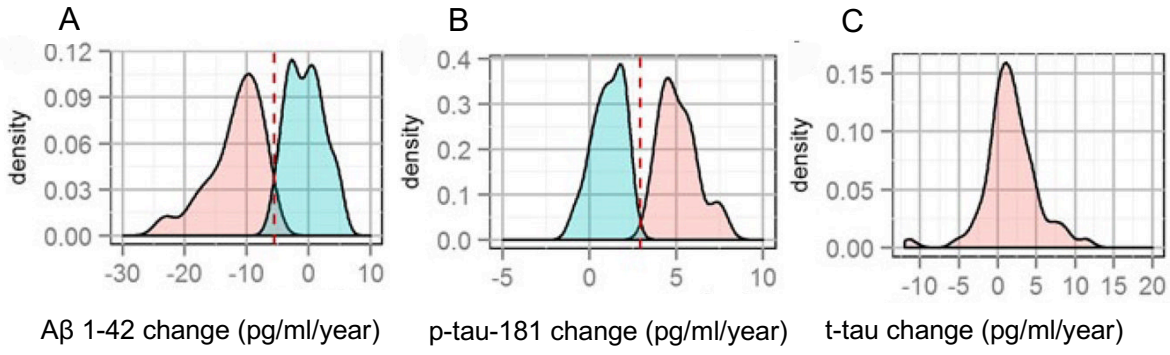


Figure 1.13: Finite mixture modelling of rates of change of CSF biomarkers in ADNI. Reproduced with permission from Toledo et al. 2013 with minimal adaptations for consistency of style; Springer Nature licence 4677700244168).
A: CSF Aβ 1-42; B: CSF p-tau-181; C: CSF t-tau

Individuals with low baseline CSF Aβ1-42 in ADNI had lower rates of decrease of CSF Aβ1-42 (implying they had reached a plateau) than those who had

higher baseline CSF Aβ1-42. However, individuals with low baseline CSF Aβ1-42 had higher baseline and greater rates of increase of CSF p-tau-181 than individuals with higher baseline CSF Aβ1-42. This bimodality in CSF Aβ1-42 rate of decline, and its association with CSF p-tau pathology, has influenced the development of biomarker-based stratification systems, such as the AT(N) framework described below (section 1.13.4.1).

1.13.4 The NIA-AA research framework 2018

1.13.4.1 AT(N) classification

Jack *et al*. [140] proposed a framework for stratification of individuals in AD research studies, based on three groups of biomarkers, which are in turn related to the core pathologies of AD: amyloid ("A"), tau ("T") and neurodegeneration ("(N)": where parentheses are used to acknowledge that (N) may capture neurodegeneration related to both AD and other pathologies but may not differentiate between these causes).Table 1.6 shows the biomarker groups.

Table 1.6: AT(N) biomarker groups according to the NIA-AA research framework. Reproduced with permission from Jack *et al.* [140], with minor adaptations.

Group	AT(N) biomarker grouping
A	Aggregated Aβ or associated pathologic state
	CSF Aβ42 or Aβ42/40 ratio Amyloid PET
T	Aggregated tau (neurofibrillary tables) or associated pathologic state
	CSF p-tau Tau PET
(N)	Neurodegeneration or neuronal injury
	MRI FDG PET CSF t-tau

This AT(N) framework incorporates the use of cut-points that binarize each element as positive or negative, yielding eight possible combinations (Table 1.7).

Table 1.7: AT(N) profiles and biomarker categories.
Reproduced with permission from Jack *et al.* [140] with minor adaptations.

AT(N) profile	**Biomarker category**	
A-T-(N)-	Normal AD biomarkers	
A+T-(N)-	Alzheimer's pathologic change	Alzheimer's continuum
A+T+(N)-	AD	
A+T+(N)+	AD	
A+T-(N)+	Alzheimer's and concomitant suspected non-Alzheimer's pathologic change	
A-T+(N)-	Non-AD pathologic change	
A-T-(N)+	Non-AD pathologic change	
A-T+(N)+	Non-AD pathologic change	

1.13.4.2 Challenges in applying the AT(N) framework

A key feature of the AT(N) framework, in which it differs from the IWG-2 research criteria, is that the former only requires A+ for an individual to be considered on the Alzheimer's continuum, whereas the latter requires the equivalent of A+T+. Both acknowledge that Aβ deposition is specific to AD, but the use of different biomarkers from the "A" group across different research studies (or indeed within the same multimodal study) could result in an individual being categorised as A+ by one biomarker and as A- by another. Efforts to harmonise cut-points are important; for CSF the development of certified reference materials and calibration of assays to the same standards is likely to help, whereas for amyloid PET the use of a common "centiloid" scale has been advocated [141].

In the “T” group, CSF p-tau has been included rather than CSF t-tau due to presumed AD-specificity of the former (see section 1.11.4.2, page 72) but in people on the Alzheimer’s continuum both p-tau and t-tau tend to rise together and may provide similar information, therefore the inclusion of t-tau in the “(N)” group may be somewhat redundant. CSF neurofilament light chain (NFL) has been proposed as an alternative fluid-based “(N)” biomarker, particularly as it encapsulates amyloid-independent neurodegeneration (see section 1.14.1, page 88). Classification concordance using different imaging biomarkers in the “(N)” group has been investigated by Jack *et al*. [142]. This study of individuals in the population-based Mayo Clinic Study of Aging (MCSA) showed that although actual correlations between continuous values of adjusted hippocampal volume, AD signature region cortical thickness and FDG PET were moderate, individuals categorised very similarly using an A(N) system incorporating PiB PET for “A” and either AD signature cortical thickness, or FDG PET, or either of adjusted hippocampal volume or FDG PET, as “(N)”.

Demographic variables also present cut-point-related challenges to AT(N) classification. For example, the prevalence of pathology in all three domains increases with age. When using age-unadjusted cut-points, in cognitively normal individuals the prevalence of A-T-(N)- decreases and the prevalence of A+T+(N)+ increases as age increases from 50 to 90 years [143]. Sex differences in “T” group biomarkers have also been observed within A+ cognitively normal individuals, with A+ women having higher CSF t-tau and p-tau levels than men [144] and higher entorhinal cortical flortaucipir SUVR than men [145]. A possible solution to this would be using age and sex-specific cut-points for determining AT(N) classification, but deriving such cut-points would

again depend upon harmonisation of multiple biomarker studies, use of common standards and ultimately *post mortem* confirmation of diagnosis.

1.13.5 The case for new fluid biomarkers

Hitherto the established biomarkers for AD as detailed in the AT(N) framework have all been derived from their relationships to the core histopathological features of the disease. However, this approach does not account for other components of AD pathology. The following sections give examples of an alternative fluid-based (N) biomarker (NFL – see section 1.14.1, page 88, and section 1.15.2.3, page 123), a synaptic marker (neurogranin: section 1.14.2, page 90), a microglial marker (soluble TREM2: section 1.14.3, page 91), and a marker of blood-brain barrier function (soluble PDGFRβ: section1.14.4, page 92).

Another key aspect of the AT(N) biomarkers is that they are either derived from CSF or from neuroimaging methods. CSF is obtained relatively invasively by lumbar puncture, which requires a skilled operator and appropriate facilities to take, process and store samples, which are sensitive to handling errors. Lumbar puncture is contraindicated in some individuals (for example, due to coagulopathy). Conversely, neuroimaging methods, particularly those involving molecular imaging, are expensive and expose individuals to ionising radiation. Both CSF sampling and molecular imaging therefore present limitations to repeated testing for tracking change over time. Blood biomarkers (see section 1.15, page 95) provide a potential solution to this problem, given their relatively non-invasive sampling methods and potentially cheaper costs. As CSF

sampling studies usually incorporate paired venous blood sampling, this provides a low-added-cost resource for searching for blood biomarkers.

1.13.6 Approaches to biomarker discovery

There are two main approaches to biomarker discovery. The first is the candidate or targeted approach. This has been employed in developing traditional assay methods, such as enzyme linked immunosorbent assays (ELISA), which were developed for all three established CSF biomarkers (Aβ peptides, p-tau and t-tau – see section 2.2.1, page 165 for method details). This approach has its limitations: it is slow and relies on *a priori* assumptions of the link between the candidate analyte and disease pathogenesis. However, this very knowledge of the relationship between the candidate and the disease process allows the context of use of the biomarker to be defined more easily, and methods based on one candidate, or a small number of candidates, are more likely to be scalable and easily generalised to different clinical and research settings.

Another approach is multiplexing, in which an untargeted screen or a large group of candidates is used to identify biomarkers without making any assumptions about their relationship to the disease process. Mass spectrometry (MS), preceded by either liquid chromatography or differential matrix or surface adsorption to enrich samples for proteins at low concentration, is an example of the application of multiplexing to biomarker discovery (see section 5.2.1.2, page 216, for an example of a liquid chromatography-mass spectrometry or LC-MS method). While this type of approach does not rely on the availability of specific antibodies to the analytes of interest, it is this very point that makes the data

noisy. The instruments and high operator dependence of mass spectrometry methods may translate into high costs and make it less easy to generalise.

Ultimately the two approaches are synergistic, in that methods like MS allow researchers to cast a wide net to identify candidates, which can then be replicated, validated and perhaps more easily scaled up using targeted testing methods like ELISA.

1.13.7 Standards for conduct and reporting of studies of diagnostic test accuracy in dementia

Studies investigating new fluid biomarkers in AD should ideally fulfil agreed quality standards, if they are to ensure rapid translation of these biomarkers into widespread research use and/or clinical practice. The QUADAS tool enables quality assessment of studies of diagnostic accuracy to be included in systematic reviews. It covers aspects of patient spectrum, reference standard, disease progression bias, verification bias, review bias, clinical review bias, incorporation bias, test execution, study withdrawals, and indeterminate results ([146]; see Box 1.1).

1. Was the spectrum of patients representative of the patients who will receive the test in practice?
2. Were selection criteria clearly described?
3. Is the reference standard likely to correctly classify the target condition?
4. Is the time period between reference standard and index test short enough to be reasonably sure that the target condition did not change between the two tests?
5. Did the whole sample or a random selection of the sample receive verification using a reference standard of diagnosis?
6. Did patients receive the same reference standard regardless of the index test result?
7. Was the reference standard independent of the index test (i.e., the index test did not form part of the reference standard)?
8. Was the execution of the index test described in sufficient detail to permit replication of the test?
9. Was the execution of the reference standard described in sufficient detail to permit its replication?
10. Were the index test results interpreted without knowledge of the results of the reference standard?
11. Were the reference standard results interpreted without knowledge of the results of the index test?
12. Were the same clinical data available when test results were interpreted as would be available when the test is used in practice?
13. Were uninterpretable/intermediate test results reported?
14. Were withdrawals from the study explained?

Box 1.1: QUADAS tool for quality assessment of studies of diagnostic accuracy to be included in systematic reviews.
Reproduced with permission from [146].

When applied to a systematic review of studies of diagnostic tests in dementia, it was revealed that the commonest areas of methodological weakness were blinding (to biomarker test result when assessing progression to dementia and *vice versa*) and uncertainty about whether patients with technically uninterpretable or intermediate results should be included in reporting the final results [147]. This led to the formulation of the STARDdem standards for reporting the objectives, methods ad results of studies of tests of diagnostic accuracy in dementia ([148]. The four key areas that should be addressed according to these standards are described further in Box 1.2.

1. Study population

Representativeness of spectrum of disorders, proportion of cases with disease, and the severity of cognitive impairment in the population to whom the test would be applied in practice should be reported relative to the study population

2. Reference standard

Consistency of application and use of different standards within the same study or between studies should be reported.

3. Circularity/incorporation bias

This applies if the index test forms part of the reference standard or the performance of the index test is unblinded to the reference standard.

4. Reliability

Intra- and inter-assay variation or intra-individual variation on repeated testing should be reported.

Box 1.2: STARDdem (Standards for Reporting of Diagnostic accuracy in studies of dementia) areas for specific attention.
Paraphrased from Noel-Storr *et al.* [148].

The second area (reference standard) and fourth area (reliability) in particular are worth considering in detail with regard to AD research. In the search for novel biomarkers, the reference or "gold" standard for validation has in recent years moved away from *post mortem* histopathology, which is difficult to obtain within the time frame of most studies or from large numbers of participants, toward the use of the "established" amyloid PET and CSF biomarkers of amyloid, t-tau and p-tau *in vivo*. This has had the effect of accelerating biomarker discovery and has been instrumental in ensuring that new biomarkers are relevant to earlier phases of the disease. It is also in keeping with a general (albeit still controversial) paradigm shift in AD research, away from its association with dementia and the almost inevitably late stages captured in histopathological studies, toward being a pathological continuum that starts in the prolonged preclinical phase.

With regards to fluid biomarker assays, test-retest reliability has been shown to be particularly important for AD research. Pre-analytical sources of variation

may have a significant impact on test results; for example, it is well-known that CSF Aβ42 measurements may be artificially reduced due to adsorption to certain tube surfaces [149]. Hence, guidelines for standardising of collection and pre-analytical processing of both CSF [150] and blood [151] have been developed, but as knowledge on novel biomarkers emerges, aspects of pre-analytical variation should be tested and these guidelines may require updating.

Building upon these approaches to biomarker and discovery validation, several studies have elucidated the potential utility of new biomarkers in CSF (section 1.14, page 88) and blood (section 1.15, page 95). Although other biofluids such as urine and saliva have also been examined, there is a relative paucity of studies utilising them, and a lack of standardised protocols for collecting and processing these biofluids at present, so they will not be discussed here.

1.14 **Emerging CSF biomarkers**

1.14.1 Neurofilament light chain

Neurofilament light chain (NFL) is a member of the class IV intermediate filament family. It is a 70 kDa protein with highly specific expression in central and peripheral nerves [17, 152], where its main physiological roles relate to axonal stability and radial growth. It is also present in synapses (the likely source of release into CSF), where it may undergo faster turnover than in axons [153]. NFL monomers (shown in Figure 1.14) assemble into dimers, tetramers and then cylindrical filaments which elongate by end-to-end annealing.

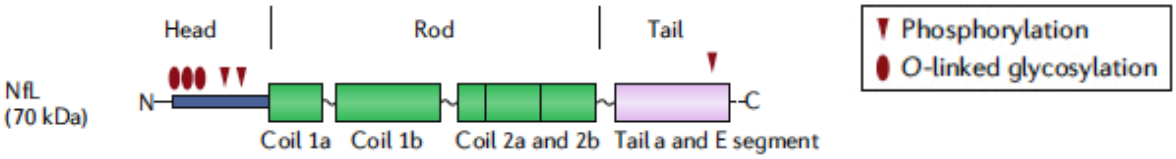


Figure 1.14: Domain structure and post-translational modifications of NFL monomers. Reproduced from Khalil *et al*. [154] with permission (Springer licence: 4590871017607).

The most widely used assays for NFL in CSF, including a commercially available ELISA from Uman Diagnostics [155], employ mouse monoclonal antibodies to conformational antigens on the rod domain. Application of such assays has demonstrated that CSF NFL increases with age [156] and is raised in many neurological disorders involving both acute or rapidly progressive neuronal injury, such as traumatic brain injury [123, 157] and prion disease [158], neuroinflammatory conditions such as multiple sclerosis [159], and neurodegenerative conditions such as Huntington's disease [160], amyotrophic lateral sclerosis [161], FTD spectrum disorders [127, 162-164] and AD (meta-analysis: [165]). In the context of differential diagnosis in dementia, CSF NFL has been shown to improve discrimination of age-matched clinically defined

FTD from early onset AD [166] and of pathology-confirmed or genetically defined FTLD from AD with an effect size (ratio of mean concentrations) of about 2.5 [164]. This is in contrast to its superior ability in discriminating prion diseases (such as either genetic or sporadic CJD) from other dementias with an effect size of 5.3-5.8 [167]. The greater rise in CSF NFL in acute brain insults and in prion disease compared to other dementias echoes a similar finding for CSF t-tau [167], suggesting that there may be a similar contrast between NFL that is released passively from dying neurons or secreted from diseased ones.

The effect size for CSF NFL in AD compared to controls was 2.3 on a meta-analysis of about 600 individuals across 10 studies [165]. This is more modest than the effect sizes for the other diseases mentioned above. As CSF NFL elevation is not specific to AD, in the AD research context NFL captures neurodegeneration in a global sense, which may reflect the effects of multiple co-pathologies. With these caveats in mind, in a group consisting of AD dementia, MCI and controls from ADNI, CSF NFL at baseline was associated with larger ventricular volumes, lower hippocampal volumes, lower FDG PET signal and worse cognition at baseline, and with greater ventricular expansion, and worsening cognition over a mean of 4 years follow-up, in both Aβ positive and negative individuals [168]. CSF NFL also predicted hippocampal atrophy in Aβ negative individuals in the same study. Another Norwegian study of cognitively normal individuals undergoing CSF sampling at the time of spinal anaesthesia showed an age-independent association of CSF NFL with hippocampal atrophy on follow-up over 2 years, regardless of Aβ status [169].

1.14.2 Neurogranin

Neurogranin (Ng), encoded by the NRGN gene on chromosome 11q24.2, is a 7.6 kDa polypeptide of 78 a.a., which is highly conserved across mammalian species and is predominantly expressed at the protein level in the cerebral cortex, hippocampus, amygdala and caudate nucleus [17, 170], where it is detected at cell bodies but concentrated at dendritic spines. Its physiological roles include sequestration of calmodulin, a modulator of intracellular calcium signalling, at post-synapses (as reviewed by Lista and Hampel [171]). This underpins synaptic processes such as long-term potentiation and long-term depression, which are held to be the neural substrates for learning. As with other synaptic markers, immunohistochemistry for Ng indicates it is reduced in brains of individuals with AD compared to controls [172]. However, several studies, using a variety of techniques such as immunoblotting, ELISA and mass spectrometry, have shown that Ng is increased in the CSF of individuals with AD compared to controls [173]; that it is specifically elevated in clinical syndromes linked to AD pathology like AD dementia, MCI-AD and logopenic aphasia but not in bvFTD, semantic dementia or Parkinsonian syndromes [174]; that the moieties raised in CSF are mostly C-terminal fragments of Ng rather than full-length Ng [175]; and that CSF Ng is highly correlated with CSF t-tau and p-tau within individuals regardless of clinical diagnosis [174, 175]. Moreover, elevation of CSF Ng in MCI-AD predicts progression to dementia and baseline CSF Ng predicts longitudinal hippocampal atrophy and FDG PET hypometabolism [176]. Longitudinal CSF sampling has shown that Ng increases significantly only in cognitively normal individuals and not those with MCI or AD dementia [177]. This indicates that the rise of CSF Ng is likely to be a preclinical phenomenon, occurring before substantial neurodegeneration. While the

mechanisms underlying the release of Ng into CSF in early AD are still being investigated, it is therefore a promising AD biomarker. Till recently CSF Ng elevation was thought to be AD-specific, however Blennow *et al.* [178] examined Ng in CJD in comparison to AD and controls, and showed that CSF Ng was higher in CJD than in AD, and even after stratifying individuals with AD by rapid (less than 2 years from symptom onset to death) or slow progression, the significance of the relative elevation in CJD was preserved. They also demonstrated a reduction of CSF Ng with disease duration in CJD, and even greater reduction of cortical and hippocampal staining for Ng on brain immunohistochemistry in CJD compared to AD, with both showing lower staining than controls.

1.14.3 sTREM2

Heterozygous rare variants in the *TREM2* gene on chromosome 6p21.1, which encodes the protein TREM2 (triggering receptor expressed on myeloid cells 2), were discovered in 2013 to be associated with increased risk of sporadic AD [57, 58]. The expression of TREM2 at RNA level in human brain is highest in white matter, medulla, substantia nigra, somewhat lower in other deep nuclei and hippocampus, and at much lower levels in cortical grey matter and cerebellum [57]. While TREM2 is expressed in both neuronal and glial cell types, it is thought that in the brain its main expression occurs in microglia. TREM2 is a transmembrane receptor protein involved in many cellular processes including phagocytosis, proliferation, survival, and regulation of production of inflammatory cytokines. However, the role of microglia and the CNS immune response in AD, is a topic of much debate and the putative mechanisms by which *TREM2* variants may confer risk are as yet unknown. Despite this, several studies have shown that the soluble form of the protein,

sTREM2, which is cleaved and shed from the ectodomain of the membrane-bound receptor, is elevated in CSF in individuals with AD pathology relative to controls. A recent quantitative meta-analysis of 8 studies by Liu *et al.* showed a standardised mean difference (SMD: the ratio of the difference in mean levels between disease and control groups to the standard deviation) of 0.48 across all studies, but the SMD was greater for the MCI-AD vs control comparison (0.77) than for the AD dementia vs control comparison (0.39) or for the preclinical AD vs control comparison (0.48) [179]. Changes in CSF sTREM2 across the disease course may be complex, as Suarez-Calvet *et al*. showed recently in ADNI [180], where sTREM2 was lower in asymptomatic individuals who were categorised by CSF as “A+T(N)-“ than those who were categorised “A-T(N)-“, or than those who are “A+T(N)+” (note that in this study the T and N domains were grouped together, as only 5% of individuals were discordant for T and N as defined by CSF p-tau and t-tau respectively).

Evidence to date suggests that sTREM2 is also a relatively AD-specific biomarker, as it is not elevated in most clinically defined FTD cases compared to controls [181]. Taken together, these findings indicate that CSF sTREM2 may be a useful and specific biomarker for staging of early symptomatic AD pathology.

1.14.4 Other emerging CSF biomarkers

Candidate approaches, employing the results of AD GWAS and the known involvement of synaptic dysfunction, neuroinflammation, and vascular dysregulation in the AD pathophysiological process, are being used to search for new CSF biomarkers. For example, in the domain of synaptic dysfunction, proteins such as visinin-like protein-1 (VILIP-1: [182, 183]), synaptosome-

associated protein -25 (SNAP-25: [183, 184]) and synaptotagmin [185] are known to be elevated in AD CSF compared to controls. In the domain of neuroinflammation and immune modulation, YKL-40 has been identified as being raised both in AD dementia compared to control CSF and also in individuals with MCI who progress to AD dementia compared to those with stable MCI [186, 187]. Vascular dysregulation has also been identified to be an early feature, with heart fatty acid binding protein (hFABP) raised in AD CSF [188]) and soluble platelet- derived growth factor receptor-β (sPDGFR-β) [189, 190] showing promise as a candidate biomarker of blood-brain barrier dysfunction, which may however be independent of the established AD biomarkers of Aβ and tau and may therefore also apply to non-AD pathologies.

In addition, less targeted approaches using transcriptomics, proteomics and metabolomics are being applied. As the methods used for these studies, such as mass spectrometry, are often highly specialised, it is likely that any biomarkers thus identified would have to be validated and translated onto simpler platforms like ELISA in order to facilitate widespread clinical use. For all of the emerging biomarkers, studies will need to evaluate the relative utility of these biomarkers above the established ones, and longitudinal data will need to elucidate how the markers change with disease trajectory. For example, a recent study of several of the novel CSF markers in ADNI showed that Ng, SNAP-25 and VILIP-1 levels (which are elevated at baseline in those with symptomatic AD relative to cognitively normal Aβ- controls) actually reduce during the progression of the symptomatic phase of AD, while YKL-40 levels increase with time in the MCI phase [183]. Ultimately, such longitudinal studies will be able to determine the context in which the use of novel biomarkers will

be appropriate, e.g. in diagnostics for disease specificity in symptomatic individuals, in prognostication or risk stratification in asymptomatic individuals, or as end-points for assessing the effects of disease or response to treatment.

A summary diagram from a review of AD fluid biomarkers by Molinuevo *et al.* ([191]; see Figure 1.15) maps some of the pathological processes involved in AD to the CSF biomarkers that are related to these pathways.

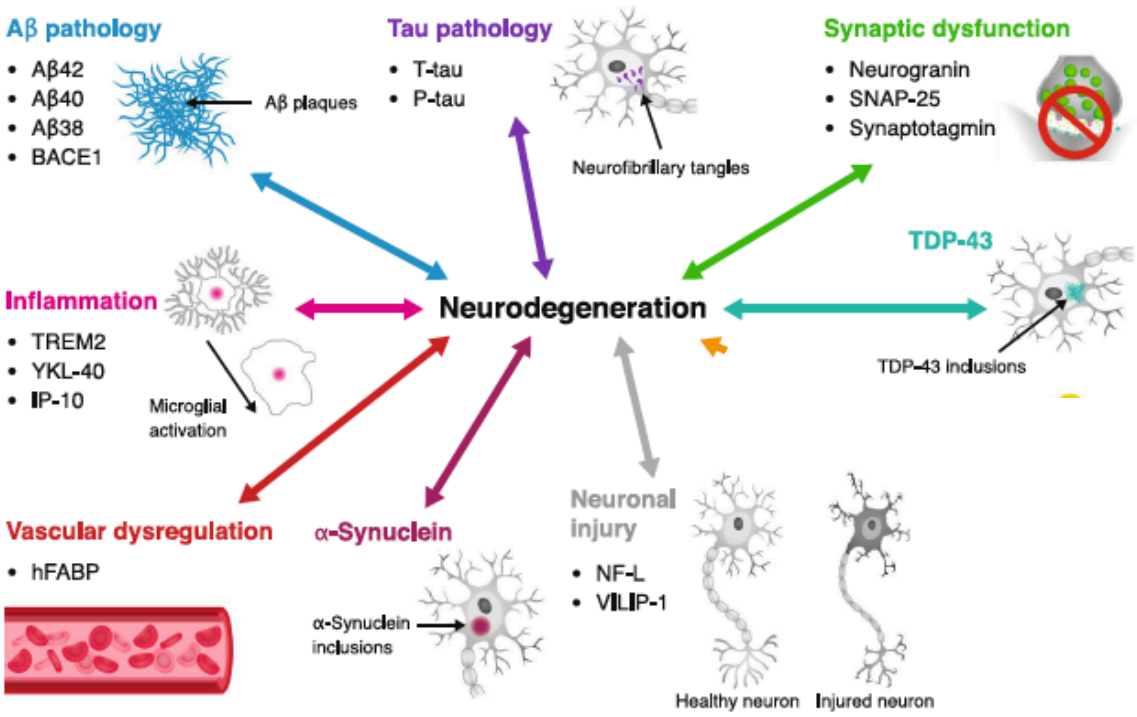


Figure 1.15: Pathological mechanisms implicated in AD and their associated fluid biomarkers. Reproduced from Molinuevo *et al.* [191] with permission, with some content modification (removal of non-fluid biomarkers). TDP-43 and α-synuclein fluid biomarkers are actively being sought and may be relevant to sporadic AD in that they may be significant co-pathologies, particularly in older populations.

1.15 **Blood biomarkers**

1.15.1 Challenges for identifying blood biomarkers

Identification of blood biomarkers that reflect central nervous system (CNS) dysfunction is challenging for many reasons. Molecules from the brain must cross the blood–brain barrier, and their concentration in blood is also likely to be much lower than that in CSF due to the much higher volume of dilution in blood. The high concentration of plasma proteins can be either a sink for secreted proteins from the brain (due to binding or enzymatic breakdown) or non-brain tissues may be a source of similar or identical proteins. Immunoassay-based methods of evaluating these biomarkers in blood may be disrupted by the presence of heterophile antibodies, which are endogenous antibodies that react with the antibodies used in the assay, but are far more abundant in blood than in CSF.

1.15.2 Candidate approaches

1.15.2.1 Amyloid-β

Given the utility of CSF Aβ peptides as biomarkers of AD, Aβ is a logical candidate to investigate in blood. However, until recently, studies using various immunoassay-based methods had provided conflicting results. Table 1.8 (page 96) summarises the results of meta-analyses of these methods performed by Olsson *et al.* [192-195], detailing the results of studies until 2016. Overall, they showed no conclusive result for plasma Aβ42, and a very small positive effect for plasma Aβ40. It was surmised that the lack of utility of blood measurements resulted from there being a substantial peripheral (non-CNS) source of these peptides. However, these studies were limited by analytical sensitivity; some used plasma and others serum; and not all of them compared the blood assays

against “gold standard” biomarker-based classifications of AD vs control individuals. The latter was also a limitation for a large retrospective nested case-control study of plasma samples, published in 2017 by Lovheim *et al.* who were unable to demonstrate classification utility for clinical diagnosis by measurement of free plasma Aβ40 and Aβ42 using the Luminex xMAP immunoassay platform [196].

Table 1.8: Meta-analyses of studies until 2016 measuring plasma Aβ peptides using immunoassay-based methods.
As detailed by Olsson *et al.* [192-195].

Contrast, study date range	**Aβ peptide measured**	**Number of studies included**	**Total number of participants**	**Effect size (95% confidence interval)** ***p***
AD dementia vs Control 2002 – 2016 [192]	Aβ40	26	AD: 2125 Control: 4265	1.065 (1.007, 1.126) *p* = 0.028
AD dementia vs Control 1999 – 2016 [193]	Aβ42	27	AD: 2336 Control: 4452	1.031 (0.962, 1.106) *p* = 0.387
MCI progressing to AD vs stable MCI 2010 – 2011 [195]	Aβ40	3	MCI progressing to AD: 308 Stable MCI: 379	1.066 (1.031, 1.103) *p* = 0.0002
MCI progressing to AD vs stable MCI 2010 – 2011 [194]	Aβ42	3	MCI progressing to AD: 308 Stable MCI: 379	0.807 (0.527, 1.236) *p* = 0.324

From 2016 onward, new ultrasensitive technologies have been applied to cohorts in which AD has been defined by CSF or amyloid PET biomarkers, yielding better evidence for the utility of plasma Aβ peptides in predicting brain fibrillar amyloid deposition. These methods included the single molecule array (Simoa – see section 2.2.2, page 167 for method details) and various targeted mass spectrometry (MS) techniques. Table 1.9 (page 98) summarises the findings of the key studies. The MS techniques appear to have superior

performance, and as discussed by Nakamura *et al.* [197] this may relate to antibody-dependent techniques experiencing interference from heterophile antibodies in the samples. Alternative explanations include pre-analytical differences between studies (as no head-to-head comparisons of the methods have yet been published) or that MS employs denaturation and therefore measures all available Aβ peptides in the sample, whereas antibody-based methods are only able to measure unbound epitopes.

Other approaches adopted by some groups have targeted the secondary structure of plasma Aβ peptides, using an antibody-coupled infrared spectroscopy method that measures an amide I band shift of all Aβ peptides due to β-sheet structure enrichment in samples [198-200], or oligomeric forms, using a multimer detection system ELISA [201] (see Table 1.10, page 105). These techniques will require replication in large cohorts and direct comparison to the more established methods, in order to determine whether they provide additional information or superior discriminant ability for predicting cerebral fibrillar amyloid deposition.

Table 1.9: Summary of studies published after 2016 using ultrasensitive quantification of plasma amyloid-β peptides in AD.

Abbreviations:
Aβ+, amyloid-β positive; Aβ-, amyloid-β negative, AD, Alzheimer's disease; ADC, Amsterdam Dementia Cohort; AIBL, Australian Imaging Biomarker and Lifestyle study; ANCOVA, analysis of covariance; *APOE* ε4, apolipoprotein E gene epsilon 4 allele; AUC, area under the receiver operating characteristics curve; BIOFINDER, Swedish study investigating Biomarkers For Identifying Neurodegenerative Disorders Early and Reliably; CDR, Clinical Dementia Rating Scale; Ctr, Control; ECL, electrochemiluminescence; EDTA, ethylenediamenetetraacetic acid; EMIF, European Medical Informatics Framework; HR, hazard ratio; IMR, immunomagnetic reduction; INSIGHT-preAD, Investigation of Alzheimer's Predictors in Subjective Memory Complainers; IP, immunoprecipitation; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; MCI, mild cognitive impairment; MS, mass spectrometry; MSD, Mesoscale Discovery; NCGG, Japanese National Center for Geriatrics and Gerontology; NTUH, National Taiwan University Hospital; PiB, 11C-Pittsburgh-B compound amyloid PET tracer; sAD, sporadic Alzheimer's disease; SCD, subjective cognitive decline; SCIENCe, Subjective Cognitive Impairment Cohort; Simoa, single molecule array; SUVR, standardised uptake value ratio; WashU, Knight Alzheimer's Disease Research Center at Washington University School of Medicine

Study authors, year	**Assay, blood fraction**	**Cohort, <u>baseline vs longitudinal blood sampling</u>, group (N)**	**Findings**
Sporadic AD (including biomarker-based definitions)			
Janelidze *et al.* 2016 [202]	Simoa, EDTA-plasma	BIOFINDER <u>Baseline (719)</u> Clinically defined cases: Ctr (274) SCD (174) MCI (214) AD dementia (57) CSF amyloid signature-defined cases:	Across all individuals, weak positive correlations were seen between plasma and CSF measurements of Aβ42, Aβ40 and Aβ42/40 ratio. Weak negative correlations were seen between ^{18}F-flutemetamol PET SUVR and either of plasma Aβ42 and Aβ42/40 ratio. All Aβ+ groups had lower plasma Aβ42 and lower plasma Aβ42/40 ratio than Aβ- Ctr. After controlling for age, sex and diagnosis, people with hypertension, ischaemic heart disease, diabetes or those taking antihypertensive/cardio-protective medications all had higher plasma Aβ42 and Aβ40 than those who did not have these vascular risk factors. The plasma Aβ42/40 ratio was also higher in those taking these medications but the other vascular risk factor groups did not show significant differences in the

		Ctr Aβ- (200) Ctr Aβ+ (74) SCD Aβ+ (60) MCI Aβ+ (121) AD Aβ+ (53)	plasma Aβ42/40 ratio. There were no significant differences in CSF measurements of Aβ42, Aβ40 or Aβ42/40 ratio according to these vascular risk factor groups.
Ovod *et al.* 2017 [203]	IP followed by LC-MS/MS, EDTA-plasma	WashU <u>Longitudinal over 24 hours (41)</u> CDR definition of cases: CDR = 0 (27) CDR > 0 (14) PET or CSF definition of cases Aβ- (23) Aβ+ (18)	This study detailed the kinetics of plasma Aβ peptides using stable isotope labelling kinetics (SILK) following from a publication by the same group detailing the application of SILK to CSF Aβ [204]. Baseline plasma Aβ42 and Aβ42/40 ratio, and the 24-hour average of each of these, were significantly lower in the Aβ+ compared to Aβ- group. The 24-hour average Aβ42/40 ratio was able to distinguish the groups with AUC of 0.887. Aβ42/40 ratio was moderately correlated in plasma and CSF within individuals. The half-life of different Aβ isoforms in plasma was similar (about 3 hours) but much shorter than that previously reported for CSF (about 9 hours). For both Aβ- and Aβ+ groups, labelled Aβ38 peaked earlier and decayed faster than Aβ40 or Aβ42 – this was unique to plasma (not seen previously in CSF). The labelled plasma Aβ42/40 ratio remained constant in the Aβ- group but dropped after 12 hours in the Aβ+ group, which indicated a faster turnover rate in these individuals, similar to that previously seen in CSF.
Nakamura *et al.* 2018 [197]	IP-MS (MALDI-TOF), EDTA-plasma	1. NCGG <u>Baseline (121)</u> Ctr (62) MCI (30) AD dementia (29) Classification by amyloid PET: Aβ- (71)	This study examined the relative performance of the plasma biomarkers Aβ1-42, Aβ1-40/1-42, APP669-771/ Aβ1-42, and a composite biomarker (the 1:1 weighted average of the latter two ratios) in two independent cohorts. Pooled analyses using Aβ status classification by different PET tracers utilised a common centiloid scale. Combining all participants and tracers within each cohort, in both cohorts all four of the above biomarkers gave significant improvements above chance in predicting PET-amyloid positivity. In unadjusted analyses, in NCGG Aβ1-40/1-42 and the composite

		Aβ+ (50) 2. AIBL <u>Baseline (252)</u> Ctr (156) MCI (67) AD dementia (29) Classification by amyloid PET: Aβ- 115 Aβ+ 137	performed similarly (AUC both 0.967) but better than APP669-771/ Aβ1-42 (0.923) or Aβ1-42 (0.872). In AIBL across all tracers, the composite performed better (AUC 0.883) than Aβ1-40/1-42 (0.837), APP669-771/ Aβ1-42 (0.828) or Aβ1-42 (0.718). Performance of all 4 biomarkers was better when analysing within the groups that had PiB PET (in both NCGG and AIBL) compared to those that were scanned using 18-F tracers (flutemetamol and florbetapir in AIBL). Analyses adjusting for age, sex, *APOE* ε4 carrier status and clinical diagnosis improved classification accuracy overall but did not change the pattern of the results. In both cohorts, classification accuracy of the composite biomarker was better within the AD dementia and MCI clinical groups than the Ctr group. All four biomarkers showed moderately high correlations with continuous PET SUVR.
Verberk *et al.* 2018 [205]	Simoa, EDTA-plasma	ADC and SCIENCe <u>Baseline (248)</u> Classification by CSF Aβ42: Aβ- (191) Aβ+ (57) Subset with amyloid PET available (69), classified by visual read: Aβ- (46) Aβ+ (23)	This was a study of SCD stratified by Aβ status. Plasma Aβ42 and Aβ42/40 were both weakly correlated with CSF Aβ42 within individuals across the cohort. Unadjusted AUC for discriminating CSF Aβ status was 0.77 for plasma Aβ42/40 and plasma 0.66 for Aβ42. Adjusted for age and *APOE* ε4 carrier status, AUC was 0.83 for plasma Aβ42/40. Unadjusted AUC for discriminating PET Aβ status was 0.68 for plasma Aβ42/40 and plasma 0.66 for Aβ42. Adjusted for age and *APOE* ε4 carrier status, AUC was 0.79 for plasma Aβ42/40. Lower plasma Aβ42/40 was associated with increased risk of clinical progression to MCI or dementia over a median of 2.8 years of follow-up (HR 2.07), and this persisted after adjustment for age and sex (HR 1.67).
Shahpasand-Kroner *et al.* 2018 [206]	Immunoprecipitation followed by MSD	Goettingen University <u>Baseline (40)</u> 23 AD dementia	No significant associations were noted for CSF and plasma values of Aβ42 or of Aβ40 within individuals but plasma Aβ42/40 was moderately highly correlated with CSF Aβ42/40 and CSF Aβ42/t-tau, and negatively correlated with CSF t-tau and p-tau-181.

	triplex ECL, EDTA-plasma	17 Other dementia CSF biomarkers available in 37 and amyloid PET in 18 individuals; all diagnoses assigned on clinical and biomarker basis	Plasma Aβ42/40 also showed a moderately high negative correlation with ^{18}F-florbetaben SUVR. AUC for AD vs other dementia was 0.87 for plasma Aβ42/40 and 0.80 for plasma Aβ42/38 (no significant difference). Adjustment for age, sex and *APOE* ε4 carrier status was not undertaken but *APOE* ε4 carriers were shown to have significantly lower plasma Aβ42/40 and Aβ42/38 than non-carriers.
Palmqvist *et al.* 2018 [207]	ECL, EDTA-plasma	BIOFINDER Baseline (850) Ctr (319) SCD (196) MCI (266) AD dementia (69)	This study examined the utility of the Roche Elecsys® plasma Aβ42/40 in prediction of CSF Aβ status. All AD dementia cases were defined using a combination of clinical criteria and CSF Aβ status (determined by testing of paired CSF samples on the same platform as used for plasma). The unadjusted AUC across the whole cohort was 0.80; adding age and *APOE* ε4 carrier status improved this to 0.86 and adding cognitive tests to the model further increased AUC to 0.88-0.89. Subgroup-specific ROC analyses in the cognitively unimpaired (Ctr + SCD) and MCI groups showed similar AUC (±0.02). In SCD and MCI participants, physicians diagnosed Aβ positivity correctly in 65% of cases pre-test. An optimal cut-point for plasma Aβ42/40 diagnosed 75% correctly, and adding age and *APOE* ε4 carrier status increased this to 79%.
Li *et al.* 2019 [208]	Simoa, EDTA-plasma	Daping Memory Clinic Baseline (84) Ctr 9 MCI 22 AD dementia 53	This study was the first to utilise amyloid PET in a Chinese memory clinic population. Plasma Aβ42/40 was able to distinguish Aβ+ from Aβ- with AUC 0.77 (logistic odds ratio 0.78 after adjusting for age, sex and *APOE* ε4 carrier status). Although Aβ42 was significantly lower in clinically diagnosed AD patients than in MCI (unadjusted $p < 0.05$) this did not translate into a significant difference when examining by Aβ status.

		Classification by PiB PET visual rating: Aβ- (36) Aβ+ (48)	
Park *et al*. 2019 [209]	Simoa, EDTA-plasma	KBASE <u>Longitudinal (76)</u> Ctr (52) MCI (9) AD (15)	This Korean study, at two timepoints two years apart, measured plasma amyloids using the Luminex xMAP platform, and t-tau and p-tau-181 using commercially available Simoa kits (however the same p-tau assay is no longer commercially available). A detailed neuroimaging protocol including 3T MRI and FDG and PiB PET was employed at baseline, and flortaucipir PET was added at 2-year follow-up. The main aim was to examine the associations of plasma tau measurements with tau PET and assess the relative predictive ability of plasma t-tau, p-tau-181, t-tau/Aβ42 and p-tau/Aβ42 for AD-associated tauopathy. Additional analyses were also presented relating to plasma Aβ42. After adjusting for age and sex, higher plasma Aβ42 was associated cross-sectionally with lower PiB SUVR, and with higher AD (temporal) region of interest tau PET signal.
Vergallo *et al.* 2019 [210]	Simoa, plasma (anticoagulant not specified)	INSIGHT-preAD <u>Baseline (276)</u> Classification by PET amyloid status at T1: Aβ- (203) Aβ+ (73) <u>Longitudinal</u> Timepoint 2 (T2: 215) Timepoint 3(T3: 134)	This was a study of SCD stratified by Aβ status determined using 18-F florbetapir PET. Plasma Aβ42 and Aβ40 both correlated with age but showed no differences between the sexes. *APOE* ε4 carriers had lower plasma Aβ42 but similar Aβ40/42 ratio as compared to non-carriers. Plasma t-tau, NFL, YKL-40 and BACE1 were also measured but only the latter two markers were significantly elevated in Aβ+ compared to Aβ-. At T1, AUC for PET amyloid status was 0.68 for plasma Aβ42 and 0.79 for Aβ40/42. A machine learning approach was also applied, utilising the longitudinal data for verification, and of all available biomarkers it selected only Aβ40/42 ratio to be a useful classifier for PET amyloid status; demographic variables of age, sex and *APOE* ε4 carrier status did not improve the derived AUC which was similar to that from simple ROC analysis based on a priori selection of Aβ40/42 ratio as a predictor.

			Although longitudinal blood data were available for a subset of 66 individuals for each of the three timepoints, they were not utilised to draw associations with baseline SUVR.
Down Syndrome (DS) AD			
Lee *et al.* 2016 [211]	IMR, plasma (anticoagulant not specified)	Taiwan DS Foundation and NTUH outpatient clinic cohort Baseline (191) Ctr 78 sAD dementia (62) DS without dementia (35) DS with dementia or its prodrome (16) Ctr and sAD were diagnosed purely clinically.	This study was formulated to examine differences between DS AD and sAD; however, it did not employ any "gold standard" biomarkers such as CSF or PET for diagnosis of sAD and Ctr. Therefore, the main utility of the study is in investigating within the DS groups. Plasma Aβ42 and Aβ42/40 were both elevated, and Aβ40 was reduced in individuals with DS with dementia or its prodrome, compared to individuals with DS without dementia. The AUC were 0.90, 0.94 and 0.90 for Aβ40, Aβ42 and Aβ42/40 respectively. However, the analyses did not adjust for age differences (mean age 35 vs 25 years in DS with and without dementia respectively).
Fortea *et al.* 2018 [212]	Simoa, plasma (anticoagulant not specified)	Catalonian Down Syndrome cohort Baseline (347): DS without dementia (192), Prodromal AD DS (39), AD dementia DS (49) Ctr (67)	Plasma Aβ42 and Aβ40 were both significantly higher in all DS compared to Ctr. Plasma Aβ42 was unable to distinguish between asymptomatic, prodromal or AD dementia groups; plasma Aβ40 was significantly raised in AD dementia DS compared to asymptomatic DS but had no other between-group differences. No significant correlation was found between CSF and plasma values of these peptides with individuals. Given that CSF Aβ42 showed the opposite pattern to plasma Aβ42 across the groups, the plasma peptide level differences might relate to altered peripheral synthesis of these peptides. This study did not report findings in relation to the plasma Aβ42/40 ratio, and although CSF was taken for Ctr they were not stratified into Aβ+ and Aβ- for analysis.

Startin *et al.* 2019 [213]	Simoa, EDTA-plasma	LonDOWNs (DS) and EMIF (Perugia and Barcelona sites) <u>Baseline (85)</u> Ctr (27) sAD with CSF Aβ42 confirmation (27) DS (31): with dementia (7), without (24)	ANCOVA adjusted for age, sex and *APOE* ε4 carrier status showed significantly higher plasma Aβ42 and Aβ40 in DS compared to sAD, and significantly lower plasma Aβ42/40 in both DS and sAD compared to Ctr, however comparisons were limited by lack of characterisation of the Ctr group according to a "gold standard" CSF or PET biomarker of AD. No clear difference in plasma Aβ42, Aβ40 or Aβ42/40 was seen in DS with dementia compared to without, but statistical testing could not be undertaken due to small sample size.

Table 1.10: Summary of studies using methods for detection of plasma amyloid-β peptide secondary structures or oligomers in AD.

Abbreviations:
Aβ+, amyloid-β positive; Aβ-, amyloid-β negative, AD, Alzheimer's disease; Aβo, amyloid-β oligomers; Alzheimer's disease; AIBL, Australian Imaging Biomarker and Lifestyle study; APOE ε4, apolipoprotein E gene epsilon 4 allele; AUC, area under the receiver operating characteristics curve; BIOFINDER, Swedish study investigating Biomarkers For Identifying Neurodegenerative Disorders Early and Reliably; CDR, Clinical Dementia Rating Scale; Ctr, Control; EDTA, ethylenediamenetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESTHER, German (Saarland) epidemiological study on chances of prevention, early detection and optimized treatment of chronic diseases in the elderly population; MCI, mild cognitive impairment.

Study authors, year	**Assay, blood fraction**	**Cohort, baseline vs longitudinal blood sampling, group (N)**	**Findings**
Nabers *et al*. 2016 [198]	Immuno-infrared, EDTA-plasma	Duisberg-Essen memory clinic Baseline (110) Disease Ctr including non-AD dementia (66) MCI-AD (11) AD dementia (33) All diagnoses were validated by CSF Aβ profile.	For discrimination of AD dementia vs disease Ctr, measurement of amide I frequency shift gave AUC of 0.90 in CSF and 0.83 in plasma. For discrimination of MCI-AD vs disease Ctr, the AUC in CSF was 0.79 and in plasma 0.71.
Wang *et al.* 2017 [201]	Multimer-detection system ELISA, heparinised plasma	Seoul and Chung-Ang University Baseline (61) Ctr (37) AD dementia (24)	Plasma Aβo were moderately negatively correlated with CSF Aβ42, and moderately positively correlated with PiB SUVR, CSF p-tau-181 and t-tau. The AUC for discriminating AD dementia from Ctr was 0.84, but the study was limited by considering only the clinical status as the reference standard and not utilising the CSF or PET standards for direct comparison.

		Diagnoses were assigned on clinical criteria despite CSF and PET availability.	The method employed also relied on spiking recombinant Aβ42 into samples, to result in a purported amplification of the Aβo present in the samples, but still required a very long incubation time of 144 hours and the actual nature of the Aβo thus detected might be heterogeneous.
Nabers *et al*. 2018 [199]	Immuno-infrared, EDTA-plasma (BIOFINDER); heparinised plasma (ESTHER)	BIOFINDER <u>Baseline (73)</u> Ctr Aβ- (37) MCI Aβ+ (36) Aβ status was defined by amyloid PET. ESTHER prospective nested case-control study <u>Baseline (312)</u> Ctr (247) AD dementia (65) Vascular Dementia (66) Mixed dementia (36)	This study extended the findings of the 2016 publication to individuals with less clinically severe AD in a biomarker discovery cohort, and to true prospective prediction of clinical AD in a population-representative cohort. <u>BIOFINDER</u> Amide I frequency shift was moderately positively correlated with CSF Aβ42 and Aβ42/40, moderately negatively correlated with CSF t-tau and SUVR, and weakly negatively correlated with CSF p-tau-181. The AUC for distinguishing Aβ+ from Aβ- was 0.76. <u>ESTHER</u> Diagnoses were defined clinically at the end 8 years of follow-up but not supported by "gold standard" biomarkers. Individuals with dementia and Ctr were matched by age, sex and education. Using plasma samples taken 8 years before diagnosis, the AUC for distinguishing AD dementia from Ctr was 0.80. Individuals with vascular dementia and mixed dementia did not display the amide I frequency shift.
Nabers *et al.* 2019 [200]	Immuno-infrared, EDTA-plasma	Duisberg-Essen memory clinic <u>Baseline (100)</u> Disease Ctr including non-AD dementia (61) AD dementia (39)	This study investigated the use of the plasma Aβ amide I frequency shift as a pre-screener to CSF Aβ and tau secondary structure-based measurements to classify AD vs non-AD dementias. Using a pre-specified cut-point for 90% sensitivity in identifying AD, 35 of 39 AD cases were correctly identified by the plasma test (but of the four that were misclassified, 3

		All diagnoses were clinical but CSF Aβ42/40, t-tau and p-tau-181 were tested to provide a gold standard of classification.	actually did not fulfil AD gold standard CSF biomarker criteria). 24 of 61 disease Ctr were falsely identified as AD. On a second diagnostic step, testing of CSF Aβ and tau secondary structure-based biomarkers was included, and the total number of false positives was reduced to only 2 of 61.

1.15.2.2 Tau

As was the case with Aβ peptides, blood measurements of t-tau (i.e. mid-region -directed immunoassays) have shown variable utility in AD. The meta-analysis by Olsson *et al.* included eight studies up to 2015 [214] incorporating measurements from 447 individuals with AD and 552 controls, and showed a combined effect size of 1.78 (95% confidence interval 1.19 – 2.69; $p = 0.006$). However, the studies included were heterogeneous, and differences in results could largely be ascribed to method sensitivity. ELISA methods generally showed small or non-significant effect sizes, while more sensitive immunomagnetic reduction (IMR) or Simoa techniques revealed effect sizes of 1.99 to 4.52.

As ultrasensitive measurement techniques such as Simoa and MS have become more widely adopted, more evidence has accumulated to support plasma t-tau as a biomarker that shows cross-sectional and longitudinal associations with measures of brain atrophy and cognitive decline, despite its poor correlation with CSF t-tau within individuals, and mixed findings regarding whether or not these associations are modified by Aβ status. The findings of relevant studies in AD and in groups at risk of AD, from 2016 onward, are summarised in Table 1.11, page 110.

T-tau in blood is not an AD-specific biomarker, as it is elevated in other neurodegenerative conditions, such as CJD [158, 215], and even in minor traumatic brain injury [216]. Plasma p-tau-181 has also been measured by some groups, each using a different technique that is yet to be replicated. One study has shown Aβ-dependent associations between plasma p-tau-181 and

flortaucipir PET, unlike t-tau where the association is not Aβ-dependent [217], therefore p-tau-181 may prove to be a more AD-specific biomarker if these findings are replicated. Nevertheless, a large population based study has recently shown that plasma t-tau may still have utility in reducing sample sizes for recruitment to a preventative trial for all-cause dementia or for AD-dementia [218].

Simoa has been the widest used technique in plasma tau research, but one group has published several papers employing IMR, in which the values of t-tau and p-tau obtained are at least one order of magnitude above those in the Simoa publications, and although the authors claim that this is due to IMR being a more “direct” method of measurement than Simoa [219], it is possible that the Simoa and IMR assays are measuring different populations of tau or its fragments.

Table 1.11: Summary of studies published after 2016 using ultrasensitive measurement of tau in blood in cohorts relevant to AD.

Abbreviations:
Aβ +, amyloid-β positive; Aβ -, amyloid-β negative, AD, Alzheimer's disease; ADC, Amsterdam Dementia Cohort; ADNI, Alzheimer's Disease Neuroimaging Initiative; AIBL, Australian Imaging Biomarker and Lifestyle study; *APOE* ε4, apolipoprotein E gene epsilon 4 allele; AUC, area under the receiver operating characteristics curve; BIOFINDER, Swedish study investigating Biomarkers For Identifying Neurodegenerative Disorders Early and Reliably; BSHRI, Banner Sun Health Research Institute; CDR, Clinical Dementia Rating Scale; Ctr, Control; DELCODE, German Center for Neurodegenerative Diseases longitudinal study on cognition and dementia; EDTA, ethylenediamenetetraacetic acid; EMIF, European Medical Informatics Framework; FHS, Framingham Heart Study; HR, hazard ratio; KBASE, Korean Brain Aging Study for the Early Diagnosis and Prediction of Alzheimer's disease; MCI, mild cognitive impairment; MCSA, Mayo Clinic Study of Aging; NTUH, National Taiwan University Hospital; PiB, 11C-Pittsburgh-B compound amyloid PET tracer; SCD, subjective cognitive decline; SCIENCe, Subjective Cognitive Impairment Cohort; Simoa, single molecule array; TIV, total intracranial volume.

Study authors, year	**Assay, blood fraction**	**Cohort, <u>baseline vs longitudinal blood sampling</u>, group (N)**	**Findings**
Sporadic AD and asymptomatic at risk			
Mattsson *et al.* 2016 [220]	Simoa, EDTA-plasma	1. ADNI <u>Baseline (563)</u> Ctr (189) MCI (195) AD dementia (179) 2. BIOFINDER Baseline (721) Ctr (274) SCD (174) MCI (212) AD dementia (61) CSF Aβ status stratification was	This study of two large and well-characterised cohorts revealed significant differences in the associations of plasma tau between the cohorts. In ADNI, baseline and longitudinal (over about four years) measures of cognition and neuroimaging were also available, and gave evidence for plasma t-tau being a biomarker of symptomatic progression but not of asymptomatic AD. Plasma t-tau values were higher in BIOFINDER than in ADNI overall but two different reagent lots were used in different laboratories, and the pre-analytical freeze-thaw cycle number was one more in ADNI than BIOFINDER. Plasma tau did not correlate with age, sex or education in either cohort. It was elevated in *APOE* ε4 carriers compared to non-carriers only in ADNI. It was weakly negatively correlated with CSF Aβ42 but not correlated with CSF t-tau or p-tau-181 in ADNI. Plasma t-tau was significantly increased in AD dementia and MCI Aβ+, compared to MCI Aβ– or all Ctr, regardless of Aβ status.

		applied and only Aβ+ AD dementia was included	Conversely, plasma t-tau was weakly positively correlated with CSF t-tau and p-tau-181 (driven by the AD dementia group) but not CSF Aβ42 in BIOFINDER. Higher plasma tau was not associated with MMSE at baseline in BIOFINDER. <u>Baseline cognitive and imaging measures in ADNI</u> Higher plasma t-tau was associated with worse ADAS-Cog and higher ventricular volume across the cohort, and with lower MMSE, worse ADAS-Cog and higher ventricular volume specifically in the AD dementia group. <u>Longitudinal cognitive and imaging measures in ADNI</u> Higher plasma t-tau was associated with steeper decline of ADAS-Cog, MMSE, hippocampal volume and FDG PET signal, and accelerated increase of ventricular volume. The ADAS-Cog, MMSE and ventricular volume findings were again likely driven by the AD dementia group. All the above associations were adjusted for age, sex, education and *APOE* ε4 carrier status.
Dage *et al.* 2016 [221]	Simoa, EDTA-plasma	MCSA <u>Baseline (539)</u> Ctr (378) MCI (161) Aβ+ status was available from PiB PET.	In this large cross-sectional community-based study, plasma t-tau was not statistically significantly higher in MCI than in Ctr. However, after adjustment for age, sex, education and *APOE* ε4 carrier status, higher plasma t-tau was associated with worse performance in tests of memory and reduced AD signature region temporal cortical thickness (which was driven predominantly by the Ctr group). FDG PET signal and hippocampal volume were not associated with plasma t-tau in either group. Association of SUVR as a continuous measure with plasma t-tau did not survive adjustment for covariates across the whole group but dichotomised Aβ+ status was associated with higher plasma t-tau within the Ctr group. Across both groups, presence of diabetes, hypertension and atrial fibrillation were associated with higher plasma t-tau. In the Ctr group, current NSAID use, current

			statin use and previous myocardial infarction were also associated with higher plasma t-tau.
Deters *et al.* 2017 [222]	Simoa, EDTA-plasma	ADNI Baseline (508) Ctr (166) MCI (174) AD dementia (168) Valid CSF measures of Aβ42, t-tau and p-tau-181 were available for 331 individuals (91 Ctr, 158 MCI, 82 AD) and CSF Aβ42 was used to determine Aβ status.	The previously quantified plasma t-tau values from Mattsson *et al*. [220] were used to probe associations with AD signature temporal cortical thickness and regional brain volumes by voxel-based morphometry, and compared these with similar associations for CSF t-tau. Across all participants no significant association was found with AD cortical thickness, but higher plasma t-tau was significantly associated with lower grey matter density in the medial temporal lobe, precuneus, striatum, thalamus middle and inferior frontal gyri after adjustment for age, sex *APOE* ε4 carrier status and TIV; when controlled for diagnosis the associations for right thalamus and bilateral striatum remained significant. After stratifying by Aβ status, no significant associations were found between plasma t-tau and grey matter density in Aβ- participants, but in Aβ+ participants there were significant associations in medial temporal lobe, precuneus, premotor cortex, pre- and post-central gyri, frontal and parietal lobes and globus pallidus. Associations with parahippocampus, premotor cortex and precuneus survived further adjustment for diagnosis. Across the whole cohort, associated grey matter density locations for plasma t-tau were mostly different from those for CSF t-tau, apart from some shared associations in the temporal pole, fusiform and angular gyrus (unadjusted for diagnosis). Within the Aβ+ group, no significant overlap was seen for grey matter density anatomical locations associated with plasma vs CSF t-tau.
Chiu *et al.* 2017 [223]	IMR, EDTA-plasma	NTUH Baseline (126)	In this small study of Taiwanese middle-aged and older cognitively normal individuals, plasma t-tau was associated with age and was higher in men and in *APOE* ε4 carriers.

		MRI data were available for 123 individuals.	In linear regression models adjusted for age, sex, *APOE* ε4 carrier status and education, there were no significant associations between plasma t-tau and hippocampal volumes, global cortical thickness or white matter hypodensities. The analysis was limited by lack of stratification by Aβ status.
Lue *et al.* 2017 [224]	IMR, EDTA-plasma	1. BSHRI <u>Baseline (36)</u> Ctr (16) AD dementia (16) 2. NTUH <u>Baseline (94)</u> Ctr (63) AD dementia (31) All diagnoses were clinically assigned.	The authors analysed each cohort individually and then combined both to do further ROC analysis but this approach was limited by differences in study populations and lack of stratification in either cohort by Aβ status. In both cohorts the AD dementia group had higher plasma t-tau and lower plasma Aβ42/t-tau ratio than the Ctr group after adjusting for age.
Mielke *et al.* 2017 [225]	Simoa, EDTA-plasma	MCSA <u>Baseline (458)</u> Ctr (335) MCI (123) Aβ+ status was available from PiB PET.	This study provided longitudinal outcome data over a median follow-up of 3 years from the cohort described by Dage *et al*. [221]. Using age as the time-scale for Cox regression, in Ctr, one log unit increase of plasma t-tau was associated with a HR of 2.5 for all-cause MCI but this was attenuated after adjusting for sex, education and *APOE* ε4 carrier status; analysis with amnestic MCI as the outcome did not achieve statistical significance. In MCI, no significant increased risk of all-cause dementia or of clinical AD dementia was seen with increase in plasma t-tau. Across the whole cohort, over the entire follow-up period, higher plasma t-tau was associated with steeper decline in global cognition, memory, attention and visuospatial

			ability, after adjusting for covariates. The relationship with decline in visuospatial ability was driven by participants with MCI and not Ctr. Over a restricted follow-up period of the first 15 months after blood sampling, higher plasma t-tau predicted cognitive decline both globally and in the visuospatial domain only in MCI and not Ctr. Adjusting for Aβ status did not confound or modify any of the above findings.
Müller et al. 2017 [226]	Simoa, EDTA-plasma	DELCODE Baseline (245) Ctr (134) SCD (111) CSF was available for only 90 individuals (50 Ctr and 45 SCD).	No significant differences in plasma t-tau (after adjustment for age, sex, education and *APOE* ε4 carrier status) were found between Ctr and SCD. Numbers for stratification by CSF Aβ status were small and there was no significant difference in plasma t-tau between Aβ- Ctr and Aβ+ SCD when employing any of three different definitions of CSF Aβ status. There was no significant association within the SCD group of plasma t-tau levels with MMSE or logical memory.
Mielke *et al.* 2018 [217]	Simoa (t-tau) and MSD (p-tau-181), EDTA-plasma	MCSA Baseline (249) Ctr (152) MCI (57) AD dementia (40)	This study utilised a homebrewed p-tau-181 MSD assay (detailed methods not provided), which has not been replicated till date, and the Simoa plasma t-tau values from Dage *et al.* [221], to investigate relationships with PiB and flortaucipir PET. All analyses were adjusted for age, sex and *APOE* ε4 carrier status. Across the whole cohort, higher plasma t-tau and p-tau-181 were both associated with either higher continuous Aβ PET SUVR or with binary Aβ PET status. They were both also associated with lower cortical thickness outcomes, and with higher entorhinal tau PET. Within diagnostic subgroups, higher plasma p-tau-181, but not t-tau, was associated with higher Aβ PET SUVR in each of the three groups, and with higher entorhinal tau PET in MCI and AD dementia. Conversely, higher plasma t-tau, but not p-tau-181, was associated with AD signature cortical thickness as a binary variable in the Ctr group and as a continuous variable in the MCI group.

			ROC analyses for Aβ PET status showed that age and *APOE* ε4 carrier status gave AUC 0.750 across the whole cohort and 0.747 across non-demented individuals (Ctr + MCI). The corresponding AUC for plasma p-tau-181 were 0.803 and 0.750, and for t-tau were 0.598 and 0.564, so p-tau-181 performed at least as well as age and *APOE* ε4 carrier status in predicting Aβ PET status. Analyses by *APOE* ε4 subgroup showed that AUC were higher for both p-tau-181 and t-tau in the *APOE* ε4 carriers (1 or 2 alleles) than in non-carriers, and p-tau-181 retained its superior predictive ability. Correlations with entorhinal tau PET by clinical and Aβ status subgroups showed that plasma p-tau-181 was correlated with entorhinal tau PET SUVR only within the Aβ+ subgroups of Ctr and MCI. Conversely, correlations between t-tau and tau PET SUVR emerged only in the Aβ- sub groups of Ctr and across both Aβ status subgroups across the whole cohort. This finding suggested that plasma p-tau-181 elevation is a more AD-specific process than plasma t-tau elevation.
Verberk et al. 2018 [205]	Simoa, EDTA-plasma	ADC and SCIENCe <u>Baseline (248)</u> Classification by CSF Aβ42: Aβ- (191) Aβ+ (57) Subset with amyloid PET available (69), classified by visual read: Aβ- (46) Aβ+ (23)	This study of individuals with SCD was designed to probe the utility of plasma markers of amyloid and tau in predicting Aβ status. All three plasma measures (Aβ42, Aβ40 and t-tau) were weakly positively correlated with each other. There was no significant association of plasma t-tau with either CSF t-tau or p-tau-181, or with CSF Aβ status. Plasma t-tau was not associated with risk of progression to MCI or AD within a median follow-up of 2.8 years.

Yang *et al.* 2018 [219]	IMR, EDTA-plasma	NTUH Baseline (73) Ctr (23) MCI (29) AD dementia (21) All diagnoses were clinically assigned.	This study described the first use of a IMR assay for p-tau-181; all measured plasma values in the study were at least 100 times above the lower limit of detection of 0.019 pg/ml. The authors also measured plasma t-tau using a similar technique. Significant unadjusted differences were found between groups with the AD dementia group having higher plasma -tau-181 than the MCI group, which in turn had higher p-tau-181 than the Ctr group. The authors sought to justify lack of age-adjustment by showing that age did not impact plasma p-tau-181 in Ctr but did not demonstrate a lack of effect of age within the other diagnostic groups. For plasma t-tau, there were no significant differences between the MCI and AD dementia groups but both had significantly higher values than Ctr. While the authors performed ROC analyses for discriminating the MCI from Ctr and AD from MCI groups for both biomarkers, these analyses were limited by lack of adjustment for age and *APOE* ε4, and by relying on clinical rather than "gold-standard" CSF or PET classification.
Park *et al.* 2019 [209]	Simoa, EDTA-plasma	KBASE Longitudinal (76) Ctr (52) MCI (9) AD (15)	This Korean study, at two timepoints two years apart, measured plasma amyloids using the Luminex xMAP platform, and t-tau and p-tau-181 using commercially available Simoa kits (however the same p-tau assay is no longer commercially available). A detailed neuroimaging protocol including 3T MRI and FDG and PiB PET was employed at baseline, and flortaucipir PET was added at 2-year follow-up. The main aim was to examine the associations of plasma tau measurements with tau PET and assess the relative predictive ability of plasma t-tau, p-tau-181, t-tau/Aβ42 and p-tau/Aβ42 for AD-associated tauopathy (increased tau PET signal in a temporal composite region). Higher values of all four biomarkers correlated with increased *in vivo* Braak stage at 2 years, after adjusting for age and sex. Logistic regressions using tau PET binarized as tau- (Braak stage ≤ II) or tau+ (Braak stage ≥ III) showed all four biomarkers were

			able to discriminate tau- from tau+. AUC was highest for plasma t-tau/Aβ42. The change in t-tau/Aβ42 over 2 years also correlated with AD region of interest tau PET signal at 2 years. Voxel-wise correlations at 2 years adjusted for age and sex showed higher plasma t-tau and p-tau-181 were associated with increased medial temporal tau PET signal. However, higher plasma t-tau/Aβ42 and p-tau/Aβ42 were additionally associated with increased tau PET signal in the cingulate, lateral temporal, frontal and parietal cortices. Baseline plasma t-tau/Aβ42 did not associate with baseline PiB SUVR, hippocampal volume or FDG PET signal. However, higher plasma t-tau/Aβ42 at 2 years was cross-sectionally associated with higher PiB SUVR, lower hippocampal volume and lower FDG PET signal at 2 years, and greater increase in plasma t-tau/Aβ42 longitudinally was similarly associated with longitudinal changes of these biomarkers in the same directions. Although cognitive measures were available at two timepoints, only the cross-sectional relationships with plasma biomarkers at 2 years were described. After adjustment for age and sex, significant correlations were observed between lower delayed verbal memory and higher plasma p-tau-181, p-tau/ Aβ42 and t-tau/ Aβ42. Although adjustment for education was not done, participants in the three groups were well-matched for education. Although this study indicated that plasma t-tau/Aβ42 was superior to p-tau/Aβ42 in predicting temporal lobe tau deposition 2 years later, it did not address whether this association was greater in Aβ+ individuals, as numbers were likely too small to be able to stratify in this way.
Pase *et al.* 2019 [218]	Simoa, plasma (anticoagulant not	1. FHS Progression to dementia sample:	The FHS population-based study evaluated the associations of plasma t-tau with several demographic and health variables, its ability to predict all-cause cognitive decline/dementia and clinical AD dementia over up to 10 years of follow-up, its cross-

	specified for either cohort)	Baseline (1453) Dementia free >65y olds (1453) - 134 individuals progressed to all-cause dementia (of whom 105 were AD dementia). Endophenotypes sample: Baseline (3832) - Detailed cognitive assessment was available in 3832 individuals aged 25 to 98y - Brain MRI for hippocampal volumes was available in 3238 individuals Neuropathology sample Baseline (42) - Autopsy data were available in 42 individuals. 2. Memento Baseline (367)	sectional associations with "endophenotypes" of cognitive function and hippocampal volume, and its ability to predict neurofibrillary tangle burden at autopsy. In the Memento memory clinic-derived cohort, the ability of plasma t-tau to predict all-cause dementia and AD dementia over up to 6 years of follow-up and the cross-sectional associations between plasma and CSF t-tau were examined. FHS Cross-sectionally, higher plasma t-tau was associated with older age, female sex and lower educational attainment. Higher plasma t-tau was also associated with a higher vascular risk factor burden after adjustment for age and sex (lower HDL cholesterol, higher BMI, treatment for hypertension, prevalent coronary artery disease, diabetes, and atrial fibrillation). No significant associations were seen with *APOE* ε4 carrier status or current smoking or systolic blood pressure. 1 log SD unit increase in plasma t-tau was associated with a significant HR of 1.29 for all-cause dementia and of 1.35 for clinical AD dementia after adjusting for age and sex across the whole cohort. HR were not significantly changed by additional adjustment for *APOE* ε4 carrier status or vascular risk factors. Stratifying by *APOE* ε4 carrier status, the HR in non-carriers were 1.53 for all-cause dementia and 1.81 for AD dementia, and in carriers were 2.08 and 1.99 respectively. The net reclassification index (NRI) was greater for non-carriers than carriers. Using NRI with a median cut-point of plasma t-tau for a hypothetical 5-year prevention trial yielded sample size reductions of 38% for an all-cause dementia end-point and 50% for an AD dementia endpoint. Using a median cut-point for plasma t-tau and including only *APOE* ε4 carriers yielded sample size reductions of 69% and 80% respectively. Higher plasma t-tau was associated with smaller hippocampal volumes, and worse performance in tests of episodic memory, verbal reasoning, visual memory, visuospatial integration, processing speed and executive function but not with an index of premorbid cognitive function, after adjusting for age, sex, time to assessment and

		Individuals with MCI (261) or SCD (106) who had both plasma and CSF t-tau measurements (140 on same day).	educational level. Associations were qualitatively unchanged by additional adjustment for vascular risk factors and *APOE* ε4 carrier status. Of the 42 individuals in the autopsy study, 11 had confirmed AD pathology. Higher plasma t-tau was associated with higher neurofibrillary tangle burden in the medial temporal lobe and higher microinfarct burden but not with cortical neuritic or diffuse plaque burden. <u>Memento</u> 1 log SD unit increase in plasma t-tau was associated with a non-significant HR of 1.14 for all-cause dementia, but with a significant HR of 1.54 for AD dementia. In the 140 participants with paired CSF and plasma on the same day, there was no significant correlation (r = 0.16, p = 0.07) between t-tau levels in both biofluids.
Li *et al.* 2019 [208]	Simoa, EDTA-plasma	Daping Memory Clinic <u>Baseline (84)</u> Ctr 9 MCI 22 AD dementia 53 Classification by PiB PET visual rating: Aβ- (36) Aβ+ (48)	This study was targeted at examining predictors of Aβ status. Neither plasma t-tau on its own nor the plasma Aβ42/t-tau ratio was able to distinguish Aβ+ from Aβ-. No significant difference in plasma t-tau was found between clinical groups. Other associations (e.g. with atrophy and cognition) were not probed.
Down Syndrome			
Lee *et al.* 2016 [211]	IMR, EDTA-plasma	Taiwan DS Foundation and university outpatient clinic cohort <u>Baseline (191)</u> Ctr 78	This study was formulated to examine differences between DS AD and sAD; however it did not employ any “gold standard” biomarkers such as CSF or PET for diagnosis of sAD and Ctr. Therefore, the main utility of the study was in investigating within the DS groups.

		sAD dementia (62) DS without dementia (35) DS with dementia or its prodrome (16) Ctr and sAD were diagnosed purely clinically.	Plasma t-tau was measured only in a subset of 21 DS (12 without and 9 with dementia or its prodrome). Plasma t-tau was unable to differentiate between the groups but it was inversely correlated with Adaptive Behaviour Dementia Questionnaire scores.
Kasai et al. 2017 [227]	Simoa, EDTA-plasma	Kyoto DS and university cohort <u>Baseline (43)</u> Ctr (22) DS (21)	Plasma t-tau was elevated in DS compared to age-matched Ctr in middle (26-42 years) and older age groups (>43 years) but not in the young age group (14-25 years). Within the DS group, t-tau was not related to either social age or to scores on a dementia screening questionnaire for individuals with intellectual disabilities. This study was limited by lack of stratification of Ctr by a “gold standard” CSF or PET AD biomarker.
Tatebe et al. 2017 [228]	Simoa, EDTA-plasma	1. Purchased cohort from USA <u>Baseline (35)</u> Ctr (15) sAD dementia (20) 2. Kyoto DS cohort <u>Baseline (42)</u> Age matched Ctr (22) DS (20)	This pilot study utilised a homebrew Simoa p-tau-181 assay, which has not been replicated till date. The lower limit of detection of the assay was 0.009 pg/ml, and 8 of the DS samples could not be quantified. In cohort 1 plasma p-tau-181 was quantifiable in all samples and the AUC for clinical AD vs Ctr was 0.79. In cohort 2, plasma p-tau-181 was significantly higher in DS than Ctr, and when a cut-point derived from cohort 1 was applied, 12 out of 20 DS but only 6 out of 22 Ctr exceeded the cut-point. There was a significant correlation between age and plasma p-tau-181 in DS but not Ctr.

		DS were not stratified by clinical dementia. 3. Paired CSF and plasma cohort <u>Baseline (8)</u> AD dementia (5) Parkinsons's disease (1) Vascular dementia (2) All diagnoses except DS were made clinically	In cohort 3 where matched CSF and plasma were available, p-tau-181 showed modest correlation between the two biofluids.
Fortea *et al.* 2018 [212]	Simoa, plasma (anticoagulant not specified)	Catalonian Down Syndrome cohort <u>Baseline (347):</u> DS without dementia (192) Prodromal AD DS (39) AD dementia DS (49) Ctr (67)	CSF and plasma t-tau were weakly correlated within individuals with DS. Plasma t-tau was significantly higher in AD dementia DS than in Ctr or in DS without dementia. The comparison to Ctr was limited by lack of stratification of the Ctr by CSF Aβ status. T-tau was unable to discriminate between DS without dementia and prodromal AD DS, but had a AUC of 0.74 for discriminating between DS without dementia and AD dementia DS; this increased and remained significant after adjustment for age, sex and APOE ε4 status.
Startin et al. 2019 [213]	Simoa, EDTA-plasma	LonDOWNs (DS) and EMIF (Perugia and Barcelona sites) <u>Baseline (85)</u> Ctr (27)	ANCOVA adjusted for age, sex and *APOE* ε4 carrier status showed no significant differences between Ctr, sAD and all DS for either plasma t-tau or Aβ42/t-tau ratio, but statistical analysis could not be undertaken to compare DS with and without dementia, due to small sample size.

		sAD with CSF Aβ42 confirmation (27) DS (31) DS with dementia (7) DS without dementia (24)	

1.15.2.3 Neurofilament light chain

A high correlation between CSF and blood measurements of NFL has been observed by many authors studying this protein across different diseases including multiple sclerosis [229, 230], progressive supranuclear palsy [231], Huntington's disease [232], amyotrophic lateral sclerosis [161], FTD [162], Down Syndrome [212], familial AD [233] and sporadic AD [234], making blood measurement an effective surrogate for CSF NFL. This close correlation is also seen in murine models of tauopathies, amyloidosis and α-synucleinopathy [235].

In both monogenetic forms (including Down Syndrome and dominantly inherited AD) and sporadic forms of AD, there has been a recent expansion of research utilising blood NFL as a biomarker for neurodegeneration. Table 1.12 (page 124) summarises the findings of key studies. These studies provide evidence for associations between:

- higher baseline blood NFL, and increasing age, worse baseline global cognition and lower whole brain and hippocampal volumes;
- greater longitudinal increase in blood NFL, and worsening longitudinal decline in global cognition, and neuroimaging measures of neurodegeneration (including hippocampal volume, entorhinal and temporal cortical thickness, and FDG PET).

Table 1.12: Summary of studies of blood neurofilament light chain in AD.

Aβ+, amyloid-β positive; Aβ-, amyloid-β negative, AD, Alzheimer's disease; ADAS-Cog 11, Alzheimer's disease assessment scale – cognitive (11 items); ADNI, Alzheimer's Disease Neuroimaging Initiative; *APOE* ε4, apolipoprotein E gene epsilon 4 allele; *APP*, amyloid precursor protein gene; ART, Alzheimer Research Trust; AUC, area under the receiver operating characteristics curve; CDR, Clinical Dementia Rating Scale; Ctr, Control; DCR, Dementia Case Register; DIAN, Dominantly Inherited Alzheimer's Network; DSBI, Down Syndrome Biomarker Initiative; ECL, electrochemiluminescence; EDTA, ethylenediaminetetraacetic acid; ES, Erlangen Score for classification of probability of AD based on CSF profile; EYO, estimated year of onset; KARVIAH, Kerr Anglican Retirement Village Initiative in Ageing Health, LonDOWNS, London Down Syndrome Consortium; MC, mutation carriers; MCI, mild cognitive impairment; MMSE, Mini Mental State Examination; MSD, Mesoscale Discovery; NC, non-carriers of dominant AD mutations, *PSEN1*, presenilin 1 gene; *PSEN2*, presenilin 2 gene; UCL FAD, University College London Familial Alzheimer's Disease.

Study authors, year	**Assay, blood fraction**	**Cohort, baseline vs longitudinal blood sampling, group (N)**	**Findings**
Sporadic AD			
Gaiottino *et al.* 2013 [236]	ECL (MSD platform), serum	Neurology patient cohort – details not given Baseline (220) Healthy Ctr (67) Neurological illness without structural CNS damage (control patients: 68) Amyotrophic lateral sclerosis (46) Guillain-Barré syndrome (19) AD dementia (20)	CSF and serum NFL measured by the same assay correlated moderately well in all disease groups but not in control patients. All disease groups had higher serum NFL than the healthy Ctr or the control patient groups but the latter two groups did not show statistically significant differences. The lower limit of quantification of this assay was 15.6 pg/ml; as blood NFL was subsequently shown to be correlated with age, this assay was therefore likely to be unable to quantify blood NFL in younger patients accurately.

		Diagnoses determined by clinical criteria	
Bacioglu et al. 2016 [235]	ECL (MSD platform), serum	Neuro-Biobank Tuebingen <u>Baseline (205)</u> Ctr (35) MCI (33) AD dementia (34) Idiopathic Parkinson's disease (32) Dementia with Lewy Bodies (20) Multiple Systems Atrophy (17) Progressive Supranuclear Palsy (24) Corticobasal syndrome (10) Diagnoses determined by clinical criteria	A moderately high correlation was seen for CSF and serum NFL within the control group; this was not assessed for the disease groups. All disease groups except MCI were demonstrated to have significantly higher CSF and serum NFL than Ctr. This was a landmark study as in addition to the human samples, paired CSF and plasma samples from murine models of cerebral β-amyloidosis, tauopathy and α-synucleinopathy were also assessed, and correlations were demonstrated not only between NFL measured in the two biofluids within individuals, but also between neuropathological load and NFL levels in either biofluid.
Mattsson *et al.* 2017 [234]	Simoa, EDTA-plasma	ADNI <u>Baseline (540):</u> Ctr (193) MCI (197) AD dementia (180)	Plasma and CSF showed a moderately high correlation within individuals across all groups. For overall differentiation of AD dementia vs control, the AUC for plasma NFL was 0.87, which was similar to those achieved by CSF Aβ42, t-tau. P-tau or NFL.

		Aβ groups determined by CSF Aβ42 common cut-point of 192 pg/ml.	The Aβ+ dementia group had significantly higher plasma NFL than the Aβ+ MCI which in turn had significantly higher plasma NFL than the Aβ+ or Aβ- Ctr groups. There was no significant difference in plasma NFL between the Aβ+ and Aβ- Ctr groups. Within the MCI group, Aβ+ individuals had higher plasma NFL than Aβ- individuals but there were no differences between stable and progressive MCI. Higher baseline plasma NFL was associated with worse baseline performance in MMSE, ADAS-Cog 11 and trail-making test B but not with logical memory or digit symbol substitution. Higher baseline plasma NFL was associated with lower baseline hippocampal volume, lower baseline AD signature region cortical thickness and higher baseline ventricular volume but not with baseline FDG PET or white matter hyperintensities. Assessing cognitive and neuroimaging measures over 4 years of follow-up, higher baseline plasma NFL was associated with steeper decline in all tested cognitive measures and greater change in all neuroimaging measures excepting white matter hyperintensities.
Pereira *et al.* 2017 [237]	Simoa, EDTA-plasma	ADNI <u>Baseline (309):</u> Ctr Aβ- (57) Ctr Aβ+ (37) MCI Aβ- (36) MCI Aβ+ (109) AD dementia Aβ- (5) AD dementia Aβ+ (65) Aβ groups determined by CSF Aβ42 common cut-point of 192 pg/ml.	This publication extended the findings of Mattsson *et al.* (2017) by examining associations between various CSF biomarkers, plasma NFL and regional cortical thickness and subcortical volumes. Plasma NFL showed moderately high correlation with CSF NFL and weaker correlations with CSF t-tau, p-tau and neurogranin. In the whole sample, plasma NFL was significantly negatively correlated with cortical thickness of the left precuneus, right middle temporal gyrus, most of the lateral surface of the brain, and with volumes of the hippocampus and nucleus accumbens.

			Analyses within groups showed significant cortical thickness correlations occurred in the MCI Aβ-, MCI Aβ+ and AD dementia Aβ+ groups. Significant subcortical volume associations occurred in the MCI Aβ- and MCI Aβ+ groups.
Chatterjee *et al.* 2018 [238]	Simoa, EDTA-plasma	KARVIAH Baseline (100): Aβ- (65) Aβ+ (35) Aβ groups determined by 18-F florbetaben PET scan	In this Australian study of cognitively normal people aged 65-90 years, after adjustment for age, higher plasma NFL was associated with worse working memory and executive function, and lower global cognitive composite scores. There was no clear association between plasma NFL and cortical amyloid load as a continuous measure, but quartile analysis showed those in the highest quartile of NFL had the highest amyloid load. No significant association was seen between plasma NFL and hippocampal volume.
Lewczuk *et al.* 2018 [239]	Simoa, EDTA-plasma	Erlangen Baseline (99) Ctr, ES ≤ 1(41) MCI-AD, ES=4 (25) AD dementia, ES=4 (33) All diagnoses were assigned on the basis of clinical presentation and CSF ES (Erlangen score) indicating likelihood of AD [240].	Plasma NFL correlated moderately positively with CSF t-tau and p-tau, and negatively with CSF Aβ42 and Aβ42/40 ratio across the whole cohort but this lost statistical significance when analysing within groups. Plasma NFL correlated inversely with MMSE scores. After adjusting for age, the AUC for plasma NFL prediction of CSF AD pathology was 0.92; given that the Ctr and AD groups were pre-selected by CSF signature, this does not mimic the likely “real world” performance of the test. The age-dependency of the optimal cut-point was assessed by linear discriminant analysis and showed that in non-age-matched groups such as in this study, the optimal cut-point actually reduced with age, and there was an age-dependent increase in sensitivity but reduction in specificity.
Lin *et al.* 2018 [241]	Simoa, EDTA-plasma	Taiwanese outpatient clinic cohort Baseline (283): Ctr (59)	After adjusting for age, sex, years of education and *APOE* ε4 carrier status, plasma NFL was elevated in AD dementia compared to each of the other groups. Plasma NFL was inversely correlated with MMSE across the whole cohort and within the AD dementia and all PD groups.

		MCI (56) AD dementia (119) PD (26) PD dementia (23) Diagnoses determined by clinical consensus criteria	No significant difference in plasma NFL was demonstrated between the MCI and Ctr groups, or between those who did and did not convert from MCI to AD dementia over about 20 months of follow-up, but these groups were purely clinically defined.
Mattson *et al.* 2019 [242]	Simoa. EDTA-plasma	ADNI <u>Longitudinal (1583):</u> Ctr (401) MCI (855) AD dementia (327) Stratification by Aβ status employed CSF cut-point of 880 ng/L Stratification by AT(N) status further employed p-tau>27 ng/L for T+ and temporal cortical thickness <2.75 mm as N+.	<u>Diagnosis and Aβ stratification</u> Rate of increase of plasma NFL was higher in AD dementia than in either MCI or Ctr. Aβ+ Ctr and MCI+ Ctr had greater rates of increase of plasma NFL compared to their Aβ- counterparts. <u>AT(N) classification</u> N+ groups (A-T+N+, A+T-N+ and A+T+N+) had increased baseline NFL. N+ groups as above, and group A-T+N- had increased rate of NFL increase. <u>Baseline biomarkers and longitudinal plasma NFL</u> After adjustment for age and sex: Baseline lower levels of Aβ42, and higher levels of p-tau and t-tau (as continuous or dichotomised variables) were associated with higher rate of NFL increase. Baseline lower hippocampal volume, temporal cortical composite thickness, FDG PET composite, MMSE, CRD sum of boxes and ADAS-Cog scores were associated with higher rate of NFL increase. Baseline higher ventricular volume was associated with higher rate of NFL increase. Baseline white matter hyperintensities were not associated with rate of NFL increase. <u>Longitudinal imaging, cognition and CSF biomarkers and longitudinal plasma NFL</u>

			After adjustment for age and sex: Steeper decrease in hippocampal volume, entorhinal cortical thickness, temporal cortical composite thickness, FDG PET composite, MMSE, CDR sum of boxes and ADAS-Cog was associated with higher rate of NFL increase. Steeper increase in ventricular volume was associated with higher rate of NFL increase. There was no significant association of rate of NFL change with changes in white matter hyperintensities, or CSF levels of Aβ42, p-tau and t-tau. All relationships were predominantly driven by findings in the Ctr and MCI groups, as length of follow-up in the AD dementia group was limited (all <3 years as opposed to maximum of 12 years in the other groups).
Ashton *et al.* 2019 [243]	Simoa, EDTA-plasma	ART and DCR cohorts Longitudinal (69) Ctr (12) AD (57) NFL immunohistochemistry and medial temporal gyrus homogenates available for 7 Ctr and 19 AD individuals	This study examined plasma NFL taken at 3 timepoints (spanning 1 to 8 years before *post mortem*) in relation to *post mortem* measures of amyloid, tau and NFL. Plasma NFL was raised in the AD group compared to Ctr at each of three timepoints prior to *post mortem.* Across all individuals, plasma NFL at timepoint 1 correlated inversely with MMSE at all three timepoints after adjustment for age. After adjusting for age at *post mortem*, *post mortem* delay and burden of other co-pathologies (such as vascular, TDP-43 and Lewy body pathology), timepoint 1 plasma NFL correlated with Braak stage. Higher timepoint 1 plasma NFL was associated with significantly lower rate of increase of NFL. Rate of increase of plasma NFL was not significantly associated with Braak stage. Timepoint 1 plasma NFL, but not rate of increase in plasma NFL, was significantly associated with tau pathology load but not with measures of Aβ1-40, Aβ41-2 or p-tau in medial temporal gyrus homogenates. Higher timepoint 3 plasma NFL, but not rate of increase or measurement at other timepoints, was associated with lower percentage NFL immunohistochemical staining in fixed medial temporal gyrus.

Down Syndrome (DS) AD			
Strydom *et al*. 2018 [244]	Simoa, heparinised plasma	LonDOWNs Baseline (94): No dementia (76) AD Dementia (18)	Median plasma NFL was significantly raised in the AD dementia group compared to the no dementia group: (63.8 pg/ml vs 20.0 pg/ml; $p < 0.001$). Plasma NFL was associated with age but not influenced by premorbid intelligence. A logistic regression model adjusting for age, sex, and *APOE* ε4 status showed that NFL level at baseline remained predictive of dementia status at 2 year follow up (available in 29 individuals).
Fortea *et al.* 2018 [212]	Simoa, plasma (anticoagulant not specified)	Catalonian Down Syndrome cohort Baseline (347): Asymptomatic DS (192), Prodromal AD DS (39), AD dementia DS (49) Ctr (67)	Plasma NFL was significantly higher in all DS groups than in Ctr even after adjusting for age, sex and *APOE* ε4 carrier status. Plasma NFL differentiated prodromal AD DS from asymptomatic DS with AUC 0.88 and AD dementia DS from asymptomatic DS with AUC 0.95. Adjusting for covariates did not alter these results. Plasma and CSF NFL were highly correlated within participants.
Shinomoto *et al.* 2019 [245]	Simoa, EDTA-plasma	Japanese DS cohort Baseline (48): DS (24) Ctr (24)	Individuals with DS had higher plasma NFL than Ctr individuals, and cross-sectionally showed a steeper relationship with age than did Ctr individuals. Within the DS group, higher baseline plasma NFL was associated with steeper decline in social age over the next 12-18 months (calculated from an adaptive behaviour score) after adjusting for age and sex. This study assessed but did not stratify according to dementia diagnosis within the DS group.
Rafii *et al.* 2019 [246]	Simoa, plasma	DSBI Baseline (12) DS (12)	This small cross-sectional exploratory study of inter-biomarker correlations in DS employed gold standard amyloid and tau PET in addition to MRI measurements of hippocampal volume, FDG PET and cognitive assessments. Plasma NFL correlated

			positively with global and regional amyloid PET. Although NFL increased with tau load overall, relationships with tau PET were inconsistent across Braak stages. Plasma NFL was significantly negatively correlated with FDG PET in most brain regions assessed, and with global cognition and paired associate learning. There no significant correlations of plasma NFL with hippocampal volume, adaptive behaviour scores and informant perceptions of memory.
Dominantly inherited AD			
Weston *et al.* 2018 [247]	Simoa, serum	UCL FAD Baseline (48) NC (11) Asymptomatic MC (19) Symptomatic MC (18)	Mean serum NFL in NC was 12.7 pg/ml, in pre-symptomatic MC 16.7 pg/ml and in symptomatic MC 46.0 pg/ml. Adjusted for age and sex, and allowing for clustering within families, there were significant differences between all 3 groups. Higher serum NFL was significantly correlated with EYO. Across all MC, higher serum NFL correlated significantly with lower MMSE, CDR sum of boxes, estimated change in IQ, recognition memory performance, baseline whole brain volume and baseline hippocampal volume. Higher serum NFL also correlated significantly with higher baseline ventricular volume and higher rate of whole brain atrophy, ventricular expansion and hippocampal atrophy.
Sánchez-Valle *et al.* 2018 [248]	Simoa, serum	Barcelona ADAD Baseline (60) NC (18) Asymptomatic MC (20) Symptomatic MC (22)	Serum NFL correlated with CSF NFL, t-tau and p-tau but not with Aβ42. It also correlated with EYO and CDR sum of boxes, and negatively correlated with MMSE within the MC group. Serum NFL was significantly increased in symptomatic MC compared to asymptomatic MC and NC; symptomatic MC were older. The analyses presented were limited by lack of adjustment for age and sex.
Preische *et al.* 2019 [233]	Simoa, serum	DIAN Baseline (405): MC (243)	Baseline CSF and serum NFL were highly correlated within participants. Both showed significant differentiation between MC and NC at -6.8 EYO. Serum NFL rate of change

		NC (162) <u>Longitudinal (196):</u> MC (133) NC (63)	showed significant differentiation between MC and NC at -16.2 EYO (and so allowed for earlier differentiation than cross-sectional serum NFL). Rates of change of NFL were highest in those MC who started as asymptomatic but converted to AD during the follow-up period, followed by symptomatic MC, followed by pre-symptomatic MC and then NC. There were no differences in NFL rate of change between mutation groups (*APP, PSEN1, PSEN2*). Retrospective pseudo-predictive analysis showed that baseline serum NFL was associated with rate of reduction of precuneus thickness, MMSE and logical memory performance. Rate of change serum NFL showed steeper associations with subsequent rates of decrease in precuneus cortical thickness and FDG PET signal in symptomatic MC compared to pre-symptomatic MC. Rate of change of serum NFL was positively associated with rate of increase of PiB PET signal, to a similar degree across all MC. A true predictive design in 35 MC replicated the findings of the retrospective baseline analysis.

1.15.3 Multiplexing and unbiased approaches

1.15.3.1 Protein blood biomarkers

In addition to studying blood biomarkers that are candidates for utilising in the "AT(N)" scheme (as considered in detail in section 1.15.2), many researchers have used less targeted approaches for AD blood biomarker discovery. In the field of proteomics, these methods include multiplexed immunoassay panels, or mass spectrometry-based methods. A selection of relevant proteomics studies is provided in Table 1.13, page 136.

The earliest of these studies used case-control designs to seek blood biomarker differences between clinically-defined AD dementia and controls, or MCI and controls. Ray *et al.* [249] used a sandwich ELISA immunoassay platform and identified an 18-analyte panel of proteins that segregated AD dementia from controls. However, when Soares *et al*. attempted to replicate these findings in ADNI, by using seven of the analytes that gave a combined diagnostic accuracy of 90% in the Ray *et al.* study, only 61% diagnostic accuracy was achieved, and the incorporation of a different 89-analyte panel increased this to only 70% [250]. Doecke *et al.* [251] cross-validated panels of biomarkers obtained from AIBL (the Australian Imaging, Biomarkers and Lifestyle study) against ADNI, and found just two biomarkers that had individual effect sizes greater than 1.5 that were common to both cohorts: insulin-like growth factor binding protein 2 and pancreatic polypeptide. However, when a multivariate model using a panel of eight plasma biomarkers including these two was added to the predictive capacity of age, sex and *APOE* genotype, the biomarker panel was only able to increase sensitivity and specificity from 77 to 83 %. A key limitation of these types of studies was the purely clinical categorisation of samples in case-control

studies; later studies turned to using endophenotypes defined by established biomarkers instead. Thus, higher plasma clusterin has been shown to be associated with concurrent lower CSF Aβ42, with longitudinal cognitive decline [252, 253], and with greater hippocampal atrophy [253]. Higher apolipoprotein E levels in plasma taken 10 years before scan was predictive of higher PET-amyloid uptake [254] and five proteins including apolipoprotein A-I and complement C3 were shown to consistently predict PET-amyloid uptake across samples taken 0, -6 and -12 years relative to scan in one longitudinal aging cohort, and this finding was replicated in plasma taken concurrently with amyloid-PET in another cohort [255].

Complex and sometimes conflicting relationships between plasma cytokine levels and AD pathology have been unearthed in multiple cohorts. For example, plasma IL-10 and IL-12 gave a very modest improvement in prediction of PET amyloid status in a subgroup of healthy controls in AIBL at 54 months into the study but not at 18 months [256]. However, investigation of a cytokine panel in a Brazilian cohort with CSF-based AD definition showed that detectable levels of these “proinflammatory” cytokines in serum were associated with lower hippocampal functional connectivity in those with MCI-AD and AD dementia but with a less AD-like CSF profile (higher Aβ42, lower p-tau) [257].

So far, studies employing plasma or serum have been described in detail, but there are more novel techniques that enrich for neuronally derived proteins. For example, neuronally-derived exosomes have yielded several candidates that are differentially affected in CSF-defined AD vs control plasma, from pathways involved in Aβ pathology, synaptic dysfunction, glucose hypometabolism/insulin

resistance and tau pathology (reviewed by Pulliam *et al.* [258]). Use of whole blood to derive platelet fractions has also been examined, as APP is known to undergo similar processing in platelets as in neurons (reviewed by Akingbade *et al.* [259]).

Table 1.13: Summary of proteomic studies utilising multiplexing or untargeted approaches for blood biomarker discovery in sporadic AD.

Abbreviations:
2DGE, 2-dimensional gel electrophoresis; Aβ+, amyloid-β positive; Aβ-, amyloid-β negative; AC, affinity chromatography; AD, Alzheimer's disease; ADC, Amsterdam Dementia Cohort; AGES-RS, Age Gene/Environmental Susceptibility – Reykjavik Study; AIBL, Australian Imaging Biomarker and Lifestyle study; *APOE* ε4, apolipoprotein E gene epsilon 4 allele; AUC, area under the receiver operating characteristics curve; BIOFINDER, Swedish study investigating Biomarkers For Identifying Neurodegenerative Disorders Early and Reliably; BLSA, Baltimore Longitudinal Study of Aging; BNP, brain natriuretic peptide; C3, complement C-3; CEA, carcinoembryonic antigen; CDR, Clinical Dementia Rating Scale; CRP, C-reactive protein; Ctr, Control; DMN FC, default mode network functional connectivity; ECL, electrochemiluminescence; EDTA, ethylenediamenetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EMIF, European Medical Information Framework; FCN2, ficolin-2; GE-067-005, Study for investigation of ^{18}F-flutemetamol in prediction of conversion of amnestic mild cognitive impairment to probable Alzheimer's disease dementia; GenADA, Genetics Alzheimer's Disease Association multi-site Canadian longitudinal study; HV, hippocampal volume; ICAM, intercellular adhesion molecule; IGFBP2, insulin-like growth factor binding protein 2; IL, interleukin; IP, immunoprecipitation; KHP-DCR, King's Health Partners Dementia Case Register UK clinic and population based study; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; MCI, mild cognitive impairment; MIP-1δ, macrophage inflammatory protein-1δ; MS, mass spectrometry; MSD, Mesoscale Discovery; NCAM, neural cell adhesion molecule; NTUH, National Taiwan University Hospital; PDGF-BB, platelet-derived growth factor BB; Penn, University of Pennsylvania cohort; PiB, 11C-Pittsburgh-B compound amyloid PET tracer; PPY, pancreatic polypeptide; ROS, Religious Orders Study; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; sRAGE, soluble receptor for advanced glycation end products; svPPA, semantic variant primary progressive aphasia; TGFβ/GDF/BMP, transforming growth factor-β/growth differentiation factor/bone morphogenetic protein; TNF-α, tumour necrosis factor-α; UCSF, University of California San Francisco; UNICAMP, University of Campinas Brazil; VCAM, vascular cell adhesion molecule; WashU, Knight Alzheimer's Disease Research Center at Washington University School of Medicine; WB, Western blot

Study authors, year	**Assay**	**Cohort, group (N)**	**Blood fraction and markers identified**	**Findings**
Zhang *et al.* 2004 [260]	LC + SDS-PAGE + MS Cross-validation with WB and ELISA	Canadian cohort (71) AD dementia (41) Ctr (30)	In serum: 12 proteins elevated in AD; 1 protein reduced in AD	3 proteins (ApoE, transthyretin and histidine-rich glycoprotein) showed specific elevations in AD but not in other disease sera. 1 protein showed specific reduction in AD: a-1 acid glycoprotein. No statements of diagnostic accuracy, sensitivity or specificity were made. This study also included sera from patients with insulin-resistant diabetes and congestive heart failure (not summarized here).

Lopez *et al.* 2005 [261]	AC + MS	ROS (302): AD dementia (62) MCI (33) Ctr (207)	In serum: 2 models generated by peptide spectral signatures	Sensitivity 83% and specificity 90–96% achieved by the two models; contribution of individual peptides not analysed.
Ray *et al.* 2007 [249]	Cytokine antibody microarray ELISA	Pooled multi-centre origin samples (259): AD dementia (85) MCI (47) Other dementia (11) Other neurological disease (22) Rheumatoid arthritis (16) Healthy Ctr (79)	In plasma: 120 cell signalling proteins probed; 18 proteins identified as associated with AD	The 18-protein predictor had 91% accuracy in predicting MCI converting to AD, 72% accuracy in predicting MCI converting to non-AD dementia or staying as MCI.
Soares *et al.* 2009 [250]	ELISA	ADNI (1013): AD dementia (61) Ctr (952)	In serum: 151 protein panel, including 8 proteins from Ray *et al.*	The panel had only 61% accuracy in classifying AD vs Ctr; unable to replicate results of Ray *et al.* [249].
Thambisetty *et al.* 2010 [254]	Discovery: 2DGE + LC-MS/MS Validation: ELISA	BLSA Baseline plasma stratified by PiB PET (57): High uptake (18) Intermediate uptake (20) Low uptake (19)	Baseline fasted plasma 10 years before PiB PET: 7 proteins identified that discriminated between high and low uptake groups, including ApoE, haptoglobin,	Of the 7 proteins identified at baseline, only one (ApoE) was taken forward for the longitudinal sample ELISA confirmation. After adjusting for age, sex and years of education, plasma ApoE was significantly positively correlated with medial temporal cortex amyloid burden and the association was driven predominantly by non-carriers. Voxel based analysis indicated that the association was greater in bilateral hippocampi and right parahippocampus and entorhinal cortex.

		Or Stratified by *APOE* ε4 carrier status (54): Carrier (17) Non-carrier (37) All participants were non-demented at baseline Longitudinal plasma (42) stratified by:	plasminogen, C3, serum albumin and and immunoglobulin γ-1 chain C Longitudinal fasted plasma 1 year before PiB PET: ApoE confirmed ApoE taken forward for validation	This study was limited by not assessing the relative predictive ability of the *APOE* genotype vs the plasma ApoE level. ROC analyses were not presented.
Doecke *et al.* 2012 [251]	Multiplexed immunoassay platform for a 151 protein panel MS for metal ions and protein panel	AIBL (961): AD dementia (207) Ctr (754) ADNI (170): AD dementia (112) Ctr (58)	In plasma: 151 analytes were probed: 138 proteins associated with AD. Of these, 8 markers were chosen: cortisol, IGFBP2, PPY, IL-17, VCAM1, VCAM2, β-2 microglobulin, EGFR and CEA	IGFBP2 and PPY each had individual effect sizes > 1.5; these and several other markers had been replicated in other studies. The 8 most predictive markers, which were flagged by each of four statistical methods, were able to increase the sensitivity and specificity of the predictive model based on age, sex, education and *APOE* genotype from 77 to 83%.
Hu *et al.* 2012 [262]	Multiplexed immunoassay platform for a 190 protein panel	Penn (267): Ctr (126) MCI (16) AD dementia (88) Other dementia (37)	In plasma in Penn and WashU (test cohorts): 17 candidate proteins identified in univariate analysis	6 markers were common to both testing cohorts for MCI/mild dementia/AD: α-1-antitrypsin, ApoE, CRP, N-terminal pro BNP, osteopontin and serum amyloid P. Individual effect sizes ranged from 0.8 to 1.1.

		WashU: Ctr (242) CDR 0.5 or very mild dementia (63) CDR 1.0 or mild dementia (28) ADNI (both clinical diagnoses and CSF Aβ status available): Ctr (58) MCI (396) AD dementia (112)	across both cohorts to be associated with clinical MCI/AD; 5 replicated by another study in serum; 6 candidates survived multivariate analysis and correction for age and sex. In fasting plasma in ADNI (validation cohort): 6 candidates identified by univariate analysis 4 of these showed correlations with disease status defined by CSF 2 of these survived multivariate analysis and correction for age and sex	In the ADNI validation cohort, when Aβ status was defined by CSF, 4 markers were identified: ApoE, BNP, CRP, and PPY. The latter 3 were independent of cholinesterase inhibitor use. BNP and PPY showed significant increases with increasing *APOE* ε4 allele number and reduced CSF Aβ42 level; PPY also showed significant positive linear correlation with the CSF t-tau/Aβ42 ratio. ApoE, BNP and CRP were therefore common to all 3 cohorts. The main limitations of the study included: 1. methodological differences across the three cohorts in the sample collection (fasting vs. non-fasting) 2. the grouping of MCI/mild dementia/AD vs. controls in the analysis, resulting in an inability to comment on the correlation between these markers and disease severity 3. small individual effect sizes and low contribution of the plasma biomarkers to explaining the variance in the CSF markers in ADNI
Björkqvist *et al.* 2012 [263]	Quantitative multiplex ECL immunoassay	Skåne University memory clinic (404) AD dementia (142) Ctr (174) Other dementia (88)	In non-fasting plasma: EGF, PDGF-BB and MIP-1δ	This study attempted unsuccessfully to replicate the findings of Ray *et al*. [249]. 3 markers were able to distinguish AD from Ctr but not from other dementias; diagnostic precision was 63%.

Hye *et al.* 2014 [253]	Luminex xMAP multiplexed immunoassay Platform	AddNeuroMed, KHP-DCR and GenADA cohorts (1148): Ctr (452) MCI progressive (51) MCI stable (169) AD dementia (476)	In plasma: 26 candidate proteins were probed based on previous literature; 16 proteins correlated with MRI measures or with cognitive decline	This study combined analyses from 3 cohorts in which cases were defined clinically: stratification by Aβ status was not available but the aim was to draw associations of plasma biomarkers with baseline endophenotypes of brain atrophy and longitudinal cognitive decline. After controlling for multiple testing, clusterin (within the all MCI group) and ApoE (within the AD group) had significant associations with baseline medial temporal lobe MRI measures. A panel of 6 proteins was able to predict 20% of variation in hippocampal volume in MCI; a different panel of 7 proteins was able to predict 12% of variation in hippocampal volume in the AD group. At baseline within the MCI group, ApoE and CRP negatively correlated with MMSE; in the AD group 6 proteins including ApoE and clusterin negatively correlated with MMSE. Three proteins were correlated with longitudinal MMSE change: NCAM and sRAGE were negatively correlated but ICAM was positively correlated with MMSE decline. A panel of 10 proteins had the greatest power for predicting conversion of MCI to AD dementia, with AUC 0.78 when applied alone and 0.84 when combined with *APOE* genotype.
Sattlecker *et al.* 2014 [252]	Slow off-rate modified aptamer-based microarray (SOMAscan)	AddNeuroMed KHP-DCR AD dementia (331) MCI (106) Ctr (211) 43 MCI patients converted to AD dementia within a year	In plasma: 1001 proteins measured; 355 proteins were associated with at least 1 of the outcome measures but only 8 passed the false discovery rate threshold	Biomarker profiles correlated with different outcome measures, including: • region-specific atrophy on MRI (e.g. higher PPY correlated with lower left HV) • rate of cognitive decline (e.g. higher clusterin and higher nucleosome assembly protein 2 correlated with steeper decline in MMSE) A 13-protein panel gave a AUC of 0.70 discriminating AD vs Ctr; 20 proteins were associated with conversion of MCI to AD dementia but none passed multiple testing corrections. These analyses were limited by lack of “gold standard” AD biomarker stratification.

		Diagnoses were made on clinical criteria only	Panel of 13 proteins selected for diagnostic classification	
Ashton *et al*. 2015 [264]	Discovery: Tandem mass-tag LC-MS/MS Technical replication and validation: ELISA	<u>AIBL</u> Stratified by PiB PET (78): Low uptake (38) High uptake (40) <u>UCSF</u> Stratified by PiB PET (79): Low uptake (47) High uptake (32)	Fasted plasma: One protein replicated across two methods in two cohorts for association with PET Aβ status	The first discovery phase identified 51 proteins associated with PiB PET load; pathway analysis showed that they were involved in pathways previously associated with complement, systemic lupus erythematosus and prion diseases. 17 of these proteins were taken forward for technical replication, and of these two proteins replicated the results of the discovery study by association with binary Aβ status: α2-macroglobulin and fibrinogen γ-chain. In the validation cohort, only fibrinogen γ-chain replicated the findings by association with both binary Aβ status and continuous SUVR.
Jaeger *et al.* 2016 [265]	Antibody microarray	<u>UCSF and Mayo Clinic samples (191):</u> AD dementia (47) Ctr (52) svPPA (92)	In plasma: 600 secreted cell signalling proteins probed	3 groups of proteins were identified that discriminated AD from Ctr and from svPPA: 'complement', 'apoptosis' and 'regulation of growth', including a new TGFβ/GDP/BMP pathway, which was then investigated in *post mortem* human brain homogenates and in murine hippocampal culture models. 39 proteins were identified that were over- or under-expressed in both AD and svPPA relative to controls. This study therefore illustrated the importance of probing putative AD biomarkers in non-AD pathologies to confirm their disease specificity. svPPA was specifically chosen as a "disease control" group as the predominant pathology in svPPA is TDP43 type C. However, in this study only about half the AD cases were confirmed by *post mortem* analysis.

Westwood *et al.* 2016 [255]	Discovery Phase 1: 2DGE + LC-MS/MS Phase 2: gel-free LC-MS/MS (low molecular weight proteins Validation: Tandem mass-tag LC-MS/MS	<u>BLSA</u> All non-demented at all 3 timepoints (54) <u>AIBL</u> Stratified by PiB PET (78): Low uptake (38) High uptake (40)	BLSA: fasted plasma at 3 timepoints – 12 years before, 6 years before and at time of PiB PET AIBL: single timepoint fasted plasma concurrent with PiB PET	This was a more extensive longitudinal investigation of the same cohort used by Thambisetty *et al*. [254], followed by validation in an independent cohort using data from Ashton *et al.* . The combined discovery phases resulted in 7 proteins being associated with PiB PET uptake across all 3 timepoints: α-2-macroglobulin, apolipoprotein A-I, C3, complement C4B, haptoglobin, immunoglobulin kappa chain C region, and serum albumin. Of these, only two (C4B and immunoglobulin kappa chain C region) were not replicated in the validation set; the other five proteins passed false discovery rate correction.
Pedrini *et al.* 2017 [256]	MSD immunoassay	<u>AIBL (665):</u> Ctr (559) MCI (39) AD dementia (67) Ctr subgroup stratified by PiB PET: At 18 months (121): Aβ- (94) Aβ+ (27) At 54 months (93):	In plasma: 22 biomarkers probed at two timepoints relative to start of AIBL study – 18 and 54 months.	The authors first performed analyses across the whole group and comparing non-converting Ctr and AD at the 18 and 54 months timepoints. Adjusted for age, sex, site and *APOE* ε4 carrier status, significant findings (but not surviving Bonferroni correction) were that peptide YY was elevated in AD compared to non-converting Ctr at 18 months at eotaxin-3 was elevated in AD compared to non-converting Ctr at 54 months. Particularly for cytokines the measurements were often near the limits of detection, and there were large coefficients of variation resulting in many individual samples being excluded from various analyses. MCI and converters to AD seemed to be excluded from final analysis due to small numbers.

		Aβ- (67) Aβ+ (26)		The next level of analysis was in those classified by PET Aβ status: there were no significant differences at 18 months but at 54 months the combination of IL-10 and IL-12-23 p40 subunit was able to increase the AUC of a ROC analysis incorporating age, sex and *APOE* ε4 carrier status only from about 0.78 to 0.81.
Magalhaes *et al.* 2018 [257]	Flow cytometry immunoassay	<u>UNICAMP (130):</u> Ctr (42) Amnestic MCI-AD (55) Mild AD dementia (33)	In serum: five "proinflammatory" cytokines probed. Cytokine results were either detectable (+) or not detectable (-).	This study examined the association of five peripheral cytokines with endophenotypes of AD CSF markers, HV and DMN FC. The MCI-AD group was defined by both clinical and CSF criteria. There were no associations between any cytokine and endophenotypes in the control group. In the amnestic MCI-AD group, IL-10+ was associated with lower p-tau-181, but TNF-α+ and IL-12+ were associated with reduced right hippocampal FC. In the mild AD dementia group, IL-10+ was associated with lower p-tau-181, left HV and bilateral hippocampal FC. IL-12+ was associated with higher CSF Aβ42 and lower t-tau. IL-1β+ was associated with lower left hippocampal FC while TNF-α+ was associated with lower bilateral hippocampal FC.
Westwood *et al.* 2018 [266]	LC-MS/MS for discovery ELISA for technical replication; Luminex xMAP and ELISA for validation:	<u>ADC</u> Discovery cohort (50): Categorisation by CSF t-tau/Aβ42 ratio: Low ratio (25) High ratio (25)	In plasma: 25 proteins were associated with high CSF t-tau/Aβ42 ratio in the LC-MS/MS study in the ADC. 3 of these proteins replicated on ELISA in the ADC.	Analysing by CSF status in the discovery cohort, higher levels of FCN2, Apolipoprotein C-IV and fibrinogen β chain were shown in LC-MS and confirmed by ELISA to correlate with higher CSF t-tau/Aβ42 ratio. In the GE-067-005 study, increased FCN2 was again shown to be associated with PET amyloid positivity, but additional significant associations were seen for complement component 3. Conversion from MCI to AD dementia was associated with levels of apolipoprotein(a), apolipoprotein A-I, ceruloplasmin and PPY.

	choice of analytes guided by discovery cohort and previous literature	ELISA technical replication (100): Low ratio (50) High ratio (50) Validation cohorts <u>GE-067-005 (173)</u> Amnestic MCI converting to probable AD dementia (52) Stable amnestic MCI over 3 years (121) Or Categorisation by PET amyloid: Aβ- (105) Aβ+ (68) <u>EMIF (492):</u> Categorisation by CSF Aβ42: Aβ − (198) Aβ + (294)	One protein replicated in GE-067-005 but not in EMIF for association with PET amyloid status.	A minimum panel of 2 proteins (Aβ40 and Apolipoprotein C4) was able to classify for PET Aβ status with AUC 0.69. In EMIF: lower α-1-antitrypsin and higher clusterin were significantly associated with Aβ +. There was no significant association of FCN2 with Aβ+. A minimum panel of 5 proteins was able to classify for CSF Aβ status with AUC 0.67.

1.15.3.2 Non-protein blood biomarkers

Other approaches utilise non-protein markers, such as small non-coding micro-ribonucleic acids (miRNAs) that are involved in regulating the translation of other target RNAs. miRNAs detected by polymerase chain reaction (PCR) in plasma, serum and whole blood that are associated with inflammation, amyloid and tau processing have been found to be dysregulated in patients with AD dementia compared to age-matched controls, but the majority of miRNAs identified have as yet unknown roles (reviewed by Swarbrick *et al.* [267]). The biosynthesis and metabolism of lipids, amino acids, neurotransmitters and hormones has also been shown using various targeted MS techniques to be significantly perturbed in the CSF and plasma of patients with AD dementia and MCI relative to controls (reviewed by Trushina and Mielke [268]).

Many of the studies employing these novel techniques have again been limited by utilising case-control designs, so researchers are now moving forward by using “gold standard” AD biomarker confirmation and endophenotype correlations. Most of these techniques are highly specialised so it is likely that replication in large longitudinal cohorts will be challenging. As noted by O’Bryant *et al*. [269], the most common context of use in which blood biomarkers have been investigated in AD is for early detection, and if any blood biomarker is be applicable in the primary care setting it would first have to meet criteria of scalability (including simplification of pre-analytic and analytic procedures), manufacturability, compatibility with existing health care infrastructures and considerations of intellectual property and regulation that are inevitably linked to the involvement of industry in bringing such a biomarker to the mainstream.

1.16 Unanswered questions, and aims of this body of work toward the doctoral thesis

While blood biomarker research has advanced using both candidate approaches and multiplexed or less targeted approaches, at the start of the work described in this thesis there remained several unanswered questions about the blood biomarkers that are linked to the core pathologies of AD (amyloid, tau and neurodegeneration). These included:

- Does the heterogeneity observed in studies, particularly of blood measurements of amyloid-β reflect true differences between the populations studied, or might it at least in part be due to differences in either modifiable (sample-related) or unmodifiable (participant-related) pre-analytical factors?
- Most studies till date had investigated these blood biomarkers using mixed cohorts of individuals with AD, MCI and controls, and many studies were limited by utilising only clinical definitions, rather than confirming diagnoses using the "gold standard" biomarkers that are derived from CSF or PET. Therefore, these studies were not focused on questions of whether these blood biomarkers could distinguish cerebral amyloid deposition and/or neurodegeneration in the preclinical phase of AD (i.e. in cognitively normal individuals).
- In the case of blood measurements of amyloid-β, till date no study had compared different assay methods in the same samples, to ascertain whether different assays have similar predictive utility for cerebral amyloid deposition.
- In the case of currently commercially available plasma t-tau assays that actually measure only tau moieties containing the mid-region, there is a

poor relationship between CSF and plasma values within individuals, and a significant overlap between the range of levels observed in those with AD and controls. Are there other tau moieties that are better able to distinguish AD from controls and do assays for these tau moieties show better correlation between plasma and CSF values within individuals?

The work described in this doctoral thesis therefore aimed to:

- use sensitive assay technology, including the Simoa digital immunoassay platform, in large and well-characterised cohorts, to examine candidate blood biomarkers linked to the core AD pathologies of amyloid, tau and neurodegeneration;
- elucidate the pre-analytical factors impacting these biomarker measurements, with a focus on modifiable variables such as choice of blood fraction and susceptibility to repeated freezing and thawing, and participant-specific variables encompassing demographic factors and factors affecting the volume of distribution of blood biomarkers;
- compare the relative ability of Simoa and liquid chromatography-mass spectrometry assays of amyloid-β to distinguish cerebral amyloid status in the Insight 46 study (the neuroimaging sub-study of the MRC National Survey of Health and Development);
- evaluate blood biomarker associations with neuroimaging markers of neurodegeneration and with cognitive metrics in the preclinical phase; and to
- collaborate to develop and validate a new Simoa-based CSF and plasma tau assay sensitive to N terminal fragments of tau.

1.17 **Specific research questions by chapter**

Chapter 3: Do multiple freeze-thaw cycles affect Simoa blood biomarkers of NFL, total tau, Aβ40 and Aβ42? Is it better to measure these in plasma or serum?

Chapter 4: How do participant-specific pre-analytical variables such as age, sex, *APOE* ε4 carrier status, serum creatinine and body mass index associate with these blood biomarkers in the Insight 46 cohort? What are the cross-sectional associations between these blood biomarkers?

Chapter 5: What are the cross-sectional associations between these blood biomarkers and amyloid PET status in the Insight 46 cohort? How do the Simoa plasma Aβ40 and Aβ42 assays perform in relation to a liquid chromatography-mass spectrometry (LC-MS) method in predicting amyloid PET status?

Chapter 6: What are the cross-sectional associations between these blood biomarkers and imaging biomarkers of neurodegeneration and cerebrovascular disease in the Insight 46 cohort?

Chapter 7: What are the cross-sectional associations between these blood biomarkers and cognitive performance in the Insight 46 cohort?

Chapter 8: Do novel tau Simoa biomarkers in CSF and plasma better differentiate Alzheimer's disease patients from controls than does "total" (mid-region) tau? Do these assays show better correlation between CSF and plasma within individuals than does the total tau assay?

2 General methods

2.1 **Cohorts**

This work has used samples from four main cohorts:

- The UCL Dementia Research Centre Clinical Cohort ("DRC clinical cohort", PI Jonathan Schott): Chapters 4 and 9,
- The Neuroimaging Sub-study of the Medical Research Council National Survey of Health and Development ("Insight 46 cohort", PI Jonathan Schott): Chapters 5, 6, 7 and 8,
- The Harvard Aging Brain Study ("HABS cohort", PI Reisa Sperling): Chapter 9 and
- The University of California San Diego Shiley-Marcos Alzheimer's Disease Research Centre cohort ("UCSD cohort", PI Douglas Galasko): Chapter 9.

The nature of the cohorts and common analytical methods used are described below.

2.1.1 DRC clinical cohort

2.1.1.1 Clinic, recruitment and ethics

The cognitive CSF day care clinic was set up at the National Hospital for Neurology and Neurosurgery (NHNN) by Dr Ross Paterson in August 2013 in conjunction with the Wolfson CSF study 12/3044 (PI Jonathan Schott, NRES Queen Square Committee reference 12_LO_1504). The cohort of patients participating in this study is henceforth referred to as the "DRC clinical cohort". The author took over the management of the clinic and cohort in October 2016 and updated the standard operating procedure in August 2017.

Patients were referred from the NHNN specialist Cognitive Disorders clinics for a clinical lumbar puncture using a standardised referral form, which the author implemented to ensure that imaging and coagulopathy safety checks were undertaken by the referring clinician prior to referral. The author telephoned each patient to book their appointment, explain the lumbar puncture procedure and ask their permission to forward the patient information leaflet for Wolfson CSF study 12/3044 with their appointment letter. The author also implemented a delegation log, managed the rota of clinicians, personally supervised and assessed the competence of all clinicians participating in the rota and trained them in the standard operating procedure. From October 2016 till present the author has performed or supervised 75% of the lumbar punctures and venepunctures.

Either informed written participant consent or informed written consultee assent was obtained on the clinic day for all participants, for collecting up to 15 ml CSF and 50 ml blood for research purposes. No additional needles were employed beyond those already being used to collect the clinical samples, which usually consisted of about 5-10 ml CSF and 5 ml blood. There is evidence to suggest that collection of total volumes of CSF up to 30 ml either has no adverse effect [270] or a slight beneficial effect on post lumbar puncture headache incidence at 24 hours, compared to collection of volumes less than 20 ml [271].

2.1.1.2 Blood and CSF sampling, pre-processing and storage

The standard operating procedure (Table 2.1, page 151) was informed by the consensus guidelines for lumbar puncture in patients with suspected

neurological disease [97] and Standards for Alzheimer's Research in Blood biomarkers (STAR-B) and Blood- Based Biomarker Interest Group (BBBIG) guidelines [151].

Table 2.1: Standard operating protocol for CSF and blood collection and processing for the DRC prospective clinical cohort.

Condition	**Standard operating protocol**
Participant status	Not instructed to fast
Timing of lumbar puncture	08:00 to 12:00
Skin preparation	As per local clinical guidelines
Local anaesthetic (to skin, subcutaneous tissues)	Up to 3mg/kg of lignocaine
Level of lumbar puncture	L2/3, L3/4, L4/5 or L5/S1
Spinal needle gauge	22G (atraumatic needle ensured from January 2017 onward)
Volume of CSF collected for research	Up to 15 ml collected at end of sampling directly into containers (without active withdrawal)
Containers for CSF collection	Polypropylene screw top (Sarstedt 62.610.018)
Timing of venepuncture	Immediately after lumbar puncture
Location of venepuncture	Upper limb peripheral vein
Tourniquet	Tourniquet used
Venepuncture needle	21G or 23G butterfly needle with BD Vacutainer adaptor
Blood collection tubes (in order of draw)	Up to 1x Lithium heparin plasma 5 ml Up to 4x SST serum 4 ml Up to 4x EDTA plasma 5 ml
Transport conditions for blood and CSF	Room temperature
Target time between sampling and centrifugation of blood and CSF	30 minutes
Centrifugation conditions for CSF	1750 g for 10 minutes at 4 °C
Centrifugation conditions for blood	1800 g for 10 minutes at 22 °C
Target time between sampling and storage at -80°C	60 minutes
Aliquot volume for CSF and blood fractions	1 ml
Aliquot container	Polypropylene screw top cryovial

2.1.2 Insight 46 cohort

A detailed description of the cohort is provided in the protocol paper [272] but information directly relevant to the experiments described here is included below.

2.1.2.1 Recruitment

Insight 46 is a prospective longitudinal two time-point (0, 24 months) sub-study of 502 members of the Medical Research Council (MRC) National Survey for Heath and Development (NSHD). Ethical approval for Insight 46 was given by the National Research Ethics Service (NRES) Committee London (REC reference 14/LO/1173, PI Jonathan Schott). All participants provided written informed consent for participation.

The original MRC NSHD has followed 5362 individuals since their birth in England, Scotland and Wales during one week in March 1946. From birth till age 70, 24 repeated waves of data collection had been undertaken. The Insight 46 recruitment criteria maximised life course data availability. Those invited to the study were selected at random from the MRC NSHD provided that they fulfilled the following criteria:

- Attendance at a study clinic visit at age 60–64
- Parental socioeconomic position: at least one indicator of occupational social class or education
- Cognition: memory and processing speed from age 60–64 AND at least one set of measures at age 8, 11 or 15
- Early physical growth trajectories: birth weight and at least one measure of height and weight at ages 4–15

- Educational attainment: highest qualification by age 26
- Mental health: teacher ratings of behaviour and temperament at age 13 or 15, and at least one measure of affective symptoms at ages 36, 43, 53 or 60–64
- Blood pressure, lung function, adult height and weight: at least one measure of each at ages 36, 43, 53 or 60–64*
- Health behaviours: at least one measure of smoking and physical exercise at ages 36, 43, 53 or 60–64*
- Blood: samples taken at either age 53 or 60–64

To enhance participation, as recruitment proceeded, criteria marked as * were relaxed, such that 1377 individuals fulfilled the remaining minimum data set requirements and were invited in batches to participate. Of those who indicated interest in participating, the following were then excluded by ascertainment by a telephone call from a study clinician:

- Those with contra-indications to MRI: known history of claustrophobia or metal implants incompatible with 3T MRI, such as pacemakers, or
- Those who had a research PET scan in the preceding 12 months (to ensure that their radiation dose for research purposes did not exceed the Administration of Radioactive Substances Advisory Committee 2018 guideline's recommended dose constraint of 10mSv) [273].

The first 502 individuals who agreed to participate and fulfilled the inclusion and exclusion criteria above were included in Insight 46. Our publication on recruitment and participation in Insight 46 [274] has detailed that while the original NSHD was a representative sample of children born in mainland Britain in 1946, Insight 46 participants were slightly biased toward higher childhood

educational attainment and higher socioeconomic position, and even when adjusting for these factors they had better self-rated health, fewer comorbidities, lower smoking prevalence and lower overweight/obesity prevalence at age 68-69 than the NSHD cohort as a whole. In contrast, sex and *APOE* genotype did not predict participation in Insight 46.

2.1.2.2 Phase 1 protocol

Phase 1 was undertaken over August 2015 to January 2018, when participants were 69 to 71 years old. Clinical and neuropsychological data, structural and functional MRI, amyloid PET imaging, and blood and urine samples were collected. The domains that are directly relevant to this project are described in detail below. The phase 1 assessment was designed to be undertaken in a single day but it 62 of the 462 individuals who had a PET-MRI scan, the scan had to be re-scheduled to a later date. In these 62 individuals the mean delay between the blood sample and the scan was 0.158 years (SD = 0.126 years).

2.1.2.2.1 Clinical assessment

Study clinicians, including the author, took a detailed history of cognitive impairment or major neurological/psychiatric illness and medications. Study clinicians also administered the Mini-Mental State Examination [277], a widely used 30-point cognitive screening tool.

The conditions fulfilling criteria for a major neurological/psychiatric illness and the criteria used for diagnosing mild cognitive impairment are listed in Box 2.1.

Box 2.1: Conditions coded as major neurological/psychiatric illnesses and determination of mild cognitive impairment (MCI) in Insight 46.

Clinical evidence of Alzheimer's disease, Parkinson's disease or other neurodegenerative disorder (not based on neuroimaging alone)
Psychiatric disorder requiring anti-psychotic medication or electroconvulsive shock therapy
Epilepsy requiring active treatment
Radiological evidence of traumatic brain injury or major neurosurgery
Clinical diagnosis or radiological features of multiple sclerosis
Radiological evidence of cortical ischaemia or haemorrhage consistent with prior cortical stroke
Radiological evidence of intracranial space-occupying lesion influencing cortical grey matter
Mild Cognitive Impairment defined as follows, based on published criteria [39]: • No clinical evidence of dementia (ICD-10)[2] • AND participant or informant concern* regarding participant's cognition • AND objective evidence of an amnestic deficit (Logical memory delayed recall score ≥1.5 standard deviations below the mean) or a non-amnestic deficit (Digit Symbol substitution score ≥1.5 standard deviations below the mean) – see section 2.1.2.2.2 below

*Significant participant cognitive concern was determined by participant report of memory or cognitive concerns more than others of the same age or prompting seeking medical attention. A structured collateral history taken either in person or via telephone using the well-validated AD8 questionnaire [275, 276] allowed study clinicians to identify significant informant cognitive concern as AD8 score ≥ 2.

30 individuals (*i.e.* 6.0% of the 502 individuals assessed) had a major neurological or psychiatric illness. These comprised 2 individuals with AD, 2 with Parkinson's disease, 1 with Parkinson's disease and epilepsy, 6 with epilepsy, 2 with bipolar disorder,1 with a consensus diagnosis of dementia on assessment in the study, 2 with depression requiring electroconvulsive therapy, 1 with traumatic brain injury, 1 with previous evacuation of subdural haemorrhage, 1 wuth hepatic encephalopathy prior to liver transplant, 1 with myotonic dystrophy, 2 with multiple sclerosis and 8 with stroke.

11 individuals fulfilled study criteria for MCI, of whom 7 (*i.e.* 1.4% of the 502 individuals assessed) had no known major neurological or psychiatric illness.

2.1.2.2.2 Neuropsychological assessment

Participants underwent a single-sitting assessment by one of the study neuropsychologists. The tests from established batteries included:

- Logical memory immediate and delayed recall from the Wechsler Memory Scale-Revised [278] – a test of free recall episodic memory;
- Digit-symbol substitution test, from the Wechsler Adult Intelligence Scale-Revised [279] – a test of executive function and psychomotor speed; and
- Matrix reasoning from the Wechsler Abbreviated Scale of Intelligence [280] – a test of non-verbal reasoning.

In addition, participants also underwent more novel tests aiming to detect subtle cognitive deficits:

- 12-item Face-Name Associative Memory Examination (FNAME-12A) [281] – a test of cued immediate and delayed recall episodic memory
- Task set switching/response inhibition
- Visual short-term memory binding
- Visuomotor integration
- Irrelevant distractor paradigm

The latter four of the above are computer-based paradigms [272] and were not used in the analyses against blood biomarkers as they have not previously been validated.

2.1.2.2.3 Blood sampling, pre-processing and storage.

The standard operating protocol for blood sampling and pre-processing in phase 1 of Insight 46 (Table 2.2, page 157) allowed for collection of samples for storage for biomarkers and genetics, in addition to samples for routine clinical

analysis. The latter included measurement of haemoglobin, platelets, urea, creatinine, thyroid stimulating hormone, vitamin B12 and blood glucose at The Doctors' Laboratory (TDL). *APOE* ε4 carrier status was derived from genotyping of the single nucleotide polymorphisms rs439358 and rs7412 available in the wider NSHD from DNA extracted from blood samples taken at age 53 [282].

Blood stored for biomarkers was pre-processed and stored by the Leonard Wolfson Biomarker Laboratory (latterly the UK Dementia Research Institute laboratory).

Table 2.2: Standard operating protocol for blood sampling and pre-processing in phase 1 of Insight 46.

Condition	**Specification in Phase 1 of Insight 46**
Participant status	Not instructed to fast
Timing of sample collection	09:30 to 11:00
Volume of blood collected	Up to 55 ml
Location of venepuncture	Upper limb peripheral vein
Tourniquet	Used
Venepuncture needle	21G or 23G butterfly needle with Vacutainer collecting system
Blood collection tubes (in order of draw)	2 x SST serum 8.5 ml^{+} 1 x SST serum 2.5 ml: routine analysis 2 x EDTA plasma 10 ml* 1 x EDTA plasma 4 ml: routine analysis and genetics
Transport conditions for blood	Room temperature
Target time between blood sampling and centrifugation	30 minutes
Centrifugation conditions for blood	2000g for 10 minutes at room temperature
Aliquot volume	0.5 ml x up to 14 serum (from $^{+}$) 0.5 ml x up to 16 plasma (from *)
Aliquot container for storage at - 80°C	Polypropylene cryovial

2.1.2.2.4 Structural brain imaging

Dynamic amyloid PET and MRI data were simultaneously acquired by performing imaging on the same Biograph mMR 3T PET/MRI scanner for all participants (Siemens Healthcare, Erlangen) at the University College Hospital

MacMillan Cancer Centre. For the MRI acquisitions, a 12-channel receiver array head coil was used with a body coil radiofrequency transmitter. The maximum gradient strength along each direction was 45mT/m. The MRI sequences were:

i. High resolution 3D T1-weighted (MPRAGE), T2-weighted (SPACE) and FLAIR (IR-SPACE) volumetric scans
ii. Resting state functional MRI
iii. Multi-shell high angular resolution diffusion-weighted MRI
iv. 3D gradient echo for simultaneous T2*-weighted/susceptibility-weighted imaging, quantitative susceptibility mapping and b0 field mapping
v. Arterial spin labelling for quantitative mapping of cerebral blood flow

An additional b0 field map was acquired to correct distortion of the resting state functional MRI and diffusion weighted MRI images. Further details of the sequences are provided in the protocol paper [272].

The analyses undertaken in this thesis utilised only the sequences in group (i) above. T1, T2 and FLAIR images were pre-processed by applying corrections for gradient non-linearity and N4-bias (brain-masked by registration of the scans to the Montreal Neurological Institute (MNI) template). Geodesic information flow (GIF) was used to apply an automated multi-region parcellation of the T1 images [283]. Further details of segmentation techniques used to derive specific outcome variables are given in section 6.2.1, page 245.

2.1.2.2.5 Amyloid PET imaging

Participants had 370 MBq of ^{18}F-florbetapir (Amyvid^{TM}) injected by a peripheral intravenous cannula. Continuous PET data acquisition allowed florbetapir uptake dynamics to be assessed. Final cerebral amyloid burden was assessed

about 50 minutes after injection over a ten-minute period, with scope for the previous ten minutes to be used if participants did not tolerate a longer scan period.

PET attenuation correction was achieved in two ways:

i. Ultra-short echo-time (UTE) sequences provided by the console vendor
ii. List-mode data reconstructions using a pseudo-CT method derived from the T1 volumetric scans [284]; this is known to improve reconstruction accuracy compared to the UTE method [285].

T1-weighted MRI data were parcellated using GIF v3 [283] and co-registered to the PET data using the Niftyreg open source package [286]. A cortical region of interest (ROI) was designed to replicate a widely used cortical composite (of the lateral and medial frontal, anterior, and posterior cingulate, lateral temporal, and lateral parietal regions) derived from statistical contrast of Alzheimer's disease patients and cognitively normal individuals [287, 288]. Global standardised uptake value ratios (SUVR) were calculated by normalising florbetapir uptake in this pre-defined cortical ROI to uptake in eroded subcortical white matter.

Amyloid status (positive/negative) was provided by Dr David Cash; it was obtained by fitting a two- component Gaussian mixture model of SUVR in all participants with adequate PET data, taking the 99^{th} centile of the lower (amyloid negative) Gaussian as the cut-point (0.6104).

2.1.2.3 Phase 2: recruitment to lumbar punctures and CSF sampling protocol

Phase 2 of Insight 46 commenced in March 2018 and is projected to complete in late 2020. In the first day of assessment, most of the measures detailed in phase 1 are repeated. An optional second half-day includes lumbar punctures (LP). The author led the writing of the LP and CSF pre-processing protocol (Table 2.3, page 162), and has performed more than 80% of the LPs to date.

The exclusion criteria for LP in Insight 46 are:

- Neuroimaging screen from phase 1 indicates not safe to perform LP (space occupying lesion with mass effect, tonsillar herniation due to Chiari malformation, or signs of raised intracranial pressure) OR clinician review of neuroimaging from phase 2 indicates not safe as per same criteria OR (if neuroimaging not done in phase 2) clinician examination from phase 2 shows concerns for raised intracranial pressure on neurological examination and/or fundoscopy,
- Known or suspected thrombocytopenia (platelets $<50x10^9$/L as known from previous testing or a recent history of easy bruising),
- Known or suspected coagulopathy (international normalised ratio 1.5 or greater as known from previous testing, or predisposing liver condition (e.g. cirrhosis) or recent history of prolonged bleeding,
- Participant taking any antiplatelet or anticoagulant medication other than aspirin 75mg daily – in this study, participants are NOT advised to discontinue or change any medication,

- Congenital spine abnormality (including but not limited to spinal dysraphism, spina bifida or major kyphoscoliosis) or lumbar fixation surgery,
- Active rash overlying proposed puncture site, or
- Allergy to lignocaine.

After ensuring that no exclusion criteria apply, participants are offered participation in LP, and to date approximately 30% of those seen in phase 2 have consented to LP. For participants who consent to LP, the blood sample is taken on day 2 immediately preceding the LP. For those who do not consent to LP, the blood sample is taken on day 1, with blood sampling and processing remaining identical across the two conditions, and performed by the same protocol as that of phase 1.

Table 2.3: Standard operating protocol for CSF and blood collection in phase 2 of Insight 46.

Condition	**Specification in phase 2 of Insight 46**
Participant status	Not instructed to fast
Timing of lumbar puncture and venepuncture	08:30 to 10:30
Skin preparation	Chlorhexidine 3% - alcohol mixture, allowed to dry fully
Local anaesthetic	Up to 3mg/kg of lignocaine to skin and subcutaneous tissues
Level of lumbar puncture	L2/3, L3/4, L4/5 or L5/S1
Spinal needle gauge	22G atraumatic needle
Volume of CSF collected	Up to 20 ml collected directly into containers (without active withdrawal)
Containers for CSF collection	Polypropylene screw top (Sarstedt 62.610.018)
Location of venepuncture	Upper limb peripheral vein
Tourniquet	Used
Venepuncture needle	21G or 23G butterfly needle with BD Vacutainer adaptor
Blood collection tubes (in order of draw)	2x SST serum 8 ml* 2x EDTA plasma 10 ml^{+} 1x EDTA plasma 4 ml^{+} 1x CPT 8 ml (lymphoid cell prep) 1x Paxgene 2.5 ml (RNA)
Transport conditions for blood and CSF	Room temperature
Target time between sampling and centrifugation of blood and CSF	30 minutes
CSF Centrifugation conditions	1750 g for 10 minutes at 4 °C
Blood centrifugation conditions (* and $^{+}$ only)	2000 g for 10 minutes at room temperature
Aliquot volume for CSF	0.5 ml x 40
Aliquot volume for blood	0.5 ml x 14 serum (from *) 0.5 ml x 18 EDTA plasma (from $^{+}$)
Containers for storage at -80 °C	Polypropylene cryovials

2.1.3 HABS and UCSD cohorts

The HABS participants [289, 290] were included as healthy controls in the test cohort described in Chapter 9. They were recruited by flyer and website advertisement via the Massachusetts Alzheimer's Disease Research Centre (https://www.madrc.org/harvard-aging-brain-study). They were assessed as cognitively normal (global Clinical Dementia Rating of 0, Mini Mental State Examination score of 27-30, and within 1 standard deviation of age and education adjusted norms for Logical Memory Delayed Recall LMIIa from the Wechsler Memory Scale-Revised [278]) and remained cognitively normal at follow up for a minimum of one year. They were all negative for cerebral amyloid deposition using PiB PET (based on a Distribution Volume Ratio cut-off of 1.2).

The UCSD cohort samples were collected from 2010 to 2018 as part of a US national study [291] with research procedures approved by the UCSD Institutional Review Board (IRB 80012). They were recruited through the UCSD AD research centre and used as a validation cohort in the experiments detailed in Chapter 9. Participants provided written informed consent and underwent detailed evaluations consisting of medical history, physical and neurological examinations, laboratory tests, and neuropsychological assessments. Control participants were volunteers in good health with no signs or symptoms suggesting cognitive impairment or neurological disease. All participants had blood sampling for DNA and underwent a research lumbar puncture to collect CSF. This study used stored CSF from selected participants with consensus

research diagnoses of cognitively normal, Mild Cognitive Impairment (MCI) or dementia consistent with probable Alzheimer's Disease (AD) [292].

Table 2.4 summarises the protocols for biofluid sampling and pre-processing in the HABS and UCSD cohorts.

Table 2.4: Standard operating protocols for CSF and blood collection and processing for the HABS and UCSD cohorts

Condition	**HABS**	**UCSD**
Participant status	Not instructed to fast	Fasted
Timing of lumbar puncture	Not specified	08:00 – 10:00
Timing of venepuncture	Within 3-6 months of LP	Immediately before LP
Spinal needle gauge	22 – 24G	24G
Volume of CSF collected for research	Not specified	15 – 20 ml
Containers for CSF collection	Polypropylene 10 ml	Polypropylene
Venepuncture needle gauge	Not specified	Not specified
Tubes for blood sampling	6 ml EDTA plasma	6 ml EDTA plasma
Transport conditions for CSF and blood	Room temperature	Room temperature
Centrifugation conditions for CSF and blood	1000 g for 10 minutes at room temperature	1500 g for 10 minutes at room temperature
Aliquot volume for CSF and plasma	1 ml	0.5 ml
Aliquot container for storage at -80 °C	Polypropylene cryovial	Polypropylene cryovial

2.2 **Experimental techniques**

2.2.1 CSF enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) are a well-established method of quantification of protein or peptide analytes in biofluids. They rely on the principle of antibody specificity to quantify a target molecule. The first (capture) antibody binds the target molecule through a specific high-affinity interaction with one epitope or region on the target. The second (detection) antibody binds the target in a similar way but via a different epitope; thus, the two antibodies form a sandwich complex with the target molecule. After sandwich complex formation, the detection antibody activates an enzyme that converts a substrate to a coloured product. The concentration of this coloured product is quantified on a plate reader by the absorption of light of a specific wavelength, and comparing this to a calibration curve generated by a set of standards allows interpolation of target molecule concentration.

The methods described here refer to ELISA used for the characterisation of CSF samples for the collaborative experiments of Chapter 9. Fujirebio INNOTEST® ELISA kits for Aβ1-42, total tau and phospho-tau-181 were used according to the manufacturer's instructions (Table 2.5, page 166).

Table 2.5**:** CSF INNOTEST® assays used in experiments of Chapter 9.

Assay	**Amyloid-β 1-42**	**Total tau**	**Phospho-tau 181**
Supplier	Fujirebio INNOTEST®		
Thaw time before plating	1 hour	3 hours	3 hours
Number of thaws	1	1	1
Capture antibody	21F12 mouse monoclonal; targets a.a. x-42 of amyloid beta peptide; bound to plate	AT120 mouse monoclonal; targets a.a. 218-224; bound to plate	HT7 mouse monoclonal; targets a.a. 159-163; bound to plate
Standards	7 provided standards (0 to 4000 pg/ml)	7 provided standards (0 to 2000 pg/ml)	7 provided standards (0 to 600 pg/ml)
Volume of CSF/ replicate	25 µL	25 µL	75 µL
Capture antibody incubation conditions	1 hour; 1000 rpm agitation; room temperature	Overnight at room temperature without agitation	Overnight in the dark at 4°C without agitation
Washes prior to detection antibody	None		
Biotinylated detection antibody	3D6; targets a.a. 1-5 of amyloid beta peptide; diluted 1 in 101; 75 µL/well	HT7 targets a.a. 159-163; BT2 targets a.a. 193-198; diluted 1 in 101; µL/well	AT270 mouse monoclonal; targets a.a. 178-184 around threonine 181-P; diluted 1 in 101; 25 µL/well
Detection antibody incubation conditions	Added at same time as samples to plate		
Washes prior to enzyme conjugate addition	5 x 400 µL/well of wash buffer		
Enzyme conjugate	Peroxidase-labelled streptavidin; 100 µL/well; room temperature		
Enzyme conjugate dilution	Diluted 1 in 101; 1000 rpm agitation		
Enzyme incubation time	30 minutes		60 minutes
Washes prior to substrate addition	5 x 400 µL/well		
Substrate	Tetramethylbenzidine (TMB) diluted 1 in 101 in 0.2% H_2O_2; 100 µL/well at room temperature on 1000 rpm plate shaker in the dark for 30 minutes		
Stop solution	0.9N sulphuric acid		
Read time	Within 15 minutes of adding stop solution		
Plate reader	BMG Labtech FLUOstar Omega multi-mode microplate reader; absorbance read at 450 nm (reference: 620 nm)		
Concentration calculation	Four-point logistic regression using Omega software		

2.2.2 Simoa digital immunoassays

Single molecule array (Simoa) technology adapts the ELISA principle by two key changes. Firstly, it uses magnetic beads as the surface for antibody-target interaction, thereby increasing the surface area and facilitating a digital readout at low target concentrations by employing the magnetic property of the beads. Secondly, the end-product of the antibody-target complex is a fluorescent molecule, so the readout is obtained as light emitted at a specific wavelength rather than light absorbed. Detailed descriptions of the technology are provided by Wilson and colleagues [293].

The Quanterix HD-1 analyser (Figure 2.1, page 168) was used for all Simoa analyses described throughout this body of work. Using the specific example of the Tau 2.0 assay, detailed steps are described in Figure 2.2 (page 169).

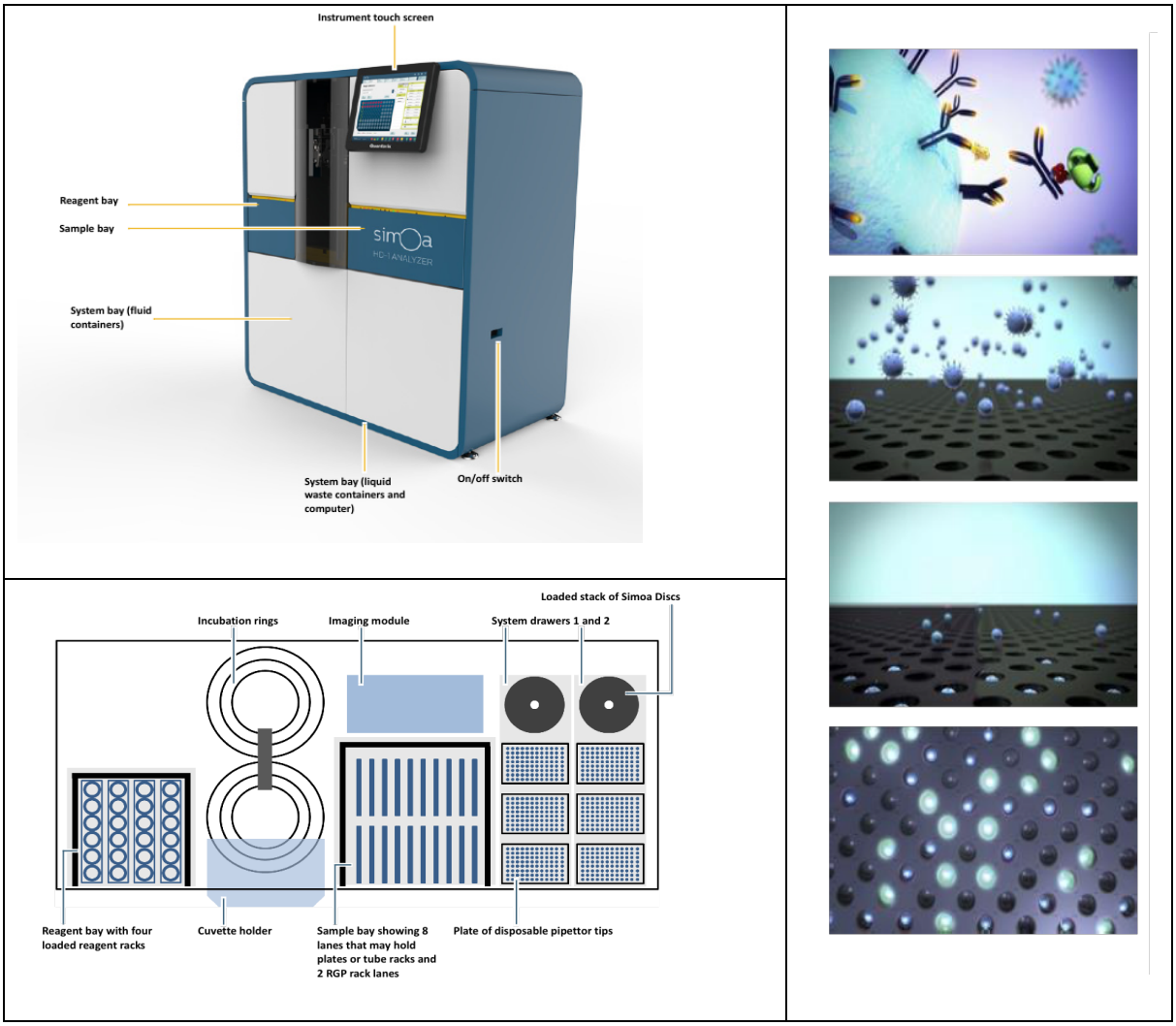


Figure 2.1: Simoa technology

Simoa technology uses antibody pairs coupled to paramagnetic beads; binding of the target molecule catalyses production of a fluorescent product which can be imaged when the beads are washed across the Simoa disc and settle into individual wells. The number of fluorescent beads reflects the concentration at the lower (digital) range of the assay and the overall fluorescence intensity reflects concentration at the higher (analogue) range.
Graphics credits: reproduced from [294, 295] with written permission, © Quanterix Corporation.

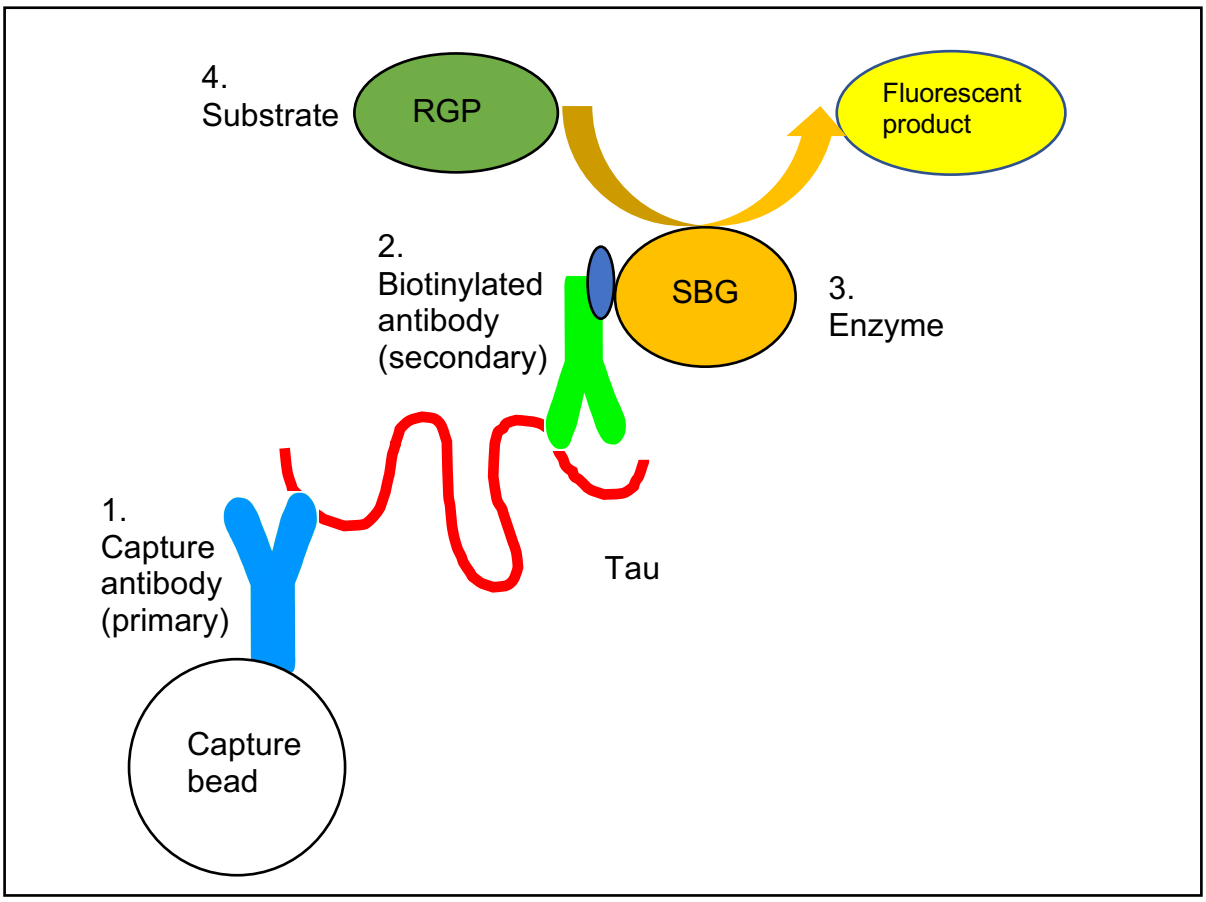


Figure 2.2: Principles of the Simoa Human total tau 2.0 Digital immunoassay.

1. Anti-tau (HT7 and BT2 mouse monoclonal; Pierce/Thermo) antibody-coated capture beads are combined with tau molecules present in the sample (which is vortexed, added to the plate and pre-diluted x4 in a diluent of phosphate buffer with bovine serum components, a heterophilic blocker, a surfactant and ProClin 300 preservative).
2. Biotinylated anti-tau (Tau 5 mouse monoclonal, Covance) detector antibodies, in phosphate buffer with bovine protein stabilisers, are added.
3. After washing, a conjugate of streptavidin-β-galactosidase (SBG) is mixed with the capture beads. By binding the biotinylated antibody, enzyme-labels the captured tau.
4. After another wash, the beads are re-suspended in resorufin β-D-galactopyranoside (RGP) solution and transferred to the Simoa disc. Individual capture beads are then sealed within micro-wells on the disc with a fluorinated polymer oil.
If tau has been captured, the β-galactosidase hydrolyses the resorufin β-D-galactopyranoside into a fluorescent product. There is enough fluorescent signal generated by a single labelled tau molecule in 30 seconds to be detected and counted by the Simoa optical system. As only one bead can fit in a well, at low tau concentrations the total percentage of beads with a positive signal is proportional to the amount of tau in the sample. At higher concentrations, when most wells contain beads with a positive signal, the total fluorescence signal is proportional to the amount of tau in the sample.

All commercially available assays used were two incubation step assays, in which the capture beads and detector antibodies were combined with the sample at the same time. Table 2.6 gives details of the antibody pairs used for the commercially available Quanterix assays used for the work in Chapters 3 to 7. The homebrew tau assays used in the study described in Chapter 8 are detailed there (section 8.2.4.3, page 287).

Table 2.6: Antibody pairs and conditions used for the Quanterix Tau 2.0, NF-light, Aβ42 and Aβ40 assays

Assay	**Tau 2.0**	**NF-Light**	**Aβ42 2.0**	**Aβ40 2.0**
Capture antibody	HT7 and BT2 mouse monoclonal (Pierce/Thermo)	47:3 (Uman Diagnostics)	6E10 (a.a. 3-8)	6E10 (a.a. 3-8)
Biotinylated detector antibody	Tau5 (Covance)	2:1 (Uman Diagnostics)	2G3 (ADx)	H31L21 (Invitrogen)
Standards	Provided with kit and tested neat: 8 standards, range 0-100 pg/ml	Diluted from calibrator concentrate: 8 standards, range 0-500 pg/ml	Diluted from calibrator concentrate: 8 standards, range 0-100 pg/ml	Diluted from calibrator concentrate: 6 standards, range 0-200 pg/ml
Sample dilution	x 4 in-line dilution			
Lower limit of quantification (from kit data sheets), pg/ml	0.061	0.174	0.137	1.23

2.3 General statistical methods

2.3.1 Software

All statistical analyses presented here were carried out by the author, with methodological advice from Dr Jennifer Nicholas. For the analyses of Chapter 4, SPSS Version 24 was used (IBM Corporation, Armonk, New York, USA). All other analyses used Stata Version 14.2 (Stata Corporation Texas, USA). Detailed statistical methods are described within each chapter but some commonly applicable techniques are addressed below.

2.3.2 Missing biomarker values

Where a given biomarker was not quantified reliably (either due to failure of assay across all replicates or due to an unacceptably high coefficient of variation between replicates) the value was discarded and the relevant individual was not included in analyses. The number of missing values was less than 10 in every case and therefore it was assumed that these values were "missing at random" but the assumption could not be tested.

2.3.3 Assessing normality of data

Most fluid biomarker values do not assume a normal distribution in the population because they are truncated at the lower limit of quantification of the assays, and they are often positively skewed. Wherever relevant to parametric analyses, and particularly when utilised as the outcome variable (e.g. Chapter 5), biomarker values were natural log-transformed. Non-parametric approaches or non-linear modelling were otherwise used to analyse raw values as appropriate.

Where linear regression was employed, assumptions of normality were checked by using plots of model residuals vs fitted values, and/or normal quantile-quantile (q-q) plots, after taking advice that more formal tests of normality (such as the Shapiro-Wilk test) would be likely to be too stringent.

2.3.4 Assessing the influence of outliers

Cook's distance is a measure of influence on both model fitted values and on regression coefficients. Model fits were tested before and after exclusion of potentially influential outliers defined by a Cook's distance of > 4/n (a widely accepted cut-off [296]) and where exclusion of these values qualitatively altered the regression coefficient for the predictor(s) of interest, this was stated.

2.3.5 Approach to multiple comparisons

Overall the number of comparisons relevant to each statistical question was small, and within each chapter/theme the various outcome variables against which biomarkers were being assessed were often themselves related. Hence the general approach throughout this thesis was not to correct for multiple comparisons, except for simple inter-biomarker correlation analyses (such as those presented in Chapter 5) in which simple Bonferroni correction was applied to *p* values.

2.3.6 Participant numbers and power calculations

Sample numbers for the studies described in Chapters 3 and 8 are discussed in those chapters. For the studies involving blood biomarker data from Phase 1 of Insight 46 (Chapters 4 to 7), all experiments were initially devised as exploratory analyses. This was because the number of individuals recruited to Insight 46

was not calculated based on the potential for identifying differences in blood biomarkers in the first instance, but determined based upon a likely 20% prevalence of PET amyloid positivity at age 70 [297], which would give 100 amyloid-positive and 400 amyloid-negative individuals. At the time of setup of Insight 46, this was deemed to be a robust sample size in which differences in neuroimaging and neuropsychological variables between the two groups could be compared. In relation to the blood biomarker variables, when these experiments were devised in 2016, data from Janelidze *et al*. in BIOFINDER [202] had shown a significant difference in Simoa plasma Aβ42/40 ratio in 74 amyloid-positive cognitively normal individuals (defined by CSF amyloid signature) compared to 200 amyloid-negative cognitively normal individuals. To detect a similar difference in plasma Aβ42/40 ratio between amyloid-positive and amyloid-negative individuals in Insight 46, using the data from Janelidze *et al.,* a difference of at least 0.011 in Simoa plasma Aβ42/40 between 100 amyloid-positive and 400 amyloid-negative individuals would be detectable at greater than 98% power, at a significance level of $p = 0.05$. Even assuming that only 80% of the recruited individuals would be cognitively normal and have no prior neurological conditions, a difference of at least 0.011 in Simoa plasma Aβ42/40 would be detectable at greater than 95% power, at a significance level of 0.05. Hence, the numbers recruited to Insight 46 would be more than sufficient to detect the differences in Simoa plasma Aβ42/40 that had been demonstrated in BIOFINDER.

For serum NFL, Weston *et al.* had shown a significant difference of 4 pg/ml (standard deviation 7.7 pg/ml, p = 0.007) in Simoa assay measurements between 19 asymptomatic dominant AD mutation carriers (MC: equivalent of

amyloid positive cognitively normal individuals) and 11 non-carriers (NC: equivalent of amyloid-negative cognitively normal individuals). Taking into account possible limitations in comparison to Insight 46 participants, who would be expected to have higher serum NFL values overall due to higher age than the participants studied by Weston *et al*., measurements in 100 amyloid-positive and 400 amyloid-negative individuals would give greater than 99% power for detecting a difference of 4 pg/ml at the $p = 0.05$ level, and greater than 95% power for detecting a difference of 3 pg/ml at the $p = 0.05$ level. Even if the total number were 400 (for example, due to restricting analyses to only to cognitively normal individuals with no prior neurological conditions), a difference of 4 pg/ml would be detectable with greater than 99% power and a difference of 3 pg/ml would be detectable with greater than 90% power at the $p = 0.05$ level.

Similar data to guide retrospective power calculations were not available for plasma t-tau in cognitively normal individuals stratified by amyloid status, as the only comparable publication at the time (by Mattsson *et al*. in ADNI and BIOFINDER [220]) had not demonstrated significant differences between amyloid status groups among cognitively normal individuals in either cohort. This publication also had not demonstrated significant linear regression coefficients for plasma t-tau against ventricular or hippocampal volumes, or for MMSE, among cognitively normal individuals in ADNI for whom those data were available. No studies were available at that time describing linear regression coefficients for Simoa biomarkers of plasma Aβ42, Aβ40 or serum NFL against brain volume or cognitive variables, and all of these analyses were therefore undertaken in Insight 46 in an exploratory manner.

3 Pre-analytical variation in Simoa blood biomarkers

3.1 Introduction

3.1.1 Publication statement

The work included in this chapter has been published previously [298] and is included here as per the publisher's policy with regards to thesis publications.

3.1.2 Background

Replication across studies of blood biomarkers in Alzheimer's disease has posed a significant challenge. One important source of variation among studies is pre-analytical factors. These include variables which cannot be controlled but can and should be recorded, such as demographics, comorbidities, diet, medications, smoking, alcohol use and activity levels. However, there are many variables which may be controlled, such as time of collection, fasting status, venepuncture needle size and location of draw, tube type and additives, tube collection order, centrifugation settings, time delay to first freeze, aliquot size and number of freeze-thaw cycles. In particular, guidelines for standardising these variables indicate that the number of freeze-thaw cycles should be minimised [151]. However, only a few AD-related publications have probed the number of freeze-thaw cycles up to which a sample may be taken before the quantification of the specific biomarker in question may be affected. Some immunoassay platform-based studies have shown that more than three freeze-thaw cycles may reduce plasma amyloid β 1-40 and 1-42 (Aβ40 and Aβ42) by 20% [299] and also reduce CSF Aβ42 [300]. There is conflicting evidence on the stability of CSF total tau (t-tau); when measuring using the same commercially available INNOTEST® ELISA, one study reported it to be stable over up to six freeze-thaw cycles [300] but another demonstrated both CSF t-

tau and phospho-tau-181 to reduce over three freeze-thaw cycles [301]. In contrast, CSF neurofilament light chain (NFL) has been shown to remain stable over up to four freeze-thaw cycles when measured by ELISA [302]. However, prior to publication of the results below, there had been no systematic examination of the stability of t-tau or NFL in blood, or of Aβ40 and Aβ42 in blood as measured on the Simoa platform. This study was undertaken primarily to investigate the effects of up to four freeze-thaw cycles on these biomarkers, as this would inform later work with these assays in the Insight 46 samples. A secondary aim was to determine whether plasma or serum would be the more suitable blood fraction in which to quantify these biomarkers.

3.2 **Methods**

3.2.1 Participants

Participants were from the DRC clinical cohort (see section 2.1.1.1, page 149, for details of recruitment and ethics).

3.2.2 Sample handling

Samples were taken and processed as per the DRC study protocol (see section 2.1.1.2, page 150) but aliquoted specifically for this study as follows. Plasma was centrifuged a median of 20 minutes after sampling, whereas serum was allowed to clot for a median of 15 minutes before centrifugation. The supernatant was pipetted in 200 µL volumes into identical polypropylene screw-top cryovials and placed in -80ºC within 60 minutes of sampling.

3.2.3 Blood fraction comparison

Paired plasma and serum from the same individuals were used to measure the same biomarker after a single thaw of 1 hour directly from -80ºC to room temperature.

3.2.4 Freeze-thaw cycles

Informed by the results of the blood fraction experiments, up to four aliquots per individual of the chosen blood fraction for each assay were used. Each aliquot was subjected to either one, two, three or four freeze-thaw cycles. Each additional freeze-thaw cycle after the first consisted of one hour of direct thaw to room temperature followed by one hour of direct replacement into -80ºC.

3.2.5 Simoa assays

In both the blood fraction and freeze-thaw experiments, after the final thaw, samples were transferred to individual 1.5 ml polypropylene centrifuge tubes and centrifuged as per the kit manufacturer's recommendation at 13 000 g for 10 minutes. The supernatant was pipetted onto the plate for analysis. For each biomarker, all aliquots from the same participant were assayed using the same batch of reagents in the same run. Details of the commercially available Simoa total tau 2.0, NF-light, Aβ40 and Aβ42 assays are given in General Methods (section 2.2.2, page 167). All measurements were conducted on the same automated HD-1 analyser (Quanterix). For NFL, Aβ40 and Aβ42, samples were assayed in duplicate. For t-tau, samples were assayed in triplicate due to greater kit availability. The mean of replicates for each sample was calculated and included in the

statistical analysis of the coefficient of variation across replicates was <15%; all samples passed this requirement.

3.2.6 Statistics

The number of samples used for this study was determined by the availability of aliquots, which were prepared specifically for this study after aliquots for the DRC study biobank were made. No formal power calculations were employed, but sample collection for testing each biomarker was deemed sufficient if at least five pairs of samples for the blood fraction comparison (each from a different individual) and ten sets of aliquots for the freeze-thaw comparison (each again from a different individual) had been collected, as testing these in duplicate would allow for the use of a single reagent kit, and this type of sample size had previously been employed in publications examining similar questions [239, 299]. The available numbers of samples slightly exceeded the planned minimum; therefore, the additional samples were tested after acquiring a second kit if necessary. The numbers of individuals for each experiment are shown in Table 3.1.

Table 3.1: Numbers of individuals in each experiment examining the effect of pre-analytical sample handling factors on Simoa blood biomarkers.
Simoa Assays: Aβ40, amyloid-β 40; Aβ42, amyloid-β 42; t-tau, tau 2.0 “total tau”; NFL, NF-light neurofilament light chain.

	Number of individuals	
Blood biomarker tested	**Blood fraction experiment**	**Freeze-thaw experiment**
NFL	5	12
t-tau	11	11
Aβ40	7	14
Aβ42	10	12

Simple pairwise correlation was used to assess the associations between serum and plasma values of analytes.

A Friedman analysis of variance (non-parametric repeated measures ANOVA) was used to assess differences within individuals across four freeze-thaw cycles (SPSS Statistics, version 24). Where differences were significant at the *p* = 0.05 level, the ratio of the concentration at a specific freeze-thaw cycle to the concentration at the first cycle was calculated across individuals.

3.3 **Results**

3.3.1 Blood fraction comparison

Figure 3.1 (page 180) shows that serum and plasma values of each of the four Simoa biomarkers were positively correlated. Values of NFL were higher in serum than plasma but values of t-tau, Aβ40 and Aβ42 were higher in plasma than serum. Therefore, for the freeze-thaw cycle experiments, serum was used to investigate effects on NFL but plasma was used for t-tau, Aβ40 and Aβ42.

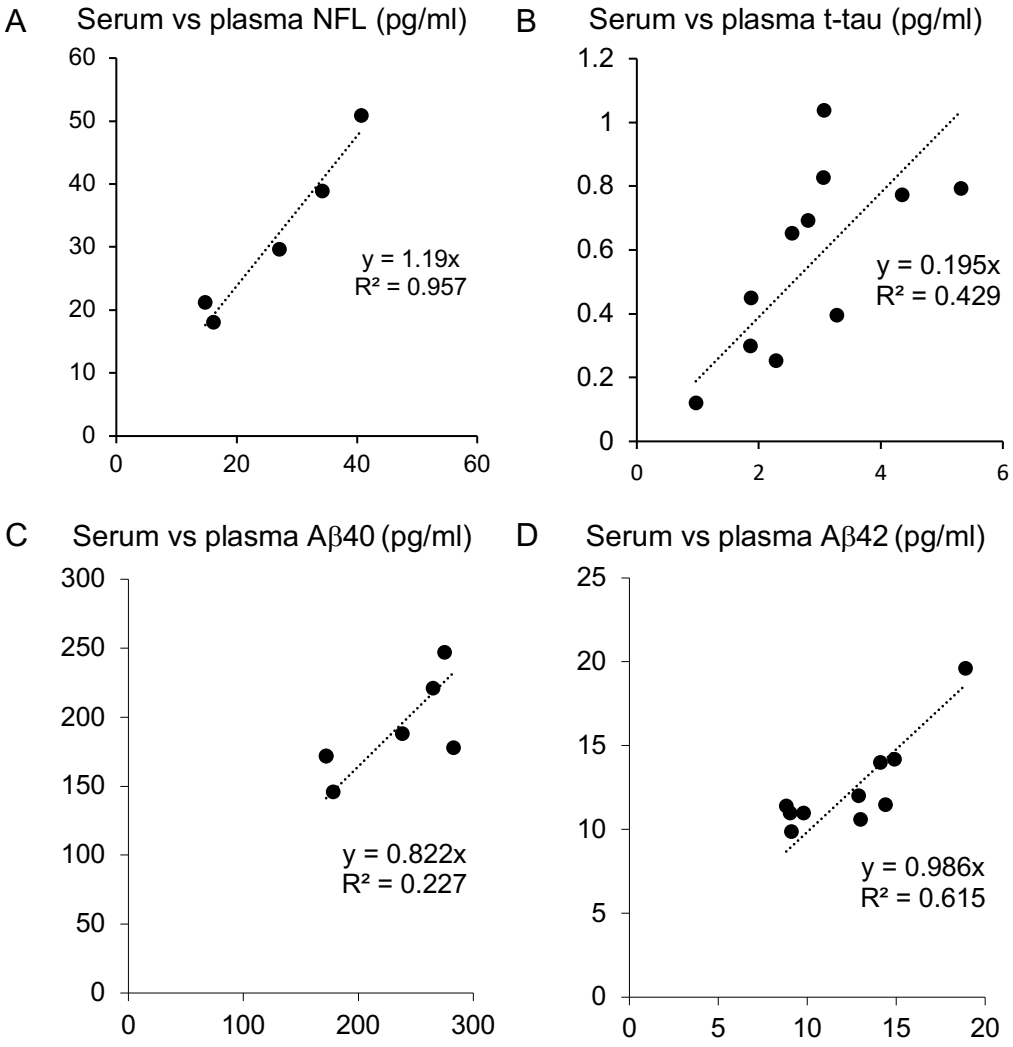


Figure 3.1: Serum (y axis) vs plasma (x axis) concentration for each Simoa biomarker.
Each result is the mean of replicates within an individual. Trend lines show linear correlation with a fixed intercept of 0.
A: NFL, n=5
B: t-tau, n=11
C: Aβ40, n=7
D: Aβ42, n=10
Simoa Assays: Aβ40, amyloid-β 40; Aβ42, amyloid-β 42; t-tau, tau 2.0 "total tau"; NFL, NF-light neurofilament light chain.
Figure reproduced with minor modifications from Keshavan *et al.* [298], with permission as per publisher's policy.

3.3.2 Freeze-thaw cycles

Figure 3.2 (page 182) shows the effect of one to four freeze-thaw cycles on the measured concentrations of the four Simoa biomarkers.

For plasma Aβ40, the median concentration ratio between the third and first cycles was 0.96 (interquartile range, 0.92 – 0.99; Friedman ANOVA $p = 0.015$). The median concentration ratio between the fourth and first cycles was 0.92 (interquartile range, 0.90 – 0.96; Friedman ANOVA $p = 0.001$), and this survived the exclusion of the one clear outlier individual.

There was no significant change in the concentrations of serum NFL, plasma t-tau or plasma Aβ42 over four cycles (Friedman ANOVA two-tailed significance $p>0.05$).

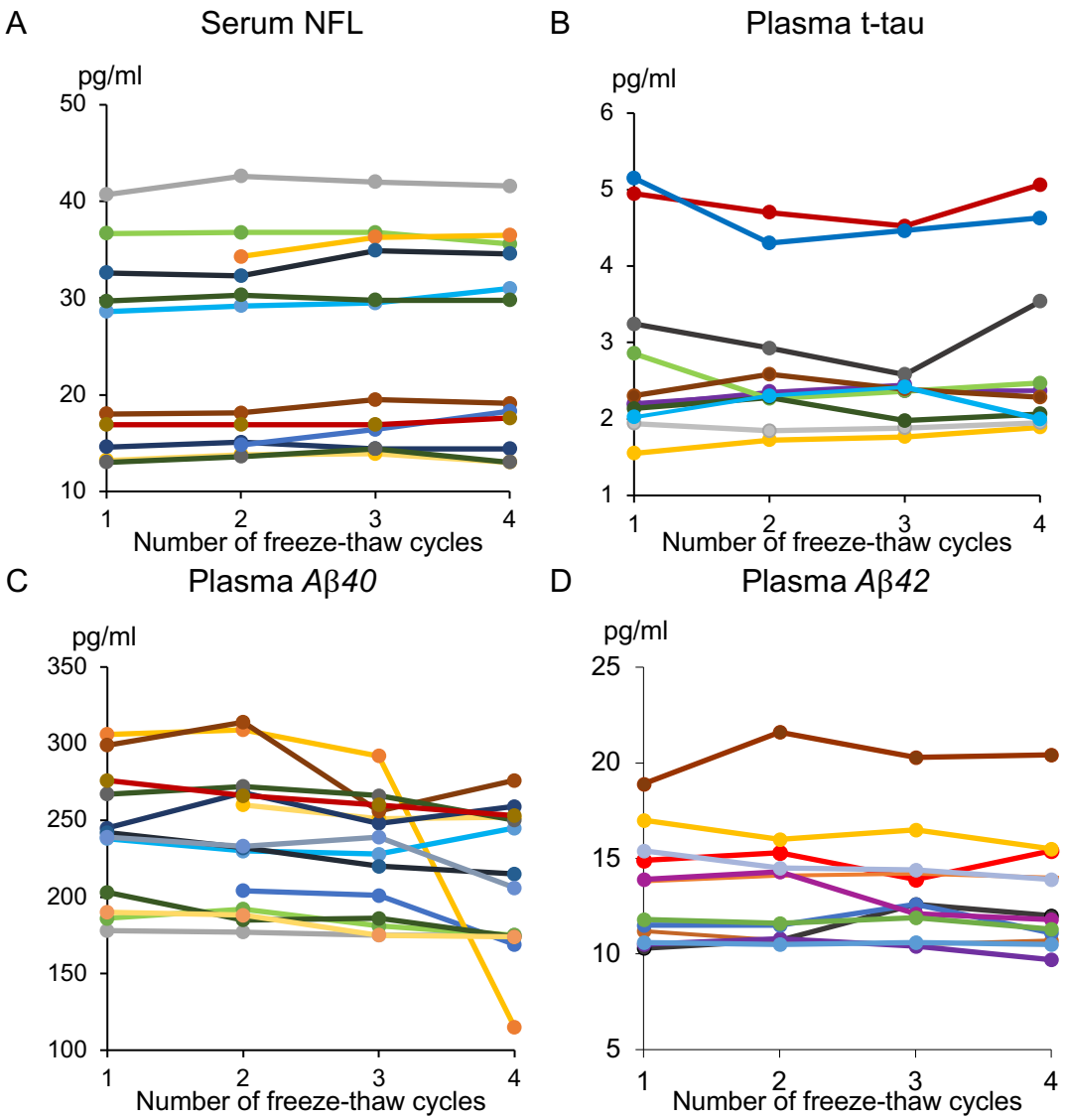


Figure 3.2: Concentration of each Simoa biomarker versus number of freeze-thaw cycles. Each result is the mean of replicates within an individual and each coloured line within a plot connects results from the same individual.
A: serum NFL, n=12
B: plasma t-tau, n=11
C: plasma Aβ40, n=14
D: plasma Aβ42, n=12.
Simoa Assays: Aβ40, amyloid-β 40; Aβ42, amyloid-β 42, t-tau, tau 2.0 “total tau”; NFL, Simoa NF-light neurofilament light chain.
Figure reproduced with minor modifications from Keshavan *et al.* [298], with permission as per the publisher’s policy.

3.4 **Discussion**

3.4.1 Blood fraction choice

These results showed a high correlation between serum and plasma NFL from the same individuals as measured on the Simoa platform, with a tendency for serum levels to be higher; this replicates the work of Kuhle *et al.* (as reported in Lu *et al.* [161]) who demonstrated a similarly high degree of correlation when measuring NFL using an electrochemiluminescence assay on the MSD platform. More modest correlations were shown between blood fractions for the other three blood biomarkers, and concentrations of t-tau, Aβ40 and Aβ42 were higher in plasma than in serum. These findings suggest that plasma is the blood fraction of choice when quantifying these latter three blood biomarkers, as less of the analytes of interest may have been removed in the pre-processing of plasma compared to that of serum.

3.4.2 Freeze-thaw stability

For up to four freeze-thaw cycles, each consisting of at least 1 hour of thawing to room temperature, in aliquot volumes of 200 µL, this series of experiments showed that there was no consistent trend for change in the concentrations of serum NFL, plasma t-tau or plasma Aβ42. These findings on stability of serum NFL contrast with those of Lewczuk *et al*. who demonstrated an upward drift of measured values that was statistically significant at the second freeze-thaw cycle, in EDTA-plasma measured on the Simoa platform [239] but the authors did not specify the duration of the freeze-thaw cycles and they used just five individuals' samples, of plasma rather than serum.

Lachno *et al.* used a Luminex bead-based immunoassay platform and a 1 hour thaw per cycle to demonstrate a reduction in plasma Aβ40 from the fourth cycle in five individuals [299]. In the experiments described here, a larger sample size showed a small but statistically significant reduction of the measured concentration of plasma Aβ40 from the third cycle, and a further reduction at the fourth cycle which survived the removal of the outlier. It is not clear whether the results from this outlier were in any way related to the freeze-thaw effect, and there were no obvious technical differences identified in sample treatment for this individual compared to the rest. Overall, in keeping with the group results, this suggests that samples in which plasma Aβ40 is to be measured should be restricted to a maximum of two freeze-thaw cycles.

After the work detailed in this chapter was published [298], Rozga *et al.* replicated the findings with respect to plasma t-tau, Aβ1-40 and Aβ1-42 measurements on the Roche Elecsys immunoassay platform, and extended them to include a detailed study of pre-analytical factors impacting measurement of these biomarkers in plasma [303]. They showed that up to three freeze-thaw cycles does not affect any of these markers, and importantly that tube transfer up to five times does not affect measurements. They also showed that citrated and heparinised plasma yield lower t-tau measurements than EDTA-plasma, which supports the choice of the latter in most studies of plasma t-tau (including our study).

The conditions chosen for this set of experiments are consistent with the treatment and aliquot volumes recommended by consensus guidelines [151] and mimic the likely conditions in large cohort studies such as Insight 46, in which it is desirable to minimise sample volumes and essential to cross-validate

results between laboratories. The ability to sub-aliquot in this manner allows for sharing samples across centres while reducing concerns about the effects of small numbers of short-duration freeze-thaw cycles on measured biomarker concentrations.

4 Factors influencing variability in Simoa blood biomarkers in phase 1 of Insight 46

4.1 Introduction

Several participant-specific pre-analytical “uncontrollable” variables may affect potential blood biomarkers (BB) of AD, including age, sex, *APOE* ε4 carrier status, and non-AD comorbidities [269]. This chapter examines the influence of sex, *APOE* ε4 carrier status and cerebral amyloid deposition on plasma Aβ42, Aβ40, Aβ42/40 ratio, t-tau and serum NFL. Large cohort studies of NFL in blood have used either plasma (as in ADNI [234]) or serum (as in DIAN [233]), but have generally used plasma for the other listed markers. The choice of blood fractions for testing in Insight 46 was therefore guided by these precedents, and by the DRC cohort plasma vs serum comparisons (Chapter 4 and [298]) indicating that serum returns higher NFL values within individuals, so we would expect that it might provide a slightly wider dynamic range than plasma. Conversely, plasma was used for testing for the other markers that were more abundant in plasma than serum (as was particularly the case for t-tau in samples from the DRC cohort).

Despite the possibility that measured BB values may be modified by the volume over which they are distributed (that is, the blood volume) and by the efficiency of their clearance mechanisms from the blood, very few studies of blood biomarkers in AD have adjusted for variables that may provide indications of blood volume or of renal clearance. In Insight 46 the body mass index (BMI) and serum creatinine were readily available as they were measured on the same day as the blood sample collection for biomarkers. This chapter therefore

also details the relationships between BB and these surrogate markers of blood volume/body size and renal function.

The questions addressed in these analyses were as follows:

- Are there differences in BB according to sex, BMI and serum creatinine?
- What are the cross-sectional associations between BB and *APOE* ε4 carrier status? Is there an interactive effect of sex on these associations?
- What are the cross-sectional associations between BB and amyloid PET SUVR? Is there an interactive effect of sex on these associations?

The main hypotheses were that all four primary BB would increase with serum creatinine, potentially due to reduced renal clearance; that they would show differences between sexes, potentially due to differences in volume of distribution; that the plasma amyloid biomarkers Aβ42 and plasma Aβ42/40 ratio would be reduced in *APOE* ε4 carriers compared to non-carriers, and that reductions in plasma Aβ42 and Aβ42/40 ratio would be associated with increased amyloid PET SUVR.

4.2 **Methods**

4.2.1 Participants

Participants were from the Insight 46 cohort (see section 2.1.2, page 152) for details of recruitment and assessments undertaken).

4.2.2 Sample handling

Blood samples for biomarkers were pre-processed as per the Insight 46 protocol (Table 2.2, page 157) and stored at -80 ºC until all phase 1 blood samples had been collected.

For serum NFL analysis: a single 500 µL aliquot of serum for each individual was thawed directly to room temperature over 1 hour and vortexed for 2 seconds to ensure thorough mixing. 200 µL was pipetted into a 1.5 ml polypropylene centrifuge tube and centrifuged at 13 000 g for 10 minutes, as per the kit manufacturer's recommendation; the remaining 300 µL was replaced into -80 ºC in the original cryovial. After the 200 µL was centrifuged, 130 µL of the supernatant was pipetted onto the plate for analysis in duplicate. If the coefficient of variation (CV) across the duplicates was >15% or no value was returned for either, the procedure above was repeated at a later date, employing one additional freeze-thaw cycle by starting with the 300 µL volume that was in the original cryovial. At this point all 500 individuals who had blood sampling had a serum NFL value quantified with a CV <15%.

For plasma Aβ40 and Aβ42 analysis: a single 500 µL aliquot of plasma for each individual was thawed directly to room temperature over 1 hour and vortexed for 2 seconds. 300 µL was pipetted into a 1.5 ml polypropylene centrifuge tube and centrifuged at 13 000 g for 10 minutes as per the kit manufacturer's recommendation; the remaining 200 µL was replaced into -80 ºC in the original cryovial. After the 300 µL was centrifuged, 100 µL of the supernatant was pipetted onto each of two plates for analysis in duplicate, capitalising on the ability to load two different reagent kits at a time on the HD-1 analyser. When

plates of samples for analysis of Aβ40 and Aβ42 were prepared in this way, the plate containing samples for Aβ40 was always analysed first, and that containing samples for Aβ42 was analysed second. The CV across the duplicates was <15% for all samples assayed for Aβ40 but for some samples assayed for Aβ42 the CV was >15% or no value was returned. In this case the procedure above was repeated at a later date, using a fresh 500 µL aliquot of plasma and pipetting out and centrifuging 200 µL then pipetting 100 µL of the supernatant onto the plate for Aβ42 analysis.

For plasma t-tau analysis: if only one aliquot of plasma had been used for Aβ42, the second (fresh) 500 µL aliquot of plasma for each individual was used for assaying total tau and the steps undertaken to prepare the sample thereafter were identical to those described above for serum NFL. However, if the Aβ42 assay was being repeated on the second aliquot, then on the same thaw of this sample, after vortexing for 2 seconds, 200 µL was pipetted into a separate 1.5 ml polypropylene centrifuge tube and used for plasma t-tau analysis in parallel. In this situation, the plate of samples for analysis of Aβ42 was analysed first and the plate for analysis of t-tau second. If the CV across duplicates was >15% on the first analysis of t-tau, the analysis was repeated at a later date, employing one additional freeze-thaw cycle by starting with the 300 µL volume that was in the original cryovial.

By this method, all samples analysed for plasma Aβ40 and Aβ42 underwent one freeze-thaw cycle; 59 samples analysed for plasma t-tau and 72 samples analysed for serum NFL underwent two freeze-thaw cycles. This procedure was deemed to be acceptable based on the evidence that up to four freeze-thaw

cycles do not affect measured plasma t-tau or serum NFL concentrations for these assays (Figure 3.2, page 182 and [298]).

4.2.3 Simoa assays

For each BB, all aliquots were assayed using the same batch of reagents excepting the last plate of samples, which were those requiring repeat analysis. Every plate was analysed according to its own calibrators (made from the stock solution provided in the kit), and included two run validation controls also made from this stock. The measured values of the two controls used in each case had an inter-plate CV of <30% so all plates were deemed acceptable for inclusion in the statistical analysis. Details of the commercially available Simoa total tau 2.0, NF-light, Aβ40 and Aβ42 assays are given in General Methods (section 2.2.2, page 167) and they were all used as per the manufacturer's instructions. All measurements were conducted on the same automated HD-1 analyser (Quanterix).

4.2.4 Statistical analysis

Individual BB results were excluded from analysis if the CV across duplicates was >15%. In addition to examining the four primary biomarkers, a ratio of plasma Aβ42 to plasma Aβ40 (henceforth known as Aβ42/40 ratio) was generated. Unadjusted differences in BB levels by sex and *APOE* ε4 carrier status were examined using non-parametric Wilcoxon rank sum tests. These differences were assessed at two levels of analysis: firstly in individuals with all available data for each BB (see Table 4.1 for numbers – page 193), and then in cognitively normal individuals with full BB data (n = 453).

To determine associations with *APOE* ε4 carrier status and with SUVR, the natural logarithm of each of the four biomarkers was then modelled in the cognitively normal individuals with full BB data, using linear regressions as shown in Box 4.1.

Model 1: ln BB ~ age, BMI, creatinine, sex, *APOE* ε4 carrier status

Model 2: ln BB ~ age, BMI, creatinine, sex, *APOE* ε4 carrier status, (sex x *APOE* ε4 carrier status)

Model 3: ln BB ~ age, BMI, creatinine, sex, SUVR

Model 4: ln BB ~ age, BMI, creatinine, sex, SUVR, (sex x SUVR)

Model 5: ln BB ~ age, BMI, creatinine, sex, *APOE* ε4 carrier status, SUVR

Box 4.1: Linear regression models used for determining associations between natural log-transformed blood biomarkers and APOE ε4 carrier status, SUVR and sex interactions with both APOE ε4 carrier status and SUVR.
All models adjusted for age, BMI and serum creatinine.
BB, blood biomarker; BMI, body mass index; SUVR, standardised uptake volume ratio from amyloid PET scan

Normal quantile-quantile plots and plots of residuals against model fitted values were used to ensure that model assumptions were fulfilled. Model fits were tested before and after exclusion of potentially influential outliers defined by a Cook's distance of > 4/n. Robust standard errors were used to calculate the 95% confidence intervals of model coefficients.

4.3 **Results**

4.3.1 Participant characteristics

Table 4.1 shows that the median values of demographic variables and blood biomarkers were similar for all individuals with available data and for those who were cognitively normal with a full set of blood biomarkers.

Table 4.1: Insight 46 phase 1 participant characteristics and blood biomarker values.

Percentages or medians (with interquartile ranges) are shown for all individuals with available data and for the cognitively normal group with a full set of blood biomarkers.
Aβ40, Simoa amyloid-β 40, Aβ42, Simoa amyloid-β 42; IQR, interquartile range; NFL, Simoa neurofilament light chain; t-tau, Simoa total tau

	All individuals with available data for the specific variable		Cognitively normal group with full set of blood biomarkers (n=453)
Variable	n		
Median age (IQR), years	502	70.7 (70.1, 71.2)	70.7 (70.1, 71.2)
Sex, %male	502	51.0	51.2
APOE ε4 carrier, % with 1 or 2 alleles	500	29.6	29.8
Median serum creatinine (IQR), μmol/L	497	74 (64, 84.5)	74 (64, 84)
Median serum NFL (IQR), pg/ml	500	18.6 (14.4, 24)	18.8 (14.4, 24)
Median plasma t-tau (IQR), pg/ml	494	2.7 (2.1, 3.3)	2.7 (2.1, 3.3)
Median plasma Aβ40 (IQR), pg/ml	496	288 (257, 323)	288 (256, 322)
Median plasma Aβ42 (IQR), pg/ml	497	19.5 (16.6, 22.7)	19.6 (16.6, 22.7)
Median plasma Aβ42/40 ratio (IQR), pg/ml	496	0.066 (0.058, 0.077)	0.066 (0.056, 0.077)

4.3.2 Unadjusted differences in BB by sex

Simple group comparisons without adjusting for other covariates showed that plasma t-tau, Aβ40 and Aβ42 were significantly higher in females than males, both in all individuals with available data and in cognitively normal individuals with full BB data (Table 4.2). Serum NFL and plasma Aβ42/40 ratio did not show significant unadjusted differences by sex.

Table 4.2: Unadjusted differences in blood biomarkers by sex.
P values from Mann-Whitney U tests are shown; those in bold are significant at the p=0.05 level (unadjusted).
Aβ40, Simoa amyloid-β 40; Aβ42, Simoa amyloid-β 42; NFL, Simoa neurofilament light chain; t-tau, Simoa total tau

	All individuals with available data			Cognitively normal individuals with full blood biomarker data (n=453)		
	Median, pg/ml (IQR) n		*p*	Median, pg/ml (IQR)		*p*
	Female	Male		Female N=221	Male N=232	
NFL	19.3 (15, 24.8) 244	18.3 (14, 23.4) 256	0.148	19.4 (15.1, 24.8)	18.3 (14, 23.2)	0.115
t-tau	2.8 (2.2, 3.7) 241	2.5 (1.9, 3.2) 253	**<0.001**	2.8 (2.2, 3.5)	2.5 (2.0, 3.2)	**0.001**
Aβ40	297 (260, 328) 241	281 (255, 315) 255	**0.005**	294 (260, 327)	283 (255, 316)	**0.034**
Aβ42	20.4 (16.7, 23.7) 242	18.8 (16.5, 21.8) 255	**0.007**	20.1 (16.6, 23.7)	18.9 (16.6, 21.8)	**0.025**
Aβ42/40 ratio	0.066 (0.057, 0.079) 241	0.066 (0.058, 0.076) 255	0.698	0.066 (0.056, 0.079)	0.067 (0.058, 0.076)	0.540

4.3.3 Correlations between blood biomarkers

As shown in Table 4.3 and Table 4.4 (page 195), plasma Aβ42, Aβ40 and t-tau were significantly correlated with each other at both levels of analysis. Plasma Aβ42/40 ratio was not significantly correlated with either serum NFL or plasma tau at either level of analysis.

Table 4.3: Pairwise correlations between natural log-transformed blood biomarkers in Insight 46 phase 1, in all individuals who had available data.
The Pearson coefficient (r), the Bonferroni-adjusted significance value for the correlation and the number of observations included in each pair are shown in each cell. Results in bold are significant at the level of $p = 0.05$.

ln t-tau	0.107 0.173 494			
ln Aβ40	**0.147** **0.010** **496**	**0.165** **0.002** **493**		
ln Aβ42	0.0780 0.822 497	**0.167** **0.002** **494**	**0.284** **<0.0001** **496**	
ln Aβ42/40 ratio	-0.007 1 496	0.071 1 493	**-0.291** **<0.0001** **496**	**0.835** **<0.0001** **496**
r Sig (Bonferroni) n	**ln NFL**	**ln t-tau**	**ln Aβ40**	**ln Aβ42**

Table 4.4: Pairwise correlations between natural log-transformed blood biomarkers in Insight 46 phase 1, in cognitively normal individuals with full blood biomarker data (n=453).
The Pearson coefficient (r) and the Bonferroni-adjusted significance value for the correlation are shown in each cell. Results in bold are significant at the level of $p = 0.05$.

ln t-tau	**0.138** **0.033**			
ln Aβ40	0.092 0.517	**0.159** **0.007**		
ln Aβ42	0.068 1	**0.153** **0.011**	**0.271** **<0.0001**	
ln Aβ42/40 ratio	0.017 1	0.064 1	**0.284** **<0.0001**	**0.846** **<0.0001**
r Sig (Bonferroni)	**ln NFL**	**ln t-tau**	**ln Aβ40**	**ln Aβ42**

4.3.4 Adjusted associations with serum creatinine and BMI: cognitively normal group

As shown in Table 4.5, higher serum creatinine was associated with higher levels of all four primary BB after adjusting for age and sex, but not with plasma Aβ42/40 ratio. Higher BMI was associated with higher plasma t-tau but with lower serum NFL after adjusting for age and sex. There were no significant associations between BMI and any of the plasma amyloid BB.

Table 4.5: Significant physiological correlates of blood biomarkers in phase 1 of Insight 46. Associations are expressed as fold change in each biomarker (and 95% confidence interval) with unadjusted *p* values for the corresponding change in the variable of interest. Bold signifies $p < 0.05$. Associations were derived by back-transforming the coefficients from linear regressions of natural log-transformed blood biomarkers, adjusted for age and sex, in the cognitively normal group with full blood biomarker data (n=453).
Aβ40, Simoa amyloid-β 40; Aβ42, Simoa amyloid-β 42; BMI, body mass index; NFL, Simoa neurofilament light chain; t-tau, Simoa total tau

	Per 10% increase in serum creatinine		Per 1 unit increase in BMI	
	Fold change (95% CI)	*p*	Fold change (95% CI)	*p*
NFL	**1.046 (1.020, 1.073)**	**<0.001**	**0.981 (0.974, 0.989)**	**<0.001**
t-tau	**1.039 (1.023, 1.055)**	**<0.001**	**1.015 (1.007, 1.024)**	**<0.001**
Aβ40	**1.031 (1.021, 1.041)**	**<0.001**	1.001 (0.997, 1.005)	0.564
Aβ42	**1.026 (1.009, 1.044)**	**0.003**	1 (0.993, 1.007)	0.968
Aβ42/40 ratio	0.996 (0.978, 1.014)	0.653	0.999 (0.993, 1.006)	0.769

4.3.5 Adjusted models: associations between BB and *APOE* ε4 carrier status in the cognitively normal group

Linear regression models for log-transformed BB were applied as described in Box 4.1. (page 192).

Table 4.6 (page 198), Table 4.7 (page 199) and Table 4.8 (page 200) show the linear regression models for ln NFL, ln t-tau and ln Aβ40 respectively. In each case model 1 showed that after adjusting for age, BMI, creatinine and sex, there was no significant association between the biomarker and *APOE* ε4 carrier status, and model 2 showed that there was no significant interactive effect of sex and *APOE* ε4 carrier status on the biomarker.

Table 4.9 (page 201) and Table 4.10 (page 202) show the linear regression models for ln Aβ42 and ln Aβ42/40 ratio respectively. For both these biomarkers, model 1 showed that being an *APOE* ε4 carrier was significantly associated with a lower value of the biomarker. Model 2 showed that females who were *APOE* ε4 carriers did not have a significantly higher value of the biomarker than females who were *APOE* ε4 non-carriers (the coefficient for the female x *APOE* ε4 carrier interaction on ln Aβ42 was 0.121, $p = 0.060$ and on ln Aβ42/40 was 0.124, $p = 0.058$).

Table 4.6: Linear regression models for ln NFL in the cognitively normal group with full blood biomarker data.
Coefficients in bold are significant at the p=0.05 level. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients, shown in brackets.
Model 1: Age + BMI + Creatinine + Sex + APOE
Model 2: Age + BMI + Creatinine + Sex + (Sex x APOE)
Model 3: Age + BMI + Creatinine + Sex + SUVR
Model 4: Age + BMI + Creatinine + Sex + SUVR + (Sex x SUVR)
Model 5: Age + BMI + Creatinine + Sex + APOE + SUVR

Model			**Age**		**BMI**		**Creatinine**		**Male**		***APOE* ε4 carrier**		**SUVR**		**Female x APOE ε4 carrier**		**Male x SUVR**	
	n	R^2	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
1	453	0.124	0.030 (-0.019, 0.079)	0.236	**-0.020 (-0.027, -0.013)**	**<0.001**	**0.007 (0.004, 0.009)**	**<0.001**	**-0.137 (-0.216, -0.059)**	**0.001**	0.057 (-0.021, 0.135	0.150						
2	453	0.129	0.031 (-0.018, 0.080)	0.216	**-0.020 (-0.027, -0.013)**	**<0.001**	**0.007 (0.004, 0.009)**	**<0.001**	**-0.099 (-0.186, -0.012)**	**0.026**	-0.003 (-0.121, 0.114)	0.954			0.131 (-0.022, 0.285)	0.093		
3	417	0.140	0.027 (-0.024, 0.077)	0.305	**-0.023 (-0.030, -0.015)**	**<0.001**	**0.006 (0.004, 0.009)**	**<0.001**	**-0.146 (-0.229, -0.064)**	**0.001**			0.374 (-0.101, 0.849)	0.123				
4	417	0.148	0.024 (-0.028, 0.075)	0.367	**-0.022 (-0.030, -0.015)**	**<0.001**	**0.006 (0.004, 0.009)**	**<0.001**	0.393 (-0.126, 0.911)	0.137			**0.883 (0.231, 1.536)**	**0.008**			**-0.959 (-1.874, -0.043)**	**0.040**
5	417	0.143	0.028 (-0.023, 0.079)	0.285	**-0.023 (-0.031, -0.015)**	**<0.001**	**0.006 (0.004, 0.009)**	**<0.001**	**-0.148 (-0.230, -0.066)**	**<0.001**	0.051 (-0.035, 0.137)	0.244	0.273 (-0.226, 0.772)	0.283				

Table 4.7: Linear regression models for ln t-tau in the cognitively normal group with full blood biomarker data.
Coefficients in bold are significant at the p=0.05 level. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients, shown in brackets.
Model 1: Age + BMI + Creatinine + Sex + APOE
Model 2: Age + BMI + Creatinine + Sex + (Sex x APOE)
Model 3: Age + BMI + Creatinine + Sex + SUVR
Model 4: Age + BMI + Creatinine + Sex + SUVR + (Sex x SUVR)
Model 5: Age + BMI + Creatinine + Sex + APOE + SUVR

Model			**Age**		**BMI**		**Creatinine**		**Male**		***APOE* ε4 carrier**		**SUVR**		**Female x APOE ε4 carrier**		**Male x SUVR**	
	n	R^2	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
1	453	0.105	0.003 (-0.045, 0.051)	0.900	**0.014 (0.006, 0.022)**	**0.001**	**0.005 (0.002, 0.007)**	**<0.001**	**-0.219 (-0.298, -0.139)**	**<0.001**	-0.022 (-0.102, 0.057)	0.580						
2	453	0.105	0.003 (-0.044, 0.051)	0.886	**0.014 (0.006, 0.022)**	**0.001**	**0.005 (0.002, 0.007)**	**<0.001**	**-0.206 (-0.292, -0.120)**	**<0.001**	-0.042 (-0.150, 0.067)	0.451			0.042 (-0.119, 0.202)	0.611		
3	417	0.085	0.010 (-0.040, 0.060)	0.694	**0.012 (0.004, 0.020)**	**0.005**	**0.004 (0.002, 0.007)**	**0.001**	**-0.203 (-0.286, -0.121)**	**<0.001**			0.239 (-0.280, 0.758)	0.366				
4	417	0.085	0.010 (-0.040, 0.061)	0.688	**0.012 (0.004, 0.020)**	**0.005**	**0.004 (0.002 0.007)**	**0.001**	-0.254 (-0.833, 0.325)	0.389			0.191 (-0.484, 0.866)	0.578			0.090 (-0.943, 1.122)	0.864
5	417	0.087	0.009 (-0.041, 0.059)	0.720	**0.012 (0.004, 0.020)**	**0.005**	**0.004 (0.002, 0.007)**	**0.001**	**-0.202 (-0.285, -0.119)**	**<0.001**	-0.041 (-0.128, 0.046)	0.354	0.319 (-0.220, 0.859)	0.245				

Table 4.8: Linear regression models for ln Aβ40 in the cognitively normal group with full blood biomarker data.
Coefficients in bold are significant at the p=0.05 level. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients, shown in brackets.
Model 1: Age + BMI + Creatinine + Sex + APOE
Model 2: Age + BMI + Creatinine + Sex + (Sex x APOE)
Model 3: Age + BMI + Creatinine + Sex + SUVR
Model 4: Age + BMI + Creatinine + Sex + SUVR + (Sex x SUVR)
Model 5: Age + BMI + Creatinine + Sex + APOE + SUVR

Model			**Age**		**BMI**		**Creatinine**		**Male**		***APOE* ε4 carrier**		**SUVR**		**Female x APOE ε4 carrier**		**Male x SUVR**	
	n	R^2	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
1	453	0.134	0.013 (-0.011, 0.036)	0.287	0 (-0.003, 0.004)	0.876	**0.004 (0.003, 0.004)**	**<0.001**	**-0.087 (-0.119, -0.054)**	**<0.001**	-0.012 (-0.048, 0.023)	0.491						
2	453	0.134	0.013 (-0.011, 0.036)	0.288	0 (-0.003, 0.004)	0.880	**0.004 (0.003, 0.004)**	**<0.001**	**-0.088 (-0.124, -0.051)**	**<0.001**	-0.011 (-0.059, 0.037)	0.660			-0.003 (-0.074, 0.067)	0.923		
3	417	0.139	0.012 (-0.012, 0.036)	0.327	0.001 (-0.003, 0.004)	0.766	**0.004 (0.003, 0.005)**	**<0.001**	**-0.081 (-0.115, 0.046)**	**<0.001**			0.066 (-0.126, 0.258)	0.499				
4	417	0.140	0.012 (-0.012, 0.036)	0.344	0.001 (-0.003, 0.004)	0.764	**0.004 (0.003, 0.005)**	**<0.001**	-0.022 (-0.246, 0.203)	0.850			0.122 (-0.179, 0.422)	0.426			-0.105 (-0.496, 0.286)	0.598
5	417	0.140	0.012 (-0.012, 0.036)	0.337	0.001 (-0.003, 0.004)	0.748	**0.004 (0.003, 0.005)**	**<0.001**	**-0.080 (-0.115, -0.046)**	**<0.001**	-0.009 (-0.046, 0.028)	0.631	0.084 (-0.116, 0.283)	0.409				

Table 4.9: Linear regression models for ln Aβ42 in the cognitively normal group with full blood biomarker data.
Coefficients in bold are significant at the p=0.05 level. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients, shown in brackets.
Model 1: Age + BMI + Creatinine + Sex + APOE
Model 2: Age + BMI + Creatinine + Sex + (Sex x APOE)
Model 3: Age + BMI + Creatinine + Sex + SUVR
Model 4: Age + BMI + Creatinine + Sex + SUVR + (Sex x SUVR)
Model 5: Age + BMI + Creatinine + Sex + APOE + SUVR

Model			**Age**		**BMI**		**Creatinine**		**Male**		***APOE* ε4 carrier**		**SUVR**		**Female x APOE ε4 carrier**		**Male x SUVR**	
	n	R^2	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
1	453	0.081	**0.075 (0.040, 0.111)**	**<0.001**	-0.001 (-0.007, 0.006)	0.867	**0.003 (0.001 0.005)**	**<0.001**	**-0.099 (-0.159, -0.040)**	**0.001**	**-0.083 (-0.147, -0.020)**	**0.010**						
2	453	0.089	**0.077 (0.041, 0.113)**	**<0.001**	0 (-0.007, 0.006)	0.951	**0.003 (0.001, 0.005)**	**<0.001**	-0.064 (-0.120, 0.003)	0.060	**-0.139 (-0.217, -0.062)**	**<0.001**			0.121 (-0.005, 0.246)	0.060		
3	417	0.076	**0.079 (0.042, 0.117)**	**<0.001**	-0.002 (-0.009, 0.005)	0.526	**0.003 (0.001, 0.005)**	**<0.001**	**-0.086 (-0.147, -0.025)**	**0.006**			**-0.529 (-0.899, -0.160)**	**0.005**				
4	417	0.077	**0.079 (0.041, 0.117)**	**<0.001**	-0.002 (-0.009, 0.005)	0.528	**0.003 (0.001, 0.005)**	**<0.001**	-0.033 (-0.459, 0.396)	0.881			-0.479 (-1.083, 0.125)	0.120			-0.095 (-0.839, 0.649)	0.802
5	417	0.082	**0.078 (0.040, 0.116)**	**<0.001**	-0.002 (-0.009, 0.005)	0.592	**0.003 (0.001, 0.005)**	**<0.001**	**-0.084 (-0.145, -0.022)**	**0.007**	-0.055 (-0.125, 0.0149)	0.123	**-0.421 (-0.816, -0.027)**	**0.037**				

Table 4.10: Linear regression models for ln Aβ42/40 ratio in the cognitively normal group with full blood biomarker data.
Coefficients in bold are significant at the p=0.05 level. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients, shown in brackets.
Model 1: Age + BMI + Creatinine + Sex + APOE
Model 2: Age + BMI + Creatinine + Sex + (Sex x APOE)
Model 3: Age + BMI + Creatinine + Sex + SUVR
Model 4: Age + BMI + Creatinine + Sex + SUVR + (Sex x SUVR)
Model 5: Age + BMI + Creatinine + Sex + APOE + SUVR

Model			**Age**		**BMI**		**Creatinine**		**Male**		***APOE* ε4 carrier**		**SUVR**		**Female x APOE ε4 carrier**		**Male x SUVR**	
	n	R^2	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*	β	*p*
1	453	0.033	**0.063 (0.025, 0.101)**	**0.001**	-0.001 (-0.007, 0.006)	0.798	0 (-0.002, 0.001)	0.632	-0.013 (-0.075, 0.049)	0.683	**-0.071 (-0.135, -0.007)**	**0.030**						
2	453	0.040	**0.064 (0.026, 0.103)**	**0.001**	0 (-0.007, 0.006)	0.883	0 (-0.002, 0.001)	0.621	0.024 (-0.046, 0.094)	0.506	**-0.128 (-0.204, -0.053)**	**0.001**			0.124 (-0.004, 0.252)	0.058		
3	417	0.043	**0.067 (0.027, 0.107)**	**0.001**	-0.003 (0.010, 0.004)	0.425	-0.001 (-0.003, 0.001)	0.433	-0.005 (-0.069, 0.059)	0.872			**-0.595 (-0.983, -0.208)**	**0.003**				
4	417	0.043	**0.067 (0.027, 0.107)**	**0.001**	-0.003 (-0.010, 0.004)	0.426	-0.001 (-0.003, 0.001)	0.434	-0.011 (-0.457, 0.436)	0.962			-0.601 (-1.252, 0.050)	0.070			0.010 (-0.774, 0.794)	0.980
5	417	0.047	**0.066 (0.026, 0.107)**	**0.001**	-0.003 (0.010, 0.004)	0.472	-0.001 (-0.003, 0.001)	0.412	0.004 (-0.068, 0.061)	0.910	-0.046 (-0.117, 0.026)	0.209	**-0.505 (-0.919, 0.092)**	**0.017**				

4.3.6 Adjusted models: associations between blood biomarkers and cerebral amyloid in cognitively normal individuals with full blood biomarker data

Model 3 from Table 4.6 (page 198) shows that there was no significant association of ln NFL with amyloid PET SUVR; however, model 4 shows that there was a significant interactive effect of sex and SUVR, such that females had a higher increase ln NFL than males for a given increase in SUVR.

Model 3 from each of Table 4.7 (page 199) and Table 4.8 (page 200) showed that there was no significant association of SUVR with either ln t-tau or ln Aβ40 respectively, and model 4 showed that there was no significant interactive effect of sex and SUVR on either of these two blood biomarkers.

Model 3 from each of Table 4.9 and Table 4.10 (depicted graphically in Figure 4.1A and B, page 204) showed that higher SUVR was associated with lower ln Aβ42 and lower ln Aβ42/40 ratio respectively, but model 4 in both cases showed no significant interactive effect of sex and SUVR on these biomarkers.

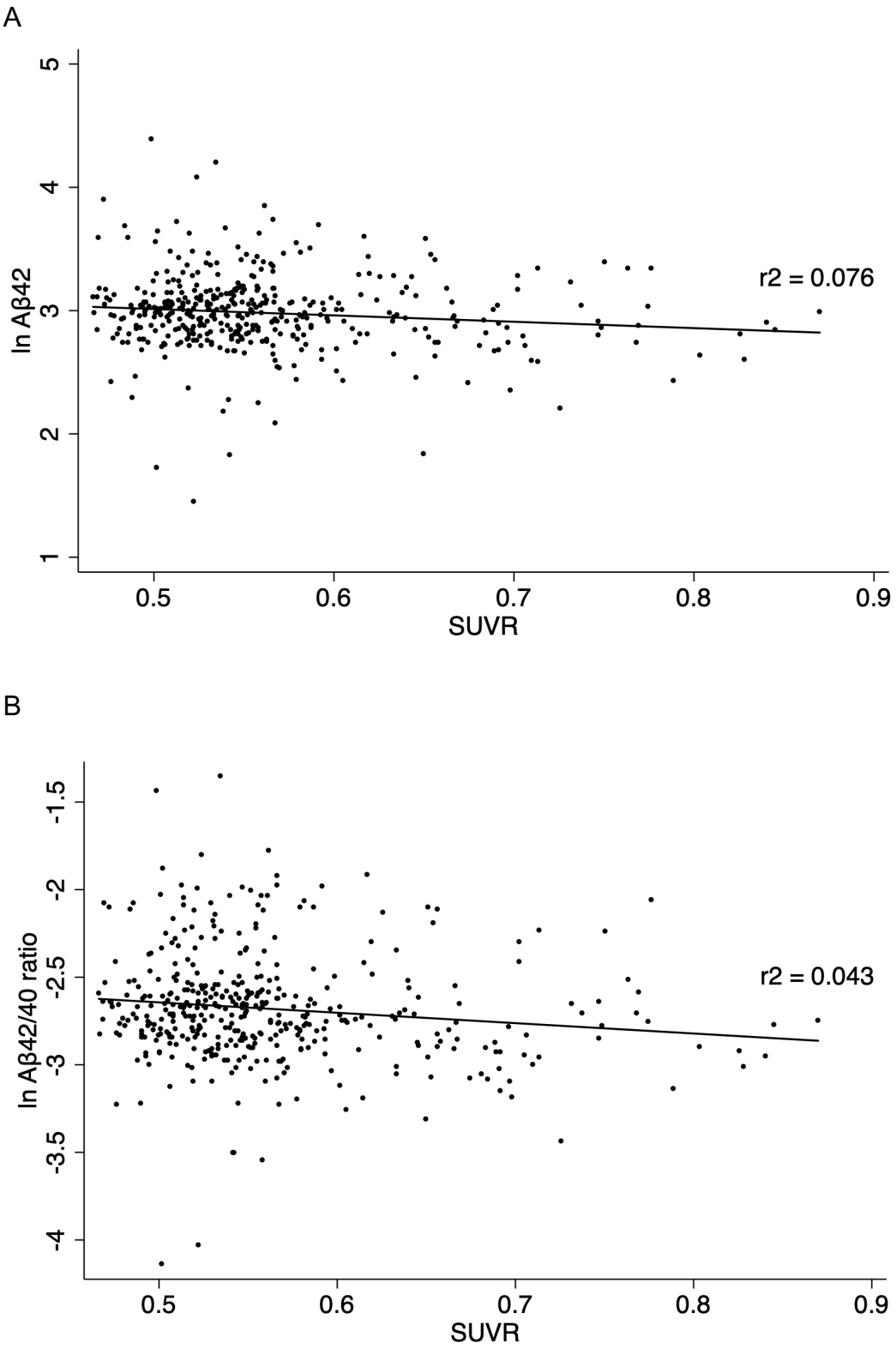


Figure 4.1: Associations between natural log-transformed plasma amyloid biomarkers and SUVR in cognitively normal individuals in Insight 46 phase 1 (n=417).
A: ln Aβ42 vs SUVR
B: ln Aβ42/40 ratio vs SUVR
Lines show linear model fits incorporating age, BMI, creatinine, sex and SUVR as predictors.

4.3.7 Adjusted associations with covariates

Higher serum creatinine was significantly associated with higher values of all four primary biomarkers across all the models detailed in Table 4.6 to Table 4.9 (pages 198 - 201). However, none of the models in Table 4.10 (page 202) showed a significant association of Aβ42/40 ratio with serum creatinine.

Higher BMI was significantly associated with lower NFL and higher t-tau across all the models detailed in Table 4.6 and Table 4.7 respectively. None of Aβ42, Aβ40 or Aβ42/40 ratio was associated with BMI (Table 4.8 to Table 4.10, pages 200 - 202).

Females had higher values than males of all four primary biomarkers, as shown in models 1, 3 and 5 of Table 4.6 to Table 4.9 (page 198 - 201). There was no significant association of Aβ42/40 ratio with sex, as shown by similar models in Table 4.10 (page 202).

Albeit within the very narrow age range of this cohort, both higher values of Aβ42 and Aβ42/40 ratio were associated with increased age, as shown consistently across all models detailed in Table 4.9 and Table 4.10 respectively (pages 201 - 202).

4.4 **Discussion**

4.4.1 Summary of results

This study showed the following cross-sectional associations in cognitively normal individuals in phase 1 of Insight 46:

- Lower values of plasma Aβ42 and plasma Aβ42/40 ratio were associated with carrying one or two *APOE* ε4 alleles, compared to not carrying *APOE* ε4;
- Lower values of plasma Aβ42 and plasma Aβ42/40 ratio were associated with higher cerebral amyloid deposition, independently of *APOE* ε4 carrier status;
- Higher values of all four measured primary blood biomarkers were associated with higher serum creatinine;
- Lower values of serum NFL and higher values of plasma t-tau were associated with higher BMI;
- Higher values of all four measured primary blood biomarkers were associated with female sex; and
- Higher values of plasma Aβ42 and Aβ42/40 ratio were associated with higher age over the very narrow age range of this cohort.

4.4.2 Blood biomarkers and *APOE* ε4 carrier status

Only a few previous studies have specifically reported on the associations between plasma amyloids and *APOE* ε4 carrier status in cognitively normal people. Using home-brewed sandwich ELISAs for detection of free plasma Aβ42 and Aβ40 in individuals who were either cognitively normal or had MCI, Lopez *et al*. reported no significant unadjusted differences between values of plasma Aβ42, Aβ40 or Aβ42/40 ratio according to *APOE* ε4 carrier status [304].

However, they did not present subgroup analyses for cognitively normal individuals, and were using assays which had lower limits of detection of 10 pg/ml for both plasma Aβ42 and Aβ40. In contrast, the Simoa assays used here for analyses in phase 1 of Insight 46 have lower limits of detection that render them 20 times more sensitive for Aβ40 and more than 200 times more sensitive for Aβ42. Using the same Simoa assays, Janelidze *et al.* found lower plasma Aβ42 but no difference in plasma Aβ40 or Aβ42/40 ratio in *APOE* ε4 carriers compared to non-carriers, in 274 cognitively normal people and 171 people with subjective cognitive decline recruited into the Swedish BIOFINDER study. However, in the 214 people with MCI and 57 people with AD also examined in this study, no association was found between any of these plasma amyloid markers and *APOE* ε4 carrier status [202]. Our study replicates the finding regarding plasma Aβ42 in cognitively normal people but also found reduced plasma Aβ42/40 ratio in cognitively normal *APOE* ε4 carriers compared to non-carriers. The likely reason for this is the significant association between plasma Aβ42 and plasma Aβ42/40 ratio, and larger number of cognitively normal individuals, in our cohort. Notably the extent of this association was not reported by Janelidze *et al.* but the percentage of *APOE* ε4 carriers in the cognitively normal group in BIOFINDER was very similar to that our cohort – 30% vs 29.8% respectively (Dr Shorena Janelidze, personal communication).

Our finding regarding the association of lower plasma Aβ42 and plasma Aβ42/40 ratio with *APOE* ε4 carrier status has biological plausibility, given that carriage of *APOE* ε4 is associated with increased cerebral amyloid deposition (as evidenced by both increases in amyloid PET signal and by reductions in

CSF Aβ42 [297]), which in turn is associated with reduced plasma Aβ42 and plasma Aβ42/40 ratio (see section 4.4.3 below).

4.4.3 Blood biomarkers and amyloid PET as a continuous measure of cerebral amyloid deposition

Several recent studies have reported correlations between plasma amyloid biomarkers and continuous measures of cerebral amyloid in cognitively normal individuals.

Park *et al.* demonstrated an association of lower plasma Aβ42 (measured by INNNO-BIA bead-based immunoassay technology) with higher PiB PET SUVR in a mixed cohort of cognitively normal/MCI/AD individuals (with 68% cognitively normal) [209]. Nakamura *et al*. [197] used a high-performance mass spectrometry based assay to perform relative quantifications of various amyloid-β peptides in two large cohorts – the Japanese National Center for Geriatrics and Gerontology (NCGG) and Australian Imaging, Biomarker and Lifestyle study of Ageing (AIBL). They showed associations of lower plasma Aβ42, higher Aβ40/42 ratio and higher APP699-711/ Aβ42 with higher PiB PET SUVR in NCGG and with higher SUVR for PiB, flutemetamol and florbetapir in different subgroups in AIBL.

In contrast, Janelidze *et al.* did not show significant associations between Simoa measures of plasma amyloids and ^{18}F-flutemetamol SUVR in cognitively normal individuals but did show significant correlations (surviving age and gender adjustment) between higher values of plasma Aβ42 and plasma Aβ42/40 ratio and higher values of their respective CSF counterparts measured

in the same individuals by a Euroimmun immunoassay [202]. This correlation between Simoa measures of plasma Aβ42, plasma Aβ42/40 ratio and CSF Aβ42 measured by INNOTEST® ELISA was replicated in individuals with subjective cognitive decline by Verberk *et al* [205].

The differences between the findings of these studies illustrate two potentially important points about plasma amyloids as a marker of cerebral amyloid deposition. Firstly, the extent of correlation may be altered by the method of assessment of cerebral amyloid deposition (either different amyloid PET tracers or CSF), and therefore comparison of the performance of different plasma amyloid assays should be undertaken only against the same "gold standard" measure of cerebral amyloid deposition. Secondly, as asymptomatic individuals who are discordant for CSF and PET amyloid measures are usually amyloid positive by CSF and negative by PET [305], CSF Aβ42 reduction may occur earlier than PET-amyloid positivity, so CSF Aβ42 or Aβ42/40 ratio may be a better "gold standard" to adopt when evaluating the utility of different plasma amyloid biomarkers, especially in cognitively normal people.

4.4.4 Blood biomarkers and renal function

Serum creatinine is a commonly used biochemical indicator of renal function. As plasma amyloids are known to be renally excreted, various authors have described significant associations between higher serum creatinine and both higher plasma Aβ42 and Aβ40 [306-308], or with Aβ40 but not Aβ42 [309], within individuals. Some authors have advocated the use of cystatin C, as a marker of glomerular function, as a covariate in analyses of plasma amyloids against AD risk [304, 310] but serum cystatin C may also be associated with AD

risk independently of glomerular filtration rate [311]. Overall, adjusting for renal function is not a common analytical approach in the recent AD blood biomarker literature, where despite extensive work on sensitive methods of detecting plasma amyloids, renal function has not been incorporated into models assessing their relationship to cerebral amyloid deposition. Notably, our study confirms the findings of others [308] that the Aβ42/40 ratio is not associated with serum creatinine, so it is a renal function-independent biomarker, at least over the range of renal function encompassed by our study. This is advantageous as it implies that further use of this biomarker (for example as a pre-screening tool for cerebral amyloid deposition – see Chapter 6) would not require adjustment for renal function.

To our knowledge, correlations between blood NFL and renal function have not previously been described. Most publications using Simoa assays for tau (for example, in the ADNI and BIOFINDER cohorts [220] and the Mayo Clinic Study of Ageing [217, 221]) have not examined renal function as potential covariate. However Pase *et al*. have described an association between higher plasma tau (assessed by the same Simoa assay as in our analyses above) and higher serum creatinine in two large prospective cohort studies [218], which our study replicates.

4.4.5 Blood biomarkers and BMI

Our confirmation of an association of higher plasma tau with higher BMI replicates the findings of Pase *et al*.; this may be related to an association of plasma tau with increased cardiovascular risk, as they also demonstrated associations of higher plasma tau with higher prevalence of atrial fibrillation,

cardiovascular disease, diabetes mellitus and treatment for hypertension, higher total cholesterol and lower HDL [218].

However, the association of higher serum NFL with lower BMI in this cognitively normal cohort is intriguing, and illustrates a clear difference in the biology of blood measures of NFL and tau, which to our knowledge has not been previously described. Speculative reasons for this may include differences in distribution volumes and the degree of central nervous system specificity of origin of NFL and tau moieties measured by these assays; while NFL is expressed only in neural tissue [17, 152], tau is expressed in a wide range of tissues, including at low to medium levels in adipose tissue and the gastrointestinal tract [17, 27]. Neurodegeneration leading to raised serum NFL might independently influence feeding behaviour or energy metabolism to result in relative weight loss, while there might be a more complex interplay between central and peripheral-origin tau and BMI.

4.4.6 Inter-blood biomarker correlations

Using a Simoa triplex assay, Verberk *et al.* reported significant correlations between plasma t-tau, Aβ40 and Aβ42 but not between plasma t-tau and Aβ42/40 ratio in people with subjective cognitive impairment [205]. Our study replicates and extends these findings both across all individuals with available data, and the cognitively normal group, in Insight 46.

4.4.7 Sex differences

There are few reports of sex differences in these blood biomarkers in the literature, most of which pertain to blood measurement of tau. No significant

plasma tau difference was demonstrable between the sexes in ADNI and BIOFINDER [220] but the analysis pooled results from controls, MCI and AD. Our result conflicts with a Taiwanese study of cognitively normal individuals by Chiu *et al*. showing males to have higher plasma tau than females [223]. However, there are many points of difference between the latter and our study, including the type of assay used (immunomagnetic reduction technique versus Simoa), the population ethnicity (Taiwanese versus Caucasian), the age range (45-95 years versus 69-71 years) and the fact that Chiu *et al.* made no assessment of cerebral amyloid, so did not adjust for it when examining sex differences. However, our plasma tau result replicates very well those of Pase *et al.,* who showed higher plasma tau in women than men, at every age group from the 20's to the 80's, in a large dataset from the Framingham Heart Study [218].

Sex differences in cerebral tau pathology have been described in CSF, where there is a higher CSF total tau in females than males and there is a superposed interactive effect of female sex and *APOE* ε4 carrier status to yield higher CSF total tau [144, 312]. More recently, Buckley *et al.* have reported increased entorhinal cortical tau PET signal in cognitively normal females compared to males in ADNI, and an interactive effect of female sex and higher amyloid PET signal to increase entorhinal cortical tau PET signal in both the HABS and ADNI cohorts [145], implying that these sex differences may be relevant to AD pathology.

However, one should be wary of assuming that the mechanisms (as yet unknown) that contribute to sex differences in cerebral tau pathology are the

same as those that underlie sex differences in plasma tau. Our results showed that females had higher values of all four of the primary biomarkers than males, which hints that there may be a shared biological mechanism for this difference. The sex differences for all four primary biomarkers survived adjustment for creatinine and BMI, indicating that they are not explained purely by differences in body mass or renal clearance between the sexes. Other possibilities include distribution effect, as females generally have an increased adiposity and therefore a reduced body water percentage compared to males, or hormonally driven differences in rates of production and clearance. We did not specifically assess the influence of adiposity as there was no direct measure of this in Insight 46, and while all females in Insight 46 can reasonably be assumed to be post-menopausal, these analyses did not probe effects of hormone replacement therapy (which some female participants may have been taking) on these blood biomarkers.

4.4.8 Blood biomarkers and age

Despite the narrow age range of our cohort, higher plasma Aβ42 and Aβ42/40 ratio were associated with increased age. It is unlikely that this was an artefact of pre-analytical variation due to time in storage, as all plasma samples were maintained at -80°C until tested. A more likely explanation for this finding is a variation in health profiles of those recruited to the study with time. We have shown that compared to the overall NSHD birth cohort, participants in Insight 46 had higher educational attainment, were more likely to have had a non-manual occupation, and at age 68-69 had higher verbal memory scores, higher self-rating of health, were less likely to be smokers or have a mental health disorder but were more likely to drink four or more units of alcohol per week [274]. It is

unknown whether variations in any of these factors would have occurred during the course of the attendance period and may have interacted with seasonal variations (that is, for example, whether those with better health profiles would have been more willing to attend a study visit in the winter, toward the end of phase 1).

4.4.9 Conclusions and impact on further work

The work described in this chapter has demonstrated that participant-related pre-analytical factors such as age, sex, BMI, and serum creatinine, in addition to factors known to be associated with AD risk (*APOE* ε4 carrier status and amyloid PET SUVR), may explain between 4 and 14% of the variance in measurements of the blood biomarkers serum NFL, and plasma t-tau, Aβ40 and Aβ42. This work therefore informed the choice of covariates for analyses of the cross-sectional relationships of these biomarkers with structural brain imaging variables (Chapter 6) and cognitive variables (Chapter 7). The lack of impact of BMI and serum creatinine on plasma Aβ42/40 ratio indicated that adjustment for these factors would not be required in analyses of the utility of this biomarker in screening for PET amyloid status (Chapter 5).

5 Cross-sectional associations between blood biomarkers and amyloid PET in Insight 46

5.1 Introduction

Longitudinal case-cohort studies have shown that reduced plasma Aβ42/40 ratio measured above age 60 predicts increased incident risk of all-cause dementia and of clinically defined AD [308, 313-315], with a meta-analysis by Chouraki *et al.* indicating hazard ratios of 1.48 and 1.53 respectively [308]. In relation to biomarker-confirmed AD, the potential use of plasma amyloid-β peptides as a screening tool for cerebral amyloid deposition has been tested in mixed cohorts of individuals with AD, MCI or cognitively normal status [197, 316] or in those with subjective cognitive decline [205, 210, 317]. However, in order to truly test the ability of plasma amyloids to function in population screening, it is important to examine their concordance with validated AD biomarkers in large numbers of cognitively normal individuals.

The main questions addressed in this chapter are therefore as follows:

- In cognitively normal individuals from Insight 46, what is the relative performance of blood biomarkers of amyloid-β measured by Simoa and by liquid chromatography-mass spectrometry (LC-MS) in detecting cerebral amyloid deposition as measured by binary amyloid-PET status? Does either measure provide a significant improvement compared to a model incorporating age, sex and *APOE* ε-4 carrier status?
- Does adding either or both of plasma t-tau or serum NFL improve the ability to detect cerebral amyloid positivity?
- How might a pre-screening blood test be used to reduce numbers of amyloid PET scans required in recruitment to a clinical trial? How is this

affected by the prevalence of PET-amyloid-positivity in the population to which the pre-screening test is applied?

- Do most individuals who are discordant for their blood and amyloid PET status show a “blood positive, PET negative state”?

5.2 **Methods**

5.2.1 Assays

5.2.1.1 Simoa Aβ40 and Aβ42 assays

These were performed as previously described in section 4.2.3, page 191.

5.2.1.2 LC-MS assay

One 500 µL aliquot of plasma from each individual in Insight 46 was pre-processed as described in section 2.1.2.2.3 (page 156). Plasma samples were stored at -80 ºC till shipping on solid carbon dioxide to Dr Josef Pannee at the University of Gothenburg, who processed them after a single thaw. A summary of the sample processing and assay steps is given below.

Calibrators were prepared using recombinant Aβ1-38, Aβ1-40 and Aβ1-42 (rPeptide) added to 8% bovine serum albumin in phosphate-buffered saline. Recombinant “heavy” peptides (^{15}N uniformly labelled Aβ1-38, Aβ1-40 and Aβ1-42 peptides; rPeptide), were added to samples and calibrators prior to sample preparation and used as internal standards. Pooled plasma samples from the University of Gothenburg were used to track assay performance over different days, and showed an inter-assay CV of <5%.

Aβ peptides were extracted from 250 µL of each sample using immunoprecipitation with anti-β-Amyloid 17-24 (4G8) and anti-β-Amyloid 1-16

antibodies (6E10, both Biolegend®) coupled to Dynabeads™ M-280 Sheep Anti-Mouse IgG magnetic beads (Thermo Fisher Scientific). Immunoprecipitation was performed using a KingFisher™ Flex Purification System (Thermo Fisher Scientific). Although some batch changes of certain reagents occurred during the study (two batches of 6E10 and two batches of magnetic beads were used), the use of heavy peptides as internal standards mitigated against potential changes of affinity. After immunoprecipitation, eluates in 100 µL each of 0.5% formic acid were vacuum centrifuged, and the dried eluates were stored at -80 ºC, pending analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Prior to analysis, the dried eluates were re-suspended in 20% acetonitrile and 4% concentrated ammonia in water, and injected into the LC-MS system (a Dionex Ultimate LC-system and a Thermo Scientific Q Exactive quadrupole-Orbitrap hybrid mass spectrometer). Chromatographic separation was achieved using basic mobile phases and a reversed-phase monolith column at a flow rate of 0.3 mL/min. The mass spectrometer, operating in parallel reaction monitoring (PRM) mode, was set to isolate the 4+ charge state precursors of the Aβ peptides. Product ions (14-15 depending on the peptide) specific for each precursor were selected and summed to calculate the chromatographic areas for each peptide and its corresponding internal standard. The area ratio of the analyte to the internal standard was used for quantification in samples and calibrators.

5.2.2 Inclusion and exclusion criteria

As shown in Figure 5.1 (page 218), analyses of inter-assay correlations were undertaken in all individuals in whom complete plasma amyloid data were

available from both assays. For analyses of the contribution of plasma amyloids to prediction of binary PET-amyloid status, individuals who had a full set of data across all assays, had PET data, had no prior neurological diagnoses and were cognitively normal were included (n = 414).

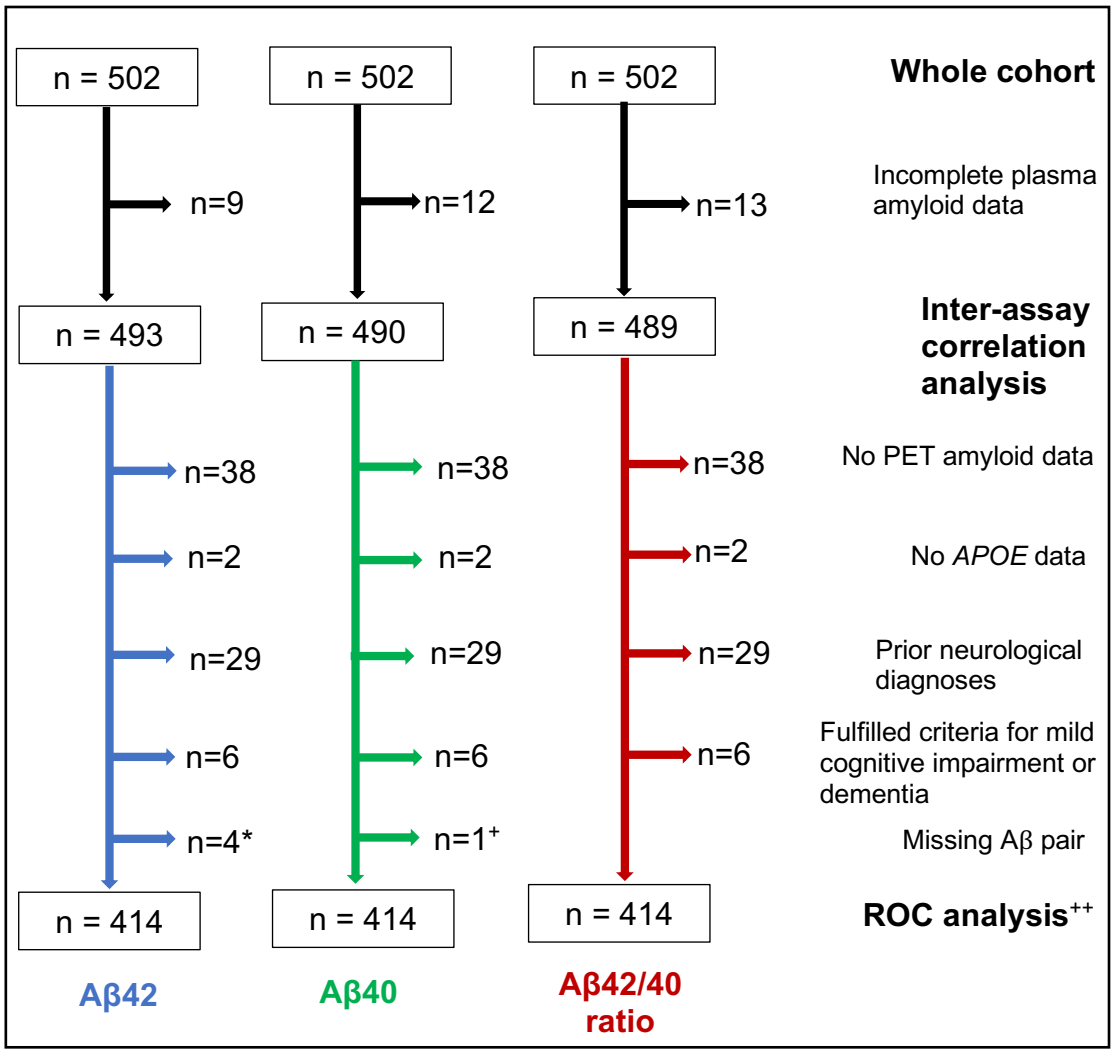


Figure 5.1: Inclusion and exclusion criteria for the two levels of analysis undertaken for plasma amyloids.
*removed as no Aβ1-40 pair available
+ removed as no Aβ1-42 pair available
++ secondary analyses also undertaken in:

1. cognitively normal individuals who had full data for plasma amyloids across both assays, plasma tau and serum NFL, n = 413
2. all individuals with full data for plasma amyloids across both assays, n = 449

5.2.3 Statistical analysis

5.2.3.1 Inter-assay correlations

Pearson correlations of natural log-transformed biomarker values were used to assess the correlations between the Simoa and MS assay measurements of Aβ42, Aβ40 and Aβ42/40 ratio. Bland-Altman analysis was used to demonstrate the systematic differences in measurement by the two assays. For these analyses, all individuals who had biomarker values measured by both assays were included.

5.2.3.2 Logistic regression and ROC analysis in cognitively normal individuals

Using binary amyloid-PET status derived from the SUVR cut-off of 0.6104 (as previously described in section 2.1.2.2.5, page 158) logistic regression models were constructed as shown in Box 5.1 (page 220). Analyses were undertaken in cognitively normal individuals with PET-amyloid data. Receiver operating characteristics (ROC) curves were constructed and the area under the curve (AUC) for the models were compared. Models were deemed to provide significantly more information than model 1 if the AUC 95% confidence interval was higher than that of model 1 and did not overlap. The covariates used in model 1 are the current standard covariates employed in clinical trial stratification. Given that the blood-to-scan delay varied across the cohort due to some individuals having to return for a scan at a later date (mostly due to failure of tracer production on the study visit day), model 1 was probed first to determine if blood-to-scan delay influenced model coefficients or fit. As blood-to-scan delay made no difference to model 1, it was not included further in these analyses.

Model 1: Binary amyloid-PET status ~ Age + sex + *APOE ε-4* carrier status

Model 2: Binary amyloid-PET status ~ (Covariates of model 1) + plasma Aβ42

Model 3: Binary amyloid-PET status ~ (Covariates of model 1) + plasma Aβ40

Model 4: Binary amyloid-PET status ~ (Covariates of model 1) + plasma Aβ42/40

Model 5: Binary amyloid-PET status ~ (Covariates of model 1) + plasma Aβ42/40 + plasma t-tau

Model 6: Binary amyloid-PET status ~ (Covariates of model 1) + plasma Aβ42/40 + plasma t-tau + serum NFL

Box 5.1: Logistic regression models for binary amyloid-PET status in the Cognitively Normal group.
Models 2, 3 and 4 were derived using either Simoa or LC-MS assay values for plasma amyloid-β peptides. Unadjusted versions (models 2u, 3u, 4u), which did not incorporate the covariates of model 1, were also derived.

5.2.4 Screening test potential

The best model derived from each assay method was selected as giving the highest AUC. Cut-off values of the model fit were chosen based firstly Youden's index for each model (the point at which the highest accuracy is achieved – where the sum of sensitivity and specificity is maximised) and then by selecting the point with a fixed sensitivity of 85%. In each case, the resulting values of sensitivity, specificity and accuracy were used to calculate the total number needed to screen (NNS) and number proceeding to scan (NPS) to identify 100 PET-amyloid-positive individuals. This was done by solving for the contents of the 2 x 2 table generated in each case, using the equations shown in Box 5.2. The relative cost savings afforded by the application of the better of the two pre-screening blood tests, at different levels of prevalence of PET-amyloid-positivity, were calculated using the equations shown in Box 5.3 (page 221).

	Scan Positive	Scan Negative
Blood Positive	100	B
Blood Negative	C	D

$$\text{Sensitivity} = \frac{100}{100 + C} \qquad \text{Specificity} = \frac{D}{B + D} \qquad \text{Accuracy} = \frac{100 + D}{100 + B + C + D}$$

$$\text{NNS} = 100 + \text{B} + \text{C} + \text{D} \qquad \text{NPS} = 100 + \text{B}$$

Box 5.2: Calculations for number needed to screen (NNS) and number proceeding to scan (NPS) using plasma amyloid models, to obtain 100 PET-amyloid-positive individuals.

$$\text{x} = Cost\ of\ individual\ PET\ scan \qquad \text{y} = Cost\ of\ individual\ blood\ test$$

$$\text{n} = \text{number of scans required without applying pre-screening blood test, to obtain 100 PET-amyloid positive individuals}$$

$$\text{NNS*y} + \text{NPS*x} = \text{Relative total cost *n*x}$$

$$\text{Relative total cost} = \frac{\left(NPS + \frac{NNS * y}{x}\right)}{n}$$

Box 5.3: Calculations for relative cost of the screening programme.
These calculations are based on specified costs of an individual PET scan and blood test, number needed to pre-screen (NNS) with blood test and number proceeding to scan (NPS), to obtain 100 PET-amyloid-positive individuals.

5.2.5 Discordance between blood test and amyloid PET

To examine whether the choice of amyloid PET ('gold standard") cut-point significantly affected the performance of the best-performing unadjusted model, the Youden's cut point for this model at a range of definitions of amyloid status utilising SUVR cut-points between 0.57 and 0.65 was examined and the corresponding sensitivity, specificity, and numbers of discordant individuals were tabulated.

5.3 **Results**

5.3.1 Summary statistics

The median and interquartile ranges of the values yielded by each assay are shown in Table 5.1.

Table 5.1: Summary statistics for Simoa and LC-MS assay values for plasma amyloid-β peptides, across all individuals.

	Simoa				**LC-MS**		
	n	Median	IQR		n	Median	IQR
				Aβ1-38 (pg/ml)	493	24.8	21.6, 28.0
Aβ40 (pg/ml)	496	287.5	257, 323	Aβ1-40 (pg/ml)	491	284	256, 314
Aβ42 (pg/ml)	497	19.5	16.6, 22.7	Aβ1-42 (pg/ml)	493	28.4	23.5, 33.4
Aβ42/40 ratio	496	0.066	0.058, 0.077	Aβ1-42/ 1-40 ratio	490	0.099	0.087, 0.113

The demographics of the cognitively normal group used for analyses of binary amyloid status are shown in Table 5.2.

Table 5.2: Demographics and summary statistics for Simoa and LC-MS assay values for cognitively normal individuals included in analyses of binary amyloid status (n = 414). Unadjusted two-tailed probabilities of equality between groups are shown.
[a] Student's t-test [b] χ^2 tests of proportions [c] Wilcoxon rank-sum test

	Total (n = 414)	PET-amyloid-negative (n = 337)	PET-amyloid-positive (n = 77)	*p*
Age, years (SD)	70.6 (0.7)	70.7 (0.7)	70.6 (0.6)	0.643[a]
Sex, female (%)	202 (48.8)	167 (49.6)	35 (45.5)	0.516[b]
APOE ε-4 carrier (1 or 2 alleles), n (%)	123 (29.7)	78 (23.2)	45 (58.4)	<0.0001[b]
Simoa Aβ40, pg/ml Median, IQR	288 (256, 319)	288 (255, 318)	287 (259, 336)	0.331[c]
Simoa Aβ42, pg/ml Median, IQR	19.6 (16.6, 22.7)	19.8 (17.1, 22.6)	18.3 (15.5, 23.5)	0.064[c]
Simoa Aβ42/40 ratio Median, IQR	0.066 (0.057, 0.077)	0.068 (0.059, 0.078)	0.062 (0.052, 0.074)	0.003[c]
LC-MS Aβ1-38, pg/ml Median, IQR	24.7 (22.0, 27.7)	24.7 (22.0, 27.6)	24.4 (21.5, 28.7)	0.982[c]
LC-MS Aβ1-40, pg/ml Median, IQR	284 (256, 313)	283 (255, 314)	286 (260, 313)	0.823[c]
LC-MS Aβ1-42, pg/ml Median, IQR	28.4 (23.5, 33.3)	29.4 (25.0, 34.2)	23.8 (20.4, 27.2)	<0.0001[c]
LC-MS Aβ1-42/1-40 ratio Median, IQR	0.099 (0.087, 0.113)	0.103 (0.093, 0.116)	0.083 (0.075, 0.090)	<0.0001[c]

In unadjusted analyses, the PET-amyloid positive group had a significantly higher percentage of APOE ε-4 carriers (58.4 vs 23.2%) and significantly lower values of Simoa Aβ42/40 ratio, LC-MS Aβ1-42 and LC-MS Aβ1-42/1-40 ratio than the PET-amyloid negative group.

5.3.2 Inter-assay correlations

Inter-assay correlations were examined in each case for the total number of pairs of results available for the Simoa and LC-MS assays for each Aβ peptide. Pearson correlations of corresponding log-transformed values from the Simoa and LC-MS assays are shown in Figure 5.2 (page 224). There was a better correlation between the Simoa Aβ40 and the LC-MS Aβ1-40 assay values (0.444, $p < 0.0001$) than between the Simoa Aβ42 and the LC-MS Aβ1-42 assay values ($r = 0.224$, $p < 0.0001$) or the Simoa Aβ4240 ratio and the LC-MS Aβ1-42/1-40 ratio values ($r = 0.175$, $p = 0.0001$). The Bland-Altman plots (Figure 5.3, page 225) show that within the ranges of values tested, the magnitude of the difference between the assays in all three cases increased as the value of the average increased.

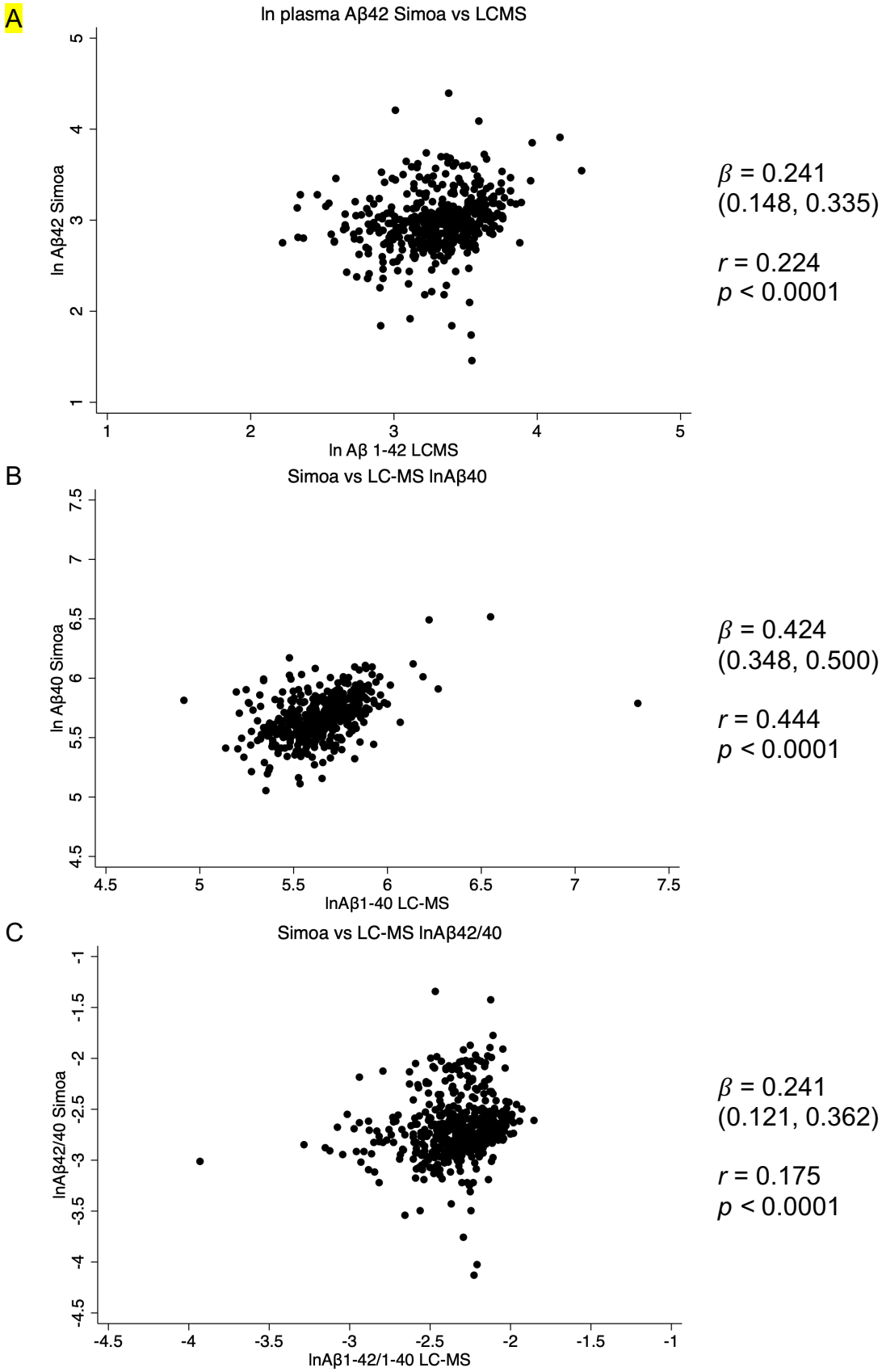


Figure 5.2: Scatterplots of log transformed LC-MS assay values (y axis) against log-transformed Simoa assay values (x axis).
The slope of the univariate linear regression β, with its 95%confidence interval in brackets, and the Pearson correlation coefficient *r*, with its p value, are indicated for each.
A: Aβ42, n=493 B: Aβ40, n=490 C: Aβ42/40 ratio, n=489

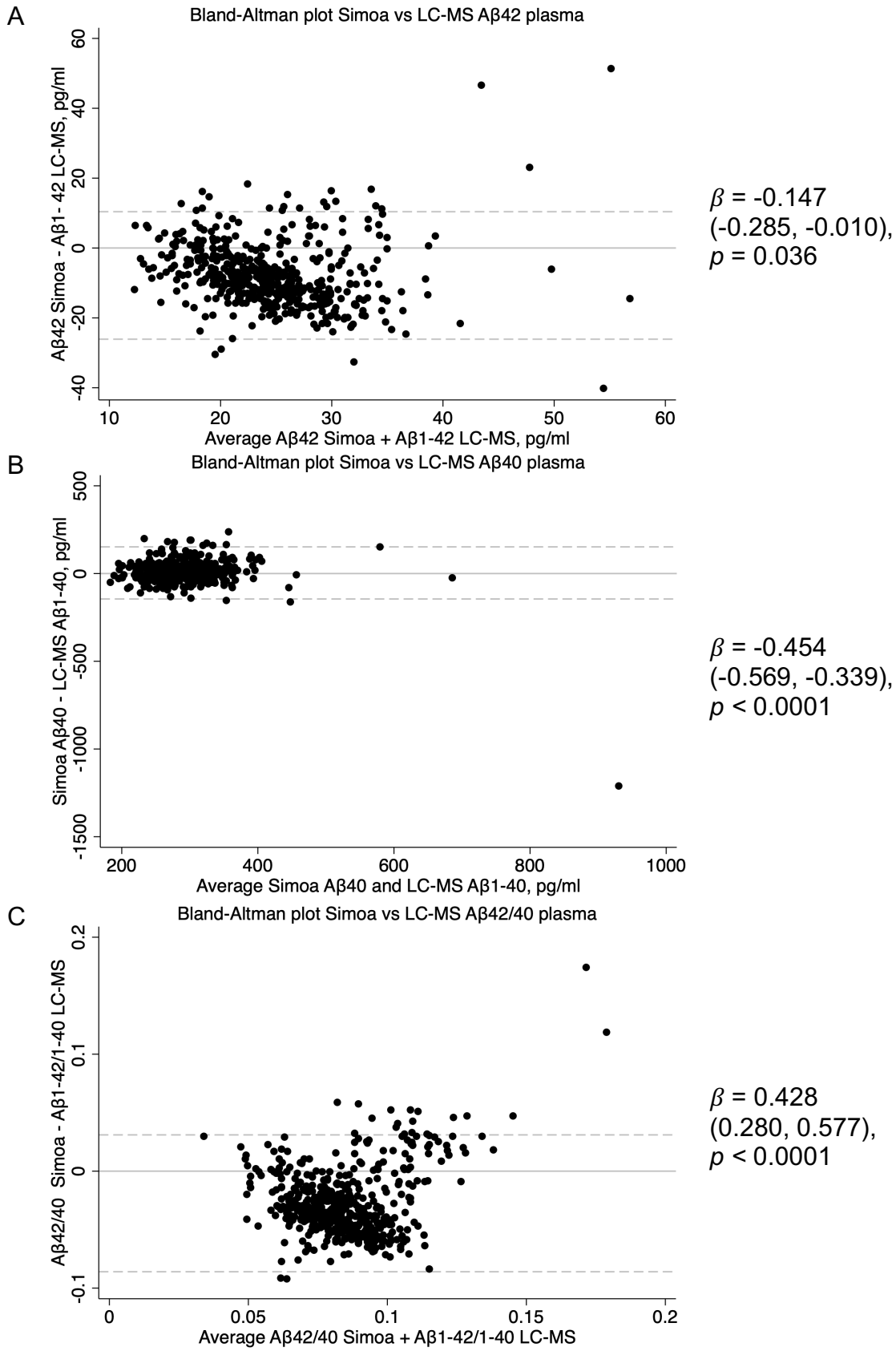


Figure 5.3: Bland-Altman plots of the difference between Simoa assay values and LC-MS assay values (y axis) against average of the two assay values (x axis).
Solid grey lines indicate zero difference; dashed grey lines indicate the 95% CI of the difference. The slope of the univariate linear regression of the difference against the average, β, is shown with its 95%confidence interval in brackets, and *p* value.
A: Aβ42, n = 493 B: Aβ40, n = 490 C: Aβ42/40 ratio, n = 489

5.3.3 Associations with binary amyloid status in the cognitively normal group

77 of 414 cognitively normal individuals were PET-amyloid-positive (*i.e.* 18.6%). The AUC from ROC analyses for unadjusted and adjusted models of PET-amyloid status are shown in Table 5.3. ROC curves from adjusted models 1-4 are shown in Figure 5.4 (A: Simoa, B: LC-MS; page 228). Both unadjusted Simoa Aβ42/40 ratio and LC-MS Aβ1-42/1-40 ratio performed better than chance, but the latter performed significantly better, and was also able to outperform the base model incorporating age, sex and APOE ε4 carrier status (model 1).

The model with the highest AUC incorporated the LC-MS plasma Aβ1-42/1-40 ratio (adjusted LC-MS model 4). None of the adjusted Simoa assay models had a significantly improved AUC compared to model 1.

Table 5.3: Areas under the curve (AUC) with their 95% confidence intervals for the receiver operating characteristics analyses for PET-amyloid status in cognitively normal individuals with complete plasma Aβ data, n = 414.
Model 1 (Base): Age + Sex + *APOE ε-4* carrier status
Model 2: Base + plasma Aβ42
Model 3: Base + plasma Aβ40
Model 4: Base + plasma Aβ42/40 ratio
u: Unadjusted models

* χ^2 p = 0.224 compared to model 1 $^{+}$ χ^2 p <0.0001 compared to model 1

	Simoa		LC-MS	
Unadjusted	AUC	95% CI	AUC	95% CI
Model 2u (Aβ42)	0.578	0.491, 0.645	0.735	0.681, 0.789
Model 3u (Aβ40)	0.536	0.463, 0.608	0.492	0.419, 0.564
Model 4u (ratio)	0.608	0.533, 0.683	0.819	0.771, 0.867
Adjusted	AUC	95% CI	AUC	95% CI
Model 1 (Base)	0.691	0.622, 0.761	0.691	0.621, 0.761
Model 2 (Aβ42)	0.692	0.622, 0.762	0.784	0.727, 0.841
Model 3 (Aβ40)	0.715	0.654, 0.776	0.691	0.621, 0.761
Model 4 (Aβ42/40 ratio)	0.722*	0.657, 0.787	0.840^{+}	0.793, 0.886

A secondary analysis in the 413 cognitively normal individuals with full plasma amyloid, plasma t-tau, serum NFL and PET data showed that addition of plasma t-tau only (model 5) or plasma t-tau and serum NFL (model 6) gave AUC 0.728 and AUC 0.729 respectively. This did not significantly improve the AUC compared to Simoa model 4 (AUC 0.723; χ^2 p = 0.780).

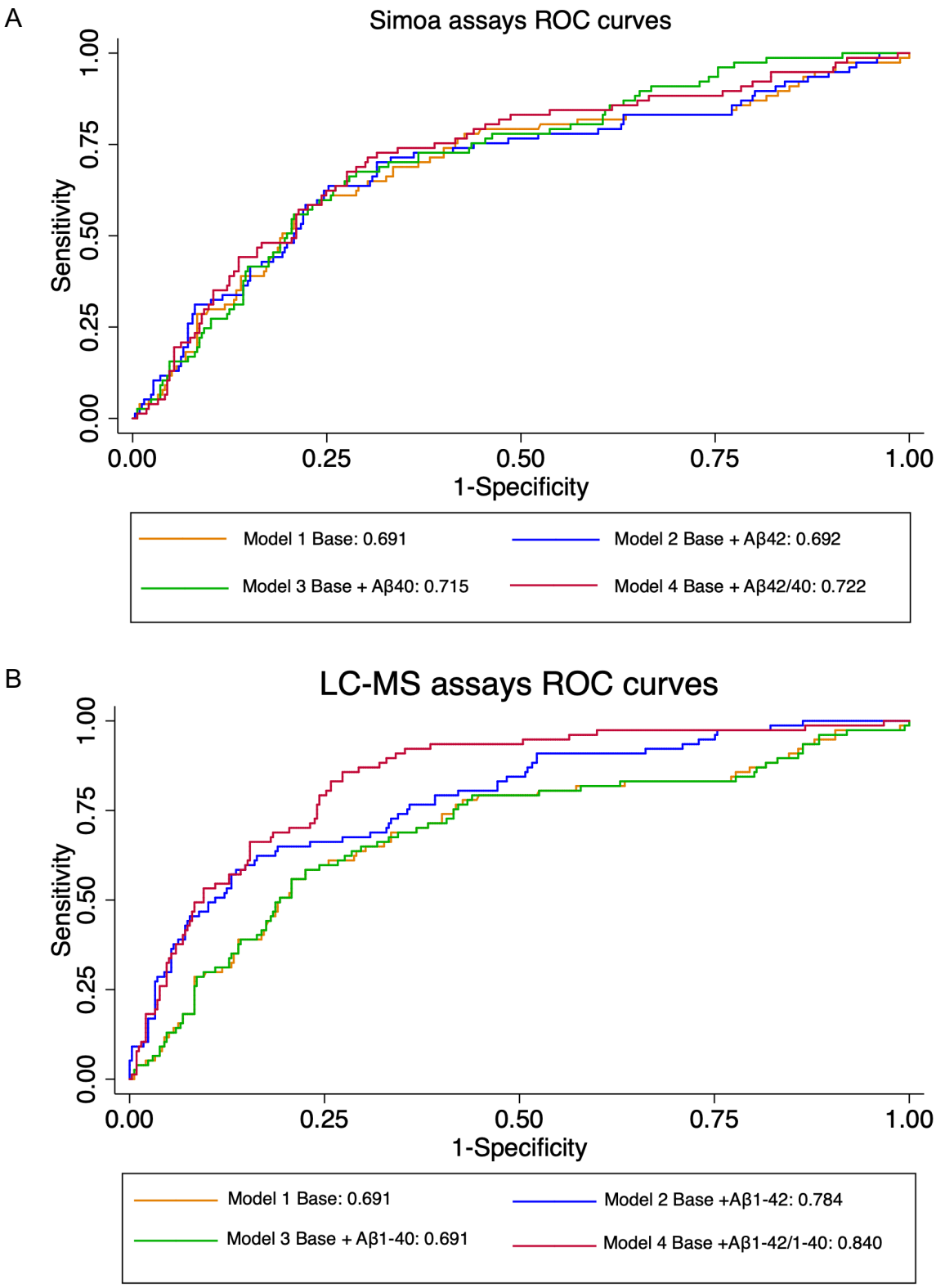


Figure 5.4: Receiver operating characteristics curves for cerebral PET-amyloid status in cognitively normal individuals with complete plasma Aβ data, n = 414.
A: Simoa B: LC-MS
Model 1 (Base): Age + Sex + *APOE ε4* carrier status
Model 2: Base + plasma Aβ42
Model 3: Base + plasma Aβ40
Model 4: Base + plasma Aβ42/40 ratio

5.3.4 Potential contributions of plasma amyloid biomarkers as a screening test prior to amyloid PET scan in cognitively normal individuals

The potential cut-points derived from model 4 in each case are shown in Table 5.4 with the associated number needed to screen (NNS) and number proceeding to scan (NPS) to obtain 100 PET-amyloid-positive individuals. Assuming the population PET-amyloid-positivity percentage to be the same as in Insight 46 (18.6%), 538 direct scans would be required to obtain 100 PET-amyloid-positive individuals. Figure 5.5 (page 230) and Figure 5.6 (page 231) demonstrate clearly that screening first with the LC-MS assay would give a better result, which would be similar whether or not adjustment for age, sex and *APOE* ε4 carrier status was undertaken.

Table 5.4: Number needed to screen (NNS) and number proceeding to scan (NPS) to yield 100 PET-amyloid positive individuals.
Youden's index cut-point from model 4 (either unadjusted, or adjusted for age, sex and *APOE* ε4 carrier status) using the Simoa plasma Aβ42/40 ratio or LC- MS plasma Aβ1-42/1-40 ratio was applied to derive the scan number reduction (SNR) afforded by each screening test, to a population where 18.6% are PET-amyloid positive.

Assay	Sensitivity %	Specificity %	Accuracy %	NNS	NPS	SNR	SNR %
Unadjusted							
Simoa	45.5	77.5	71.5	1183	317	221	41
LC-MS	87.0	71.8	74.6	617	242	296	55
Adjusted for age, sex and APOE ε*4* carrier status							
Simoa	72.7	68.6	69.3	746	292	246	45
LC-MS	85.7	72.7	75.1	627	239	299	56

5.3.5 ROC analyses across all individuals with available plasma amyloid-β and PET-amyloid data

A secondary analysis was undertaken in the 449 individuals who had full Simoa and LC-MS plasma amyloid-β and PET-amyloid data (Appendix Table 12.1, page 327) and AUC comparison confirmed that model 4 incorporating the LC-MS Aβ1-42/1-40 values performed better than model 4 incorporating the Simoa Aβ42/40 values (AUC 0.839 vs 0.736 respectively, χ^2 $p < 0.0001$).

Figure 5.5: Scan number reduction afforded by using the unadjusted Simoa or the LC-MS assay to screen prior to amyloid PET scan, assuming a population prevalence of 18.6%.
In each case the Youden's index cut-point was used for assigning blood amyloid status.

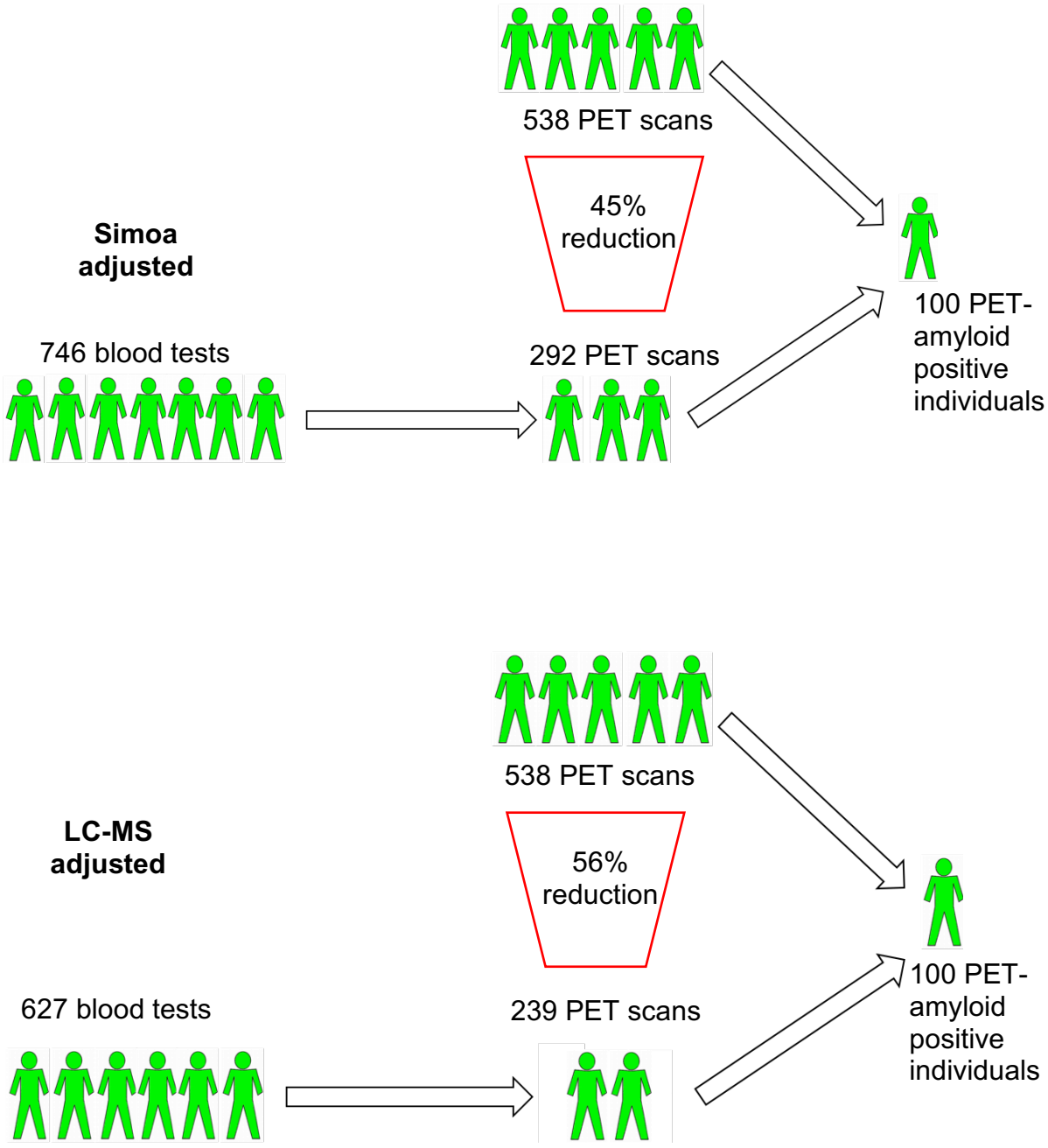
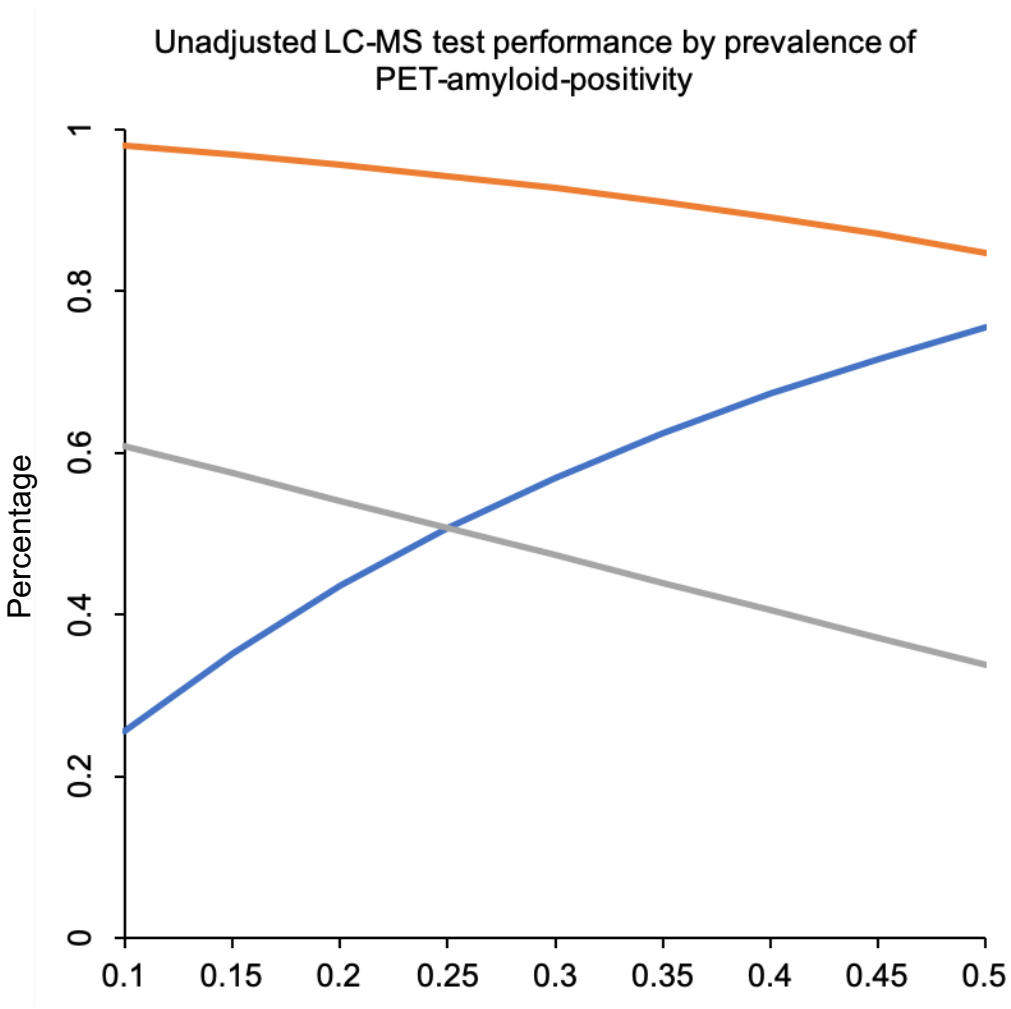


Figure 5.6: Scan number reduction afforded by using the Simoa or the LC-MS assay (adjusted for age, sex and *APOE* ε4 carrier status) to screen prior to amyloid PET scan, assuming a population prevalence of 18.6%.
In each case the Youden's index cut-point was used for assigning blood amyloid status.

5.3.6 Predictive value of the LC-MS test as a pre-screener in populations of varying prevalence of PET-amyloid-positivity

Once a cut-point is chosen, sensitivity and specificity are fixed properties of a screening test, but predictive value depends on prevalence of the underlying condition. Therefore, the Youden's cut-point for the unadjusted LC-MS Aβ1-42/1-40 model was used to derive the positive and negative predictive values of applying this model for screening prior to scan, over a range of population prevalence of PET-amyloid-positivity from 10 to 50% (Figure 5.7, page 233). This shows that if the population prevalence of PET-amyloid-positivity is 10%, as might be expected at age 50 [297], only 26% of those who are blood test-positive will be PET-amyloid-positive, but 98% of those who are blood test-negative will be PET-amyloid-negative. Conversely, if the prevalence of PET-amyloid-positivity is 50%, as might be expected over the age of 90 years [297], 76% of those who are blood test-positive will be PET-amyloid-positive but 85% of those who are blood test-negative will be PET-amyloid negative. The overall accuracy of the test improves as prevalence increases but the relative number of scans saved by the pre-screening reduces.



Prevalence of PET-amyloid-positivity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Relative scan number reduction (%)
10	73.3	25.5	98.0	60.8
20	74.9	43.6	95.7	54.1
30	76.3	56.9	92.8	47.3
40	77.9	67.3	89.2	40.6
50	79.4	75.5	84.7	33.8

Figure 5.7: Positive predictive value, negative predictive value and relative scan number reduction afforded by using the unadjusted LC-MS Aβ1-42/1-40 model as a pre-screening blood test.
Positive predictive value: blue
Negative predictive value: orange
Relative scan number reduction: grey
PET-amyloid positivity is defined by a florbetapir SUVR cut-point of 0.6104.

5.3.7 Relative cost savings afforded by application of the LC-MS test as a pre-screener

Using the equations in Box 5.3 (page 221), the cost of an individual blood test as a fraction of the cost of an individual amyloid PET scan is shown in Figure 5.8, as a function of the relative total cost of the screening programme using the unadjusted LC-MS blood test compared to without the blood test, at different levels of population prevalence of PET-amyloid-positivity. This shows that as prevalence increases, for the same relative cost of an individual blood test, the relative total cost of the screening programme also increases (i.e. the costs savings afforded by the introduction of the blood test to the screening programme reduce).

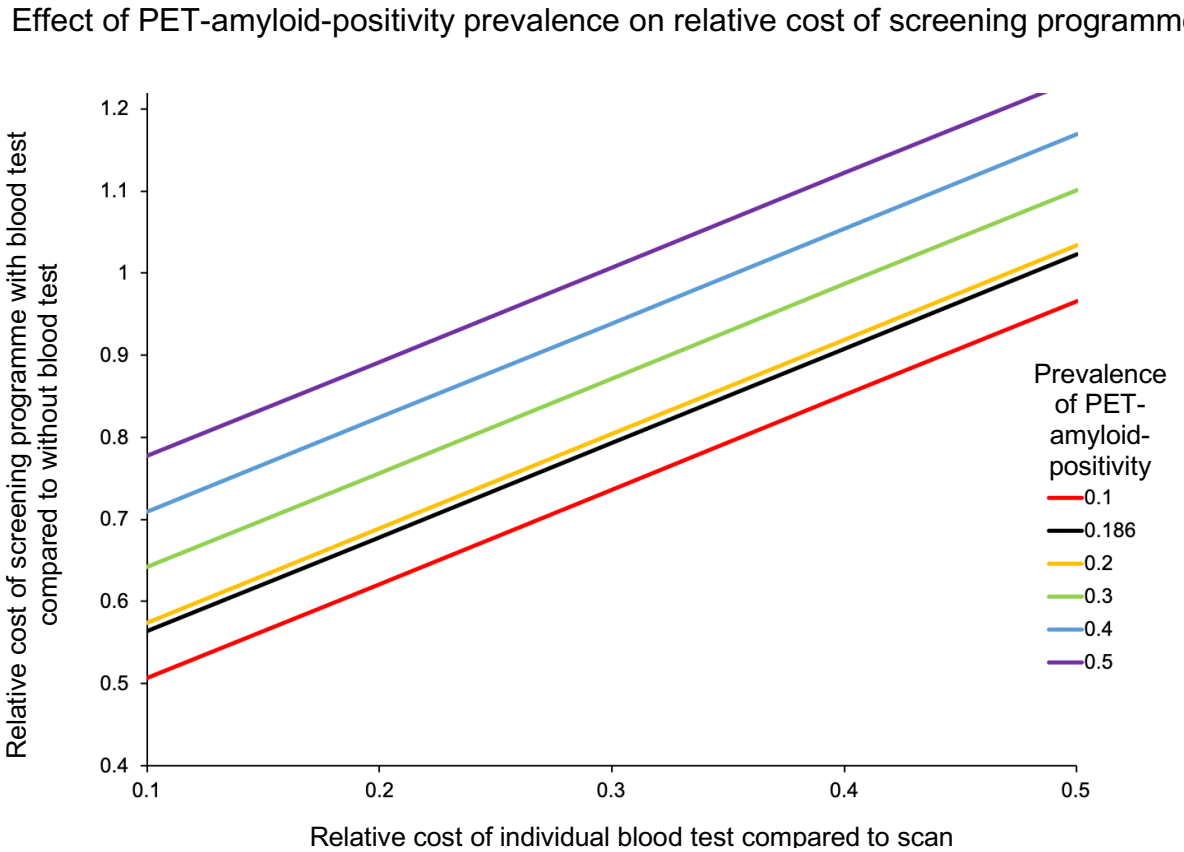
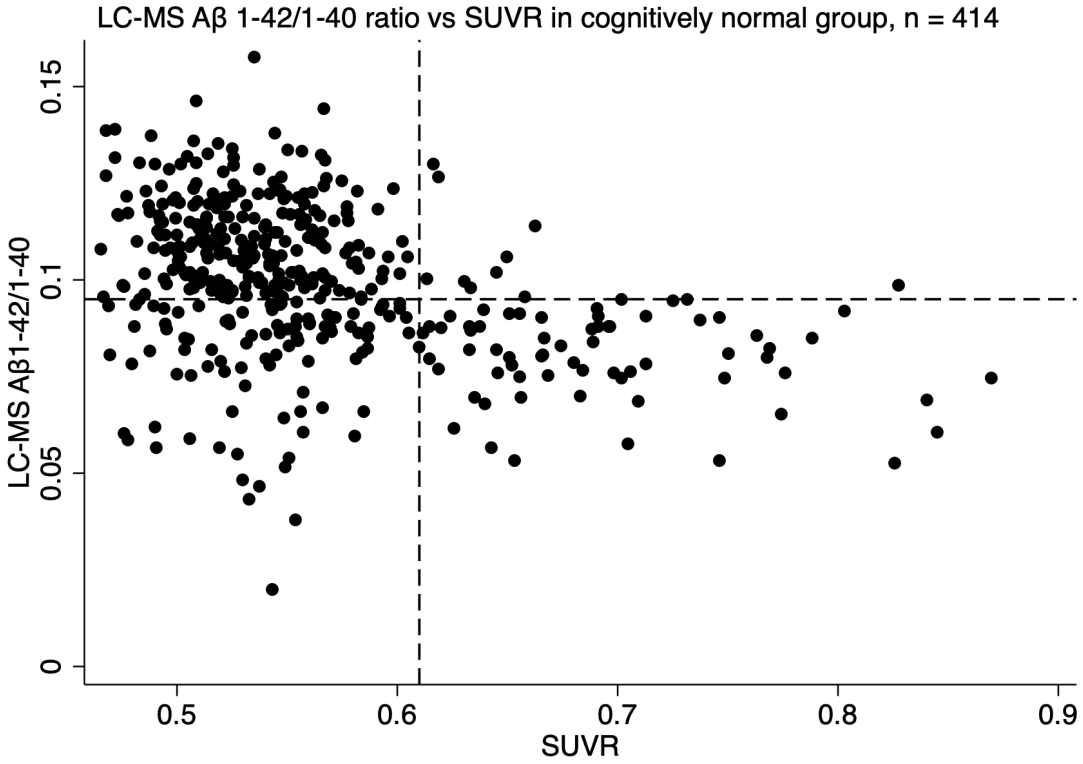


Figure 5.8: Effect of prevalence of PET-amyloid-positivity on relative cost of a screening programme incorporating the unadjusted LC-MS Aβ1-42/1-40 blood test.
Coloured lines show relative costs with increasing prevalence of PET-amyloid-positivity; the black line shows the relative costs in a population with equivalent prevalence to that seen in Insight 46 (18.6%).

5.3.8 Discordance for LC-MS blood test and PET scan

The unadjusted LC-MS Aβ1-42/1-40 values were used to examine discordance between blood amyloid status (as defined by a blood test Youden's index cut-point of 0.095) and PET-amyloid status, as previously defined by the SUVR cut-point of 0.6104. Figure 5.9 shows that out of 108 discordant individuals, 98 (*i.e.* 91%) were "blood positive, PET negative".



Amyloid Status	PET positive (% of total)	PET negative (% of total)
Blood positive	67 (10.9)	98 (23.7)
Blood negative	10 (2.4)	239 (57.7)

Figure 5.9: Examining discordance between the LC-MS Aβ1-42/1-40 assay and amyloid PET SUVR in classifying cognitively normal individuals in Insight 46 (total *n* = 414).

An amyloid PET cut-point of 0.6104 was used to assign PET status and a LC-MS Aβ1-42/1-40 cut-point of 0.095 was used to assign blood status.

The influence of altering the SUVR cut-point used to define PET-amyloid status was next examined (Figure 5.10 below and Table 5.5, page 237). As the SUVR cut-point increased from 0.57 to 0.65, the unadjusted LC-MS Youden's index cut-point remained similar at 0.095, but the sensitivity of the assay increased from 71% to 94%, while the specificity reduced from 74% to 67%. While the total percentage of discordant individuals remained similar (between 26% and 29%), the proportion of these who were "blood positive, PET negative" increased.

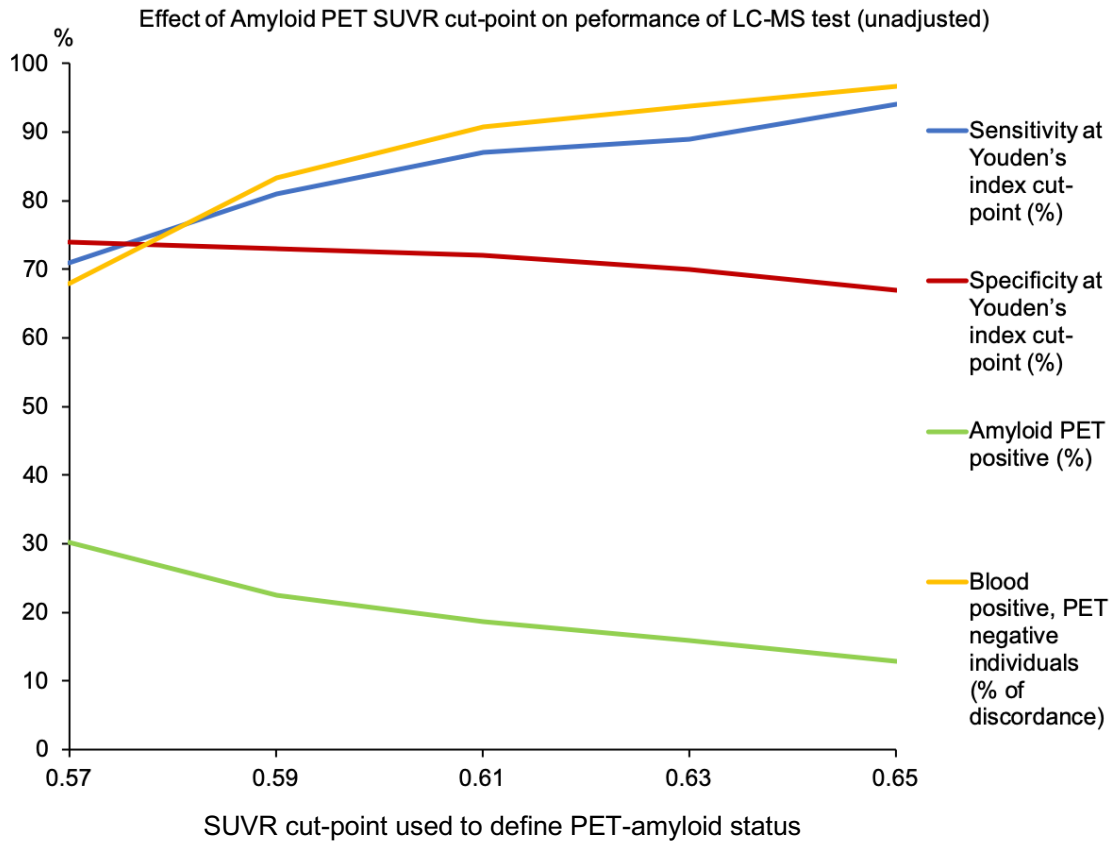


Figure 5.10: Effect of altering the SUVR cut-point used to define PET-amyloid status on the performance of the LC-MS blood test (unadjusted) in cognitively normal individuals in Insight 46 (total n = 414).
The percentage of PET-amyloid-positive individuals, the sensitivity and specificity at the Youden's index cut-point of the blood test are shown along with the percentage of discordant individuals who would be identified as "blood positive, PET negative".

Table 5.5: Influence of SUVR cut-point for amyloid status on LC-MS Aβ1-42/1-40 assay performance in cognitively normal individuals in Insight 46 (total *n* = 414). Across this range of SUVR cut-points, the Youden's index cut-point for the LC-MS Aβ1-42/1-40 assay (used to assign blood status) remained constant at 0.095.

SUVR cut-point	0.57		0.59		0.61		0.63		0.65	
Amyloid PET positive (%)	30.2		22.5		18.6		15.9		12.8	
Amyloid status	**PET positive (%)**	**PET negative (%)**	**PET positive (%)**	**PET negative (%)**	**PET positive (%)**	**PET negative (%)**	**PET positive (%)**	**PET negative (%)**	**PET positive (%)**	**PET negative (%)**
Blood positive	89 (21.5)	76 (18.4)	75 (18.1)	90 (21.7)	67 (10.9)	98 (23.7)	59 (14.3)	106 (25.6)	49 (11.8)	116 (28.0)
Blood negative	36 (8.7)	213 (51.4)	18 (4.3)	231 (55.8)	10 (2.4)	239 (57.7)	7 (1.7)	242 (58.5)	4 (1.0)	245 (59.2)
Concordance (% of total)	72.9		73.9		73.9		72.7		71.0	
Discordance (% of total)	27.1		26.1		26.1		27.3		29.0	
Blood positive, PET negative individuals (% of discordance)	67.9		83.3		90.7		93.8		96.7	
Sensitivity at Youden's index cut-point (%)	71.0		81.0		87.0		89.0		94.0	
Specificity at Youden's index cut-point (%)	74.0		73.0		72.0		70.0		67.0	

5.4 **Discussion**

5.4.1 Summary of results

The questions formulated in section 5.1 (page 215) were addressed as follows:

- The LC-MS plasma Aβ1-42/1-40 ratio performed better than the Simoa plasma Aβ42/40 ratio in detecting PET-amyloid-positivity and gave additional information above a model incorporating age, sex and *APOE* ε4 carrier status.
- Addition of plasma t-tau or serum NFL did not improve the result.
- In a population with similar PET-amyloid-positivity prevalence to Insight 46, after adjusting for age, sex and *APOE* ε4 carrier status, application of the LC-MS plasma Aβ1-42/1-40 ratio as a pre-screening test would reduce the required number of PET scans by 56%, as opposed to the Simoa plasma Aβ42/40 ratio which would reduce the required number of scans by 45%.
- As estimated population prevalence of PET-amyloid-positivity increases, as occurs with age, the positive predictive value and overall accuracy of the LC-MS plasma Aβ1-42/1-40 ratio increase, but the negative predictive value and the relative scan number reduction afforded by the test decrease. This would result in reduced relative cost savings from introduction of the LC-MS plasma Aβ1-42/1-40 ratio blood test into a screening programme.
- Most individuals who were discordant for LC-MS blood and PET amyloid status were “blood positive, PET negative”; this remained the case even if slightly lower or higher SUVR cut-points were used to define PET amyloid status.

5.4.2 Plasma amyloid-β and cerebral amyloid deposition

The literature on plasma amyloid-β peptides and cerebral amyloid deposition has previously been discussed (section 4.4.3, page 208); it demonstrates a heterogeneity of results partly due to methodological differences in quantification of plasma amyloid-β peptides. This study reports the first direct comparison of two different assay methods in the same set of plasma samples, and demonstrates the superiority of the LC-MS method over the commercial Simoa assays in detecting PET-amyloid-positivity. A larger plasma amyloid-β peptide "round robin" study is currently being facilitated by the Alzheimer's Association Biofluid Biomarkers Professional Interest Area, utilising samples from the DRC cohort, among others, in collaboration with the laboratories that have reported on individual methods previously.

Ovod *et al.* [203] performed a plasma amyloid-β peptide stable isotope labelling kinetics (SILK) study based on a LC-MS/MS method, which showed that the half-life of plasma Aβ1-38, Aβ1-40 and Aβ1-42 is around 3 hours, but there is a faster decay of the labelled plasma Aβ1-42/1-40 ratio after 12 hours in PET-amyloid positive individuals compared to negative individuals, which is not present when examining the labelled plasma Aβ1-40/1-38 ratio. These plasma decay kinetics mirror those of the same peptides in CSF [204] and provide associative evidence that cerebral amyloid deposition is responsible for the faster decay in Aβ1-42 in both biofluids in the PET-amyloid positive individuals. However, both CSF and plasma SILK studies indicate that some individuals classified as amyloid negative by PET display the same altered decay kinetics, which echoes the finding in ADNI that cognitively normal individuals who are amyloid positive by CSF Aβ1-42 but negative by PET display the greatest

longitudinal gain in amyloid PET SUVR [305]. This indicates that in cognitively normal individuals with underlying AD pathology, CSF Aβ1-42 changes may occur earlier than PET changes, and so future studies of plasma amyloid-β as a pre-screener may benefit from using CSF Aβ1-42 or Aβ1-42/1-40 ratio as the "gold standard", rather than PET. It also implies that some of those who turn out to be "false positives" by applying a blood test actually are on the trajectory toward developing cerebral amyloid deposition. Indeed, our data show that in Insight 46 the individuals who are discordant for blood and PET amyloid are mostly "blood positive, PET negative". With follow-up, it will be possible to examine whether these individuals show the greatest increment in amyloid PET SUVR in phase 2 of Insight 46. Phase 2 sampling will also allow for assessment of concordance between LC-MS plasma and CSF Aβ1-42/1-40 ratio in comparison to that of LC-MS plasma Aβ1-42/1-40 ratio and amyloid PET SUVR.

5.4.3 Considerations for population screening for cerebral amyloid-β deposition

5.4.3.1 The influence of population prevalence in screening test application

The premise of screening for cerebral amyloid deposition is that it is a necessary and early step in the pathophysiology of AD. However, only 17-37% of people who are amyloid positive in their late sixties to early seventies progress to developing MCI or AD within the next three years [318] so it is important to continue to develop other "gold standard" *in vivo* biomarkers that are more proximate to symptom onset, but still applicable in the asymptomatic phase.

The contribution of screening tests in AD is also heavily dependent upon population prevalence of the "gold standard" against which the screening is applied. For example, extrapolating from our data, as assumed population prevalence of PET-amyloid positivity increases (as might be expected with age), the positive predictive value and overall accuracy of classification of the LC-MS plasma Aβ1-42/1-40 ratio increase, but the negative predictive value and the relative scan number reduction decrease. Jansen *et al*.'s meta-analysis estimated that the prevalence of cerebral amyloid pathology (as assessed by *in vivo* CSF or PET biomarkers) increases with age, from 10% at age 50, to 23% at age 70, and 44% at age 90 for cognitively normal individuals [297]. In this regard, the percentage of amyloid positivity in Insight 46 (18.6% at age 70) is slightly lower than predicted. This observed difference may be related either to the cognitively normal individuals included in the meta-analysis having been recruited predominantly by advertisement (rendering the meta-analysis potentially susceptible to positive selection bias) or to a potential negative selection bias in Insight 46 (which would be in keeping with our findings that participants in this sub-study had better health measures in a variety of indicators, than those in the overall NSHD [274]).

APOE genotype also heavily influences amyloid prevalence, with ε4 carriers estimated to have a 48% prevalence at age 70, compared to non-carriers with 17% prevalence [297]. While our adjusted LC-MS plasma Aβ1-42/1-40 model gave a very similar AUC to the unadjusted model (0.84 vs 0.82), given these differences in prevalence of amyloid positivity according to *APOE* genotype, it is likely that gains from including the plasma Aβ1-42/1-40 ratio in a pre-screening

blood test will come predominantly from identifying at-risk individuals who are non-carriers of *APOE* ε4, who would not be selected as "at-risk" by employing *APOE* genotyping alone.

5.4.3.2 Refinements to the screening test

In addition to *APOE* genotyping, polygenic risk scores (PRS) [61] and age-specific genetic risk-derived polygenic hazard scores (PHS) [319] for AD have also been proposed as potential screening tests, with a version of PHS recently having been made commercially available to consumers on the internet. In the smaller TGen (Translational Genomics Research Institute) dataset, PRS for AD confirmed on *post mortem* pathology have also been described [320]. This AD PRS is currently being derived for the Insight 46 cohort and we plan to test the ability of the LC-MS plasma Aβ1-42/1-40 ratio to provide information to predict cerebral amyloid above a model incorporating age, sex, *APOE* and the AD PRS.

Nakamura *et al.* [197] advocated the use of a composite plasma amyloid score, derived from the 1:1 weighted average of z-scores of the Aβ1-40/1-42 ratio and the APP669-711/Aβ1-42 ratio, as being superior to using the Aβ1-40/1-42 ratio alone in predicting PET amyloid status, but a closer examination of their data indicates that in cognitively normal individuals this was borne out only in the AIBL cohort and not in the NCGG cohort. We also plan to examine whether this composite biomarker has superior predictive ability to the LC-MS Aβ1-42/1-40 ratio in Insight 46.

5.4.3.3 Use of different "gold standard" definitions

In this study, a florbetapir PET SUVR cut-point of 0.6104 was used as the "gold standard for determination of amyloid status, but as described in section 5.4.2 (page 239), reductions in CSF Aβ42 may occur earlier than accumulation of the amount of amyloid that would be required to cross the PET scan threshold. It is possible that the concordance of the plasma and CSF values of Aβ1-42/1-40 would therefore be better than that of plasma and amyloid PET. This prediction will be able to be tested in phase 2 of Insight 46, when paired plasma and CSF samples from the same individuals will be available.

If the chosen amyloid PET cut-point in this study were too conservative (i.e. too high) then it would be expected that the concordance between the LC-MS Aβ1-42/1-40 and PET-amyloid status would drastically improve if the amyloid PET cut-point were lowered. However, examining Table 5.5 (page 237) reveals that slight downward or upward shifts of the amyloid PET cut-point would not affect the concordance of the LC-MS Aβ1-42/1-40 assay (for which the Youden's index cut-point does not change with the re-classification of PET-amyloid status using these new cut-points). Moreover, the majority of discordance would still arise from "blood positive, PET negative" individuals.

5.4.3.4 Cost and other practical considerations

Practical considerations for the implementation of a blood biomarker for screening or early detection of AD pathology have been discussed previously (page 145 and [269]). At present, given that this specific LC-MS Aβ1-42/1-40 assay is available only on a research basis at the University of Gothenburg, the cost of reagents and operator time per sample is estimated at approximately

£300 (Prof Kaj Blennow, personal communication). Contrasting with an estimated cost of £1500 per person for an amyloid PET scan, and taking into account the relationship between population prevalence of PET-amyloid-positivity and relative cost savings (see Figure 5.8, page 234), recruitment of 100 PET-amyloid-positive individuals from a population with a prevalence of 18.6% (such as Insight 46) by using a PET-scan only would cost £807 000. However, if using the unadjusted LC-MS Aβ1-42/1-40 blood test as a pre-screener the cost would drop to £548 100, giving a relative cost saving of 32.1%. Although the projected relative cost savings assume that the assay itself performs in the same way with varying population prevalence, this is an extrapolation from data from a group in which age is very tightly controlled, so this assay (and the associated cost projections) will require validation in cohorts with a wider age range.

Actual costs are also likely to be associated not just with the tests themselves but also with the infrastructure associated with a screening programme. The LC-MS technique is currently dependent upon appropriate pre-processing and cold storage of samples, until they are transported to and analysed in a highly specialist laboratory. While this technique may therefore be appropriate for use in the context of screening for recruitment to clinical trials, if a therapeutic agent for AD were to meet its primary endpoint for efficacy and the demand for the screening test were to increase, it is unlikely that this technique would be able to be upscaled to the level that would be required for application to the general population in the primary care setting. The search for a more scalable test (which would be more likely to be an automated immunoassay) therefore continues.

6 Cross-sectional associations between blood biomarkers and structural brain imaging in Insight 46

6.1 Introduction

As detailed in Chapter 3, Insight 46 participants had simultaneous 3T MRI and ^{18}F-florbetapir PET. One of the key aims of the work detailed in this thesis was to assess the blood biomarkers measured in phase 1 of Insight 46 against validated measures of brain volumes, cortical thickness and white matter lesion load. Cross-sectional associations between blood biomarkers and structural brain imaging measures were investigated with the following predictions:

- Higher plasma t-tau and serum NFL (but not plasma Aβ42, Aβ40 or Aβ42/40 ratio) would associate with lower whole brain volume and higher ventricular volume.
- Higher serum NFL would associate with lower hippocampal volume and lower cortical thickness in an AD signature region previously described by Jack et al. [142], and this association would be more significant in amyloid positive individuals.
- Higher plasma Aβ40 and Aβ42 (but not Aβ42/40 ratio) would associate with higher white matter hyperintensity volume, as previously reported by Janelidze *et al*. [202].

6.2 Methods

6.2.1 Outcome variable derivation

6.2.1.1 Brain volume variables

From the T1-weighted images, whole brain volumes (WBV) and ventricular volumes (VV) were extracted by an automated Brain Multi-Atlas Propagation and Segmentation (BMAPS) technique [321] and hippocampal volumes were

extracted by Similarity and Truth Estimation for Propagated Segmentations (STEPS) [322], then underwent manual checking and editing by the Insight 46 Imaging team. Mean hippocampal volume (HV - the simple average of the left and right hippocampal volumes) was used in analyses.

6.2.1.2 White matter hyperintensity volume

The automated Bayesian Model Selection (BaMoS) algorithm [323], using the T1-weighted and FLAIR images, was used to estimate global white matter hyperintensity volume (WMHV) in the subcortical white matter, excluding the infratentorial regions. Visual quality control was applied by Dr Christopher Lane, and subsequently confirmed by neuroradiologist review, to exclude individuals with white matter lesions characteristic of demyelination or large cortical infarcts inappropriately segmented as subcortical white matter hyperintensities.

6.2.1.3 Alzheimer's signature region cortical thickness

Automated surface-based reconstruction was performed by Dr Thomas Parker using Freesurfer 6.0 (http://surfer.nmr.mgh.harvard.edu/) followed by cortical thickness estimation using the mean distance between the grey matter/white matter and grey matter/CSF surfaces in a validated method [324]. Two modifications to the automated pipeline were made: skull stripping accuracy was improved by using a locally generated and manually edited brain mask, and segmentation accuracy was improved by using both the T1- and T2-weighted images as inputs (https://surfer.nmr.mgh.harvard.edu/fswiki/recon-all#UsingT2orFLAIRdatatoimprovepialsurfaces). The Alzheimer's signature region cortical thickness (CTh) was calculated as the surface weighted average

of the fusiform, middle/inferior temporal and entorhinal cortical thickness values [142].

6.2.2 Covariate derivation

Total intracranial volume (TIV) was calculated from the T1-weighted sequences using Statistical Parametric Mapping software (SPM12) [325].

6.2.3 Inclusion and exclusion criteria

For the analyses of WBV, VV and HV, individuals with complete blood biomarker (BB), *APOE* ε4 carrier status and PET-amyloid status data (group 1 data) whose T1 scans passed BMAPS segmentation quality control were included.

For the analyses of WMHV, individuals with group 1 data who had T1 and FLAIR scans passing quality control for BaMoS were included.

For the analyses of CTh, individuals with group 1 data whose T1 and T2 scans passed quality control for Freesurfer segmentation were included.

Analyses for each of these variables were conducted in two groups:

- the “Full Data” group, which met the above criteria and
- the “Cognitively Normal” group, which excluded those with prior neurological diagnoses and those fulfilling study criteria for MCI or dementia.

Figure 6.1 shows the numbers of individuals included in each set of analyses in the full data and cognitively normal groups.

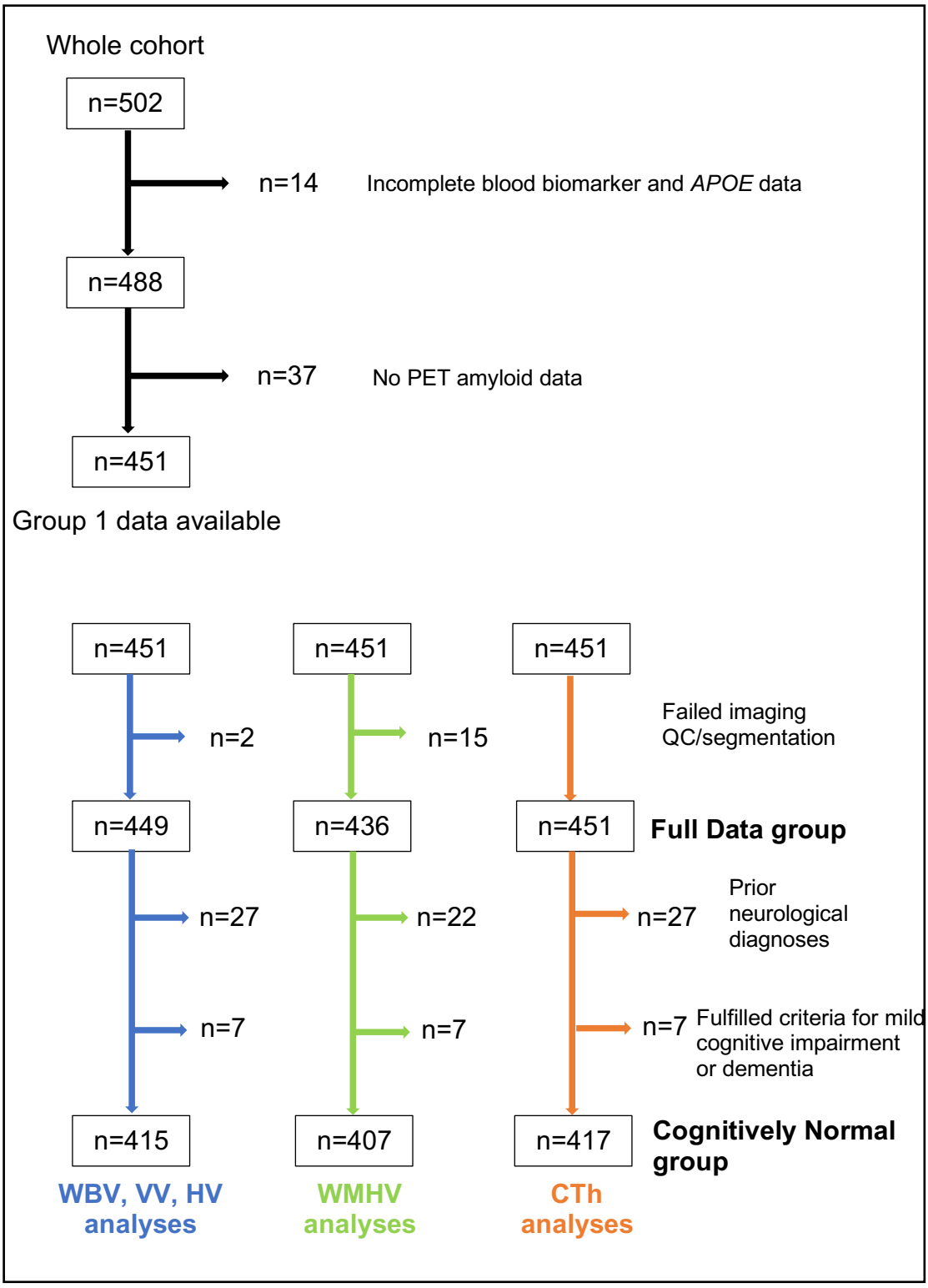


Figure 6.1: Inclusions and exclusions for analyses of associations between blood biomarkers and brain volume and cortical thickness variables in phase 1 of Insight 46.
CTh, Alzheimer's signature region cortical thickness; VV, ventricular volume; WBV, whole brain volume; WMHV, white matter hyperintensity volume.

6.2.4 Statistical analyses

6.2.4.1 Choice of covariates

Age at blood sampling, sex, *APOE* ε4 carrier status, PET-amyloid status and TIV were included as covariates in all models of WBV, VV, HV and WMHV. TIV was not included as a covariate in the models of CTh, as per the method described by Jack *et al.* [142] and after confirming that it made no difference to model fit or BB coefficients. Blood-to-scan delay was not included in final analyses as it too made no difference to model fit or BB coefficients for any of the structural brain imaging outcomes examined. Given the contributions of serum creatinine and BMI to blood biomarker variance (Chapter 5), the impact of introducing each or both of these variables into a base model without the blood biomarkers was first assessed, for each structural brain imaging outcome. If there was a significant improvement in model fit (as evidenced by lowering of the Akaike Information Criterion, AIC, by ≥ 10), and/or if there was a significant coefficient for creatinine or BMI, the adjusted coefficients derived from models incorporating blood biomarkers with and without creatinine or BMI were compared. Box 6.1 summarises the covariates included in the various base models for each structural brain imaging outcome.

Model 1: Structural brain imaging outcome ~ age + sex + *APOE* ε4 carrier status + amyloid status (+ total intracranial volume if appropriate)

Model 2: Structural brain imaging outcome ~ (covariates of model 1) + serum creatinine

Model 3: Structural brain imaging outcome ~ (covariates of model 1) + BMI

Model 4: Structural brain imaging outcome ~ (covariates of model 1) + serum creatinine + BMI

Model 5: Structural brain imaging outcome ~ (covariates of model 1) + (amyloid status x blood biomarker interaction) *

Box 6.1: Covariates for statistical models of structural brain imaging outcomes.
* For NFL and t-tau only

6.2.4.2 Choice of models

Natural log-transformed WBV, VV, HV and CTh were modelled using linear regression, incorporating natural log-transformed BB variables and the above covariates. QQ plots and scatter plots of residuals against model fit results were assessed to ensure that model assumptions were met. Potentially influential outliers were considered by deriving Cook's distance for every individual for each model and re-running the models only in those individuals in whom the Cook's distance was less than 4/n.

As WMHV had a positively skewed distribution, generalised linear models using a gamma distribution with log link were used to determine associations with BB. Exponentiated coefficients were reported as ratio changes for a 1 unit rise in BB. Scatter plots of Anscombe residuals against model fit results were used to assess model assumptions.

Robust standard errors were used to calculate the 95% confidence intervals of the model coefficients. The coefficients for BB were back-transformed and reported as ratio changes for a 10% BB rise. For example, in a linear regression model of lnWBV incorporating lnNFL:

$$lnWBV = constant + \beta_1(\text{age}) + \beta_2(\text{sex}) + \beta_3(APOE\ \varepsilon4\ \text{carrier status}) + \beta_4(\text{amyloid status}) + \beta_5(\text{TIV}) + \beta_6(\text{lnNFL})$$

the ratio change in WBV for a 10% increase in NFL is given by

$$Ratio\ change\ in\ WBV\ for\ 10\%\ increase\ in\ NFL = (1.1)^{\beta_6}$$

Similarly, in the generalised linear model with log link of WMHV incorporating NFL, the ratio change in WMHV for a 10% increase in NFL is given by

$$Ratio\ change\ in\ WMHV\ for\ 10\%\ increase\ in\ NFL = e^{(\beta_6 * 1.1)}$$

6.3 **Results**

6.3.1 Significance of serum creatinine and BMI as covariates

Appendix Table 12.2 (page 328) shows model 4 (as per Box 6.1, page 249) for each brain imaging outcome, before adding BB, in the Cognitively Normal group. Adding either serum creatinine or BMI to any model did not give an improvement in AIC by ≥ 10. Higher serum creatinine showed a significant association with higher WBV, CTh and WMHV. Higher BMI had a significant association only with higher HV.

6.3.2 Associations of blood biomarkers with structural MRI measures

6.3.2.1 Cognitively Normal Group

Table 6.1 (page 253) shows that associations between blood biomarkers and structural MRI measures remained similar with and without adjustment for serum creatinine and BMI. A 10% increase in plasma tau was associated with a 0.1% reduction in WBV, while a 10% increase in serum NFL was associated with a 1.2% increase in VV. After outliers were excluded from analysis in the model of VV incorporating Aβ42, a 10% increase in Aβ42 was associated with a 1.4% increase in VV. This association was strengthened in a secondary analysis using the LC-MS Aβ1-42 values; a 10% increase in LC-MS Aβ1-42 was associated with a 2% increase in VV and a 10% increase in LC-MS Aβ1-42/1-40 ratio was associated with a 2.1% increase in VV (Appendix Table 12.6, page 332).

After removal of potentially influential outliers a 10% increase in serum NFL was associated with a 0.2% lower HV. A 10% increase in plasma tau or a 10% increase in serum NFL was associated with a 0.1% reduction in CTh; removal

of outliers did not impact these results. There were no significant associations between any blood biomarker and WMHV.

There was no significant interactive effect of amyloid status with either plasma tau or serum NFL on any of the five structural brain imaging variables (model 5: Appendix Table 12.3, page 329; and Table 12.4, page 330).

A graphical summary of the blood biomarker coefficients within model 1 for each structural MRI measure is shown in Figure 6.2 (page 256).

Table 6.1: Associations between Simoa blood biomarkers and structural MRI measures in the Cognitively Normal group.
Coefficients were transformed and presented as ratio change for a 10% rise in the respective blood biomarker. Robust standard errors of the coefficients were used to calculate the 95% confidence intervals (brackets). Values in bold were statistically significant at the level of $p = 0.05$.
Model 1: Structural brain imaging outcome ~ age + sex + *APOE* ε4 carrier status + amyloid status (+ total intracranial volume if appropriate)
Model 2: Cognitive outcome ~ (covariates of model 1) + serum creatinine
Model 3: Cognitive outcome~ (covariates of model 1) + BMI
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; Simoa neurofilament light chain; t-tau, Simoa total (mid-region) tau.
*After removing potentially influential outliers (determined by Cook's distance≥4/415), this association became statistically significant: ratio change 1.015 (1.004, 1.027), p =0 .008, n = 386
[+] After removing potentially influential outliers (determined by Cook's distance≥4/415), this association became statistically significant: ratio change and 95% confidence interval remained the same but p = 0.048.

	Whole brain volume (n = 415)														
	Model 1					Model 2					Model 3				
BB	Ratio change	95% CI	p	AIC	r^2	Ratio change	95% CI	p	AIC	r^2	Ratio change	95% CI	p	AIC	r^2
None	-	-	-	-1502	0.808	-	-	-	-1504	0.808	-	-	-	-1501	0.807
Aβ42	1	0.999, 1.002	0.593	-1501	0.807	1	0.999, 1.001	0.797	-1502	0.808	1	0.999, 1.002	0.588	-1499	0.807
Aβ40	1.001	0.999, 1.003	0.588	-1501	0.807	1	0.998, 1.002	0.907	-1502	0.808	1.001	0.999, 1.003	0.594	-1499	0.807
Aβ42/40	1	0.999, 1.001	0.801	-1501	0.807	1	0.999, 1.001	0.741	-1502	0.808	1	0.999, 1.001	0.792	-1499	0.807
t-tau	**0.999**	**0.998, 1**	**0.024**	-1506	0.809	**0.999**	**0.998, 1**	**0.007**	-1510	0.812	**0.999**	**0.998, 1**	**0.019**	-1505	0.810
NFL	1	0.999, 1.001	0.965	-1501	0.807	1	0.999, 1.001	0.687	-1502	0.808	1	0.999, 1.001	0.899	-1499	0.807

Cont'd

	Ventricular volume (*n* = 415)														
	Model 1					Model 2					Model 3				
BB	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2
None	-	-	-	493	0.239	**-**	-	**-**	491	0.238	-		-	492	0.237
Aβ42	1.010*	0.996, 1.024	0.150	491	0.239	1.009	0.995, 1.023	0.204	492	0.241	1.010	0.996, 1.024*	0.151	492	0.241
Aβ40	1.006	0.981, 1.031	0.660	494	0.231	1.001	0.976, 1.027	0.956	493	0.238	1.006	0.981, 1.031	0.639	494	0.237
Aβ42/40	1.008	0.995, 1.021	0.231	491	0.238	1.008	0.995, 1.021	0.216	492	0.240	1.008	0.995, 1.021	0.243	493	0.239
t-tau	1.003	0.994, 1.013	0.480	492	0.236	1.002	0.993, 1.012	0.646	493	0.238	1.004	0.995, 1.014)	0.392	493	0.238
NFL	**1.012**	**1.001, 1.023**	**0.033**	488	0.245	1.011	1, 1.022	0.055	489	0.245	1.011	**1, 1.022**	**0.046**	490	0.245
	Mean hippocampal volume (*n* = 415)														
	Model 1					Model 2					Model 3				
BB	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2
None		-	-	-803	0.271		-	-	-802	0.265		-	-	-804	0.268
Aβ42	1	0.997, 1.002	0.777	-800	0.262	0.999	0.997, 1.002	0.607	-800	0.265	1	0.997, 1.002	0.802	-802	0.268
Aβ40	1.001	0.996, 1.006	0.725	-800	0.262	1	0.994, 1.005	0.909	-800	0.265	1.001	0.996, 1.006	0.759	-802	0.268
Aβ42/40	0.999	0.997, 1.002	0.626	-800	0.262	0.999	0.997, 1.002	0.667	-800	0.265	0.999	0.998, 1.002	0.668	-802	0.268
t-tau	0.999	0.997, 1.002	0.458	-800	0.262	0.999	0.997, 1.001	0.300	-801	0.266	0.999	0.997, 1.001	0.309	-802	0.270
NFL	0.998+	0.996, 1	0.087	-803	0.267	**0.998**	**0.995, 1**	**0.045**	-804	0.273	0.999	0.996, 1.001	0.244	-803	0.271

Cont'd

	Alzheimer's signature region cortical thickness (*n* = 417)														
	Model 1					Model 2					Model 3				
BB	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2	Ratio change	95% CI	*p*	AIC	r^2
None		-	-	-1709	0.006		**-**	**-**	-1711	0.017		-	-	-1708	0.008
Aβ42	1	0.999, 1.001	0.616	-1707	0.007	1	0.999, 1.001	0.836	-1701	0.024	1	0.999, 1.001	0.588	-1698	0.017
Aβ40	0.999	0.997, 1.001	0.225	-1708	0.009	1	0.998, 1.001	0.732	-1701	0.024	0.999	0.989, 1.001	0.285	-1699	0.018
Aβ42/40	1	0.999, 1.001	0.889	-1707	0.006	1	0.999, 1.001	0.974	-1701	0.024	1	0.999, 1.001	0.982	-1698	0.016
t-tau	**0.999**	**0.998, 1**	**0.015**	-1713	0.019	**0.999**	**0.999, 1**	**0.036**	-1705	0.033	**0.999**	**0.998, 1**	**0.021**	-1703	0.028
NFL	**0.999**	**0.998, 1**	**0.015**	-1713	0.020	0.999	0.998, 1	0.051	-1705	0.034	**0.999**	**0.998, 1**	**0.006**	-1706	0.034
	White matter hyperintensity volume (*n* = 407)														
	Model 1					Model 2					Model 3				
BB	Ratio change	95% CI	*p*	AIC		Ratio change	95% CI	*p*	AIC		Ratio change	95% CI	*p*	AIC	
None		-	-	-3871			-	-	-3867			-	-	-3870	
Aβ42	1	0.980, 1.020	0.995	-3871		0.995	0.976,1.014	0.587	-3867		1	0.981,1.021	0.967	-3871	
Aβ40	1.001	0.999, 1.003	0.545	-3871		0.999	0.997, 1.002	0.650	-3867		1.001	0.998,1.003	0.563	-3871	
Aβ42/40	0.769	0.005,128.801	0.920	-3871		0.695	0.005,90.36	0.884	-3867		0.870	0.005,148.581	0.958	-3871	
t-tau	0.975	0.881, 1.080	0.633	-3871		0.958	0.868, 1.057	0.393	-3867		0.970	0.877, 1.074	0.559	-3871	
NFL	1.005	0.997, 1.013	0.228	-3870		1.004	0.995, 1.014	0.381	-3866		1.006	0.998, 1.014	0.165	-3870	

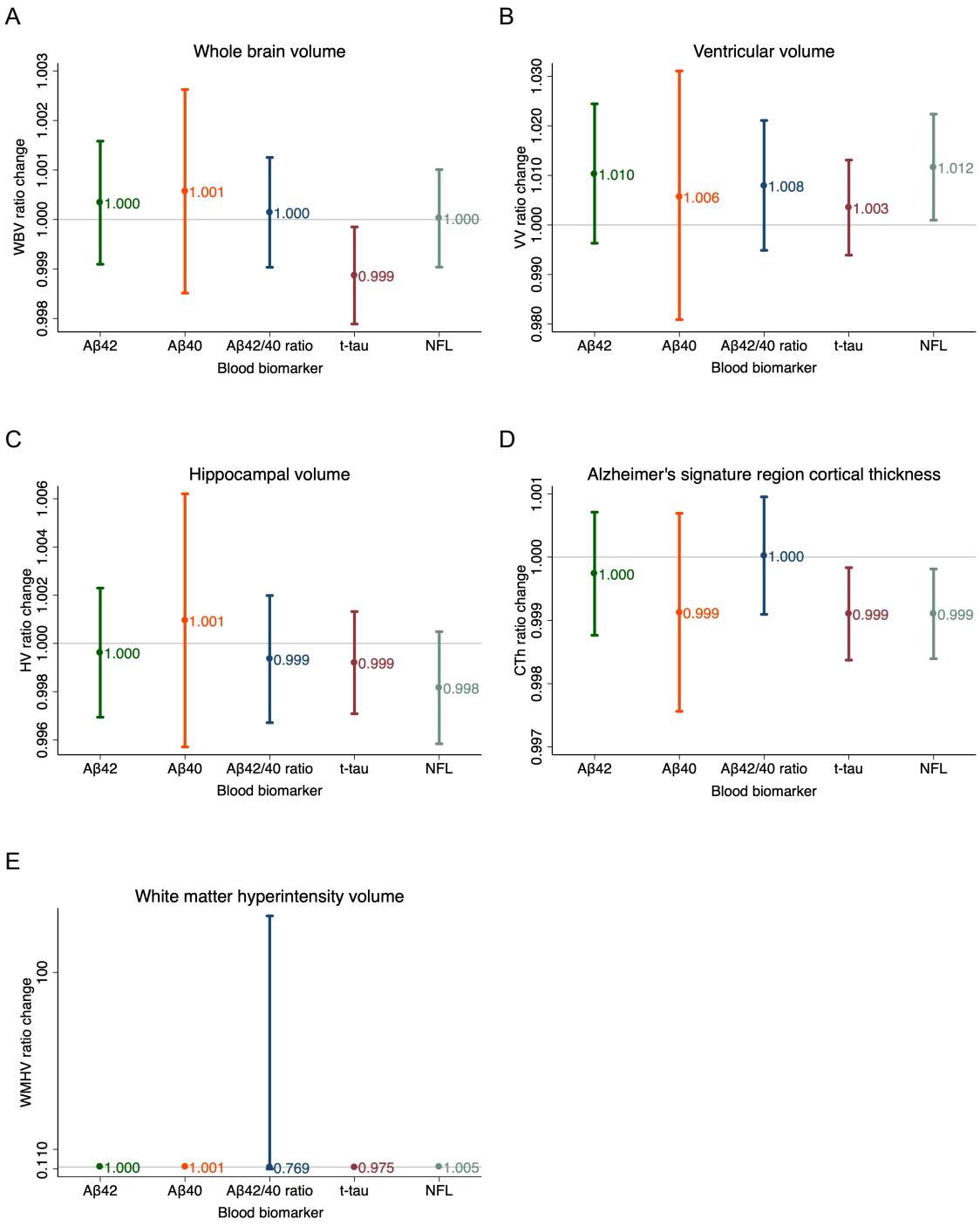


Figure 6.2: Dot-whisker plots showing the 95% confidence intervals of the coefficients of ratio change for each structural MRI measure for a 10% increase in each blood biomarker, derived from model type 1.
A: Whole brain volume
B: Ventricular volume
C: Hippocampal volume
D: Alzheimer's signature region cortical thickness
E: White matter hyperintensity volume
Horizontal grey lines show a ratio change of 1.

6.3.2.2 Full data group

In the Full Data group (Appendix: Table 12.5, page 331) the coefficients were overall similar to those derived from the analyses in the Cognitively Normal group. Associations retained statistical significance between higher plasma tau and lower WBV, and higher serum NFL and higher VV. Statistical significance was also achieved by the association between serum NFL and HV (a 0.3% reduction in HV for a 10% increase in NFL) and between Aβ42 and VV (a 1.5% increase in VV for a 10% increase in Aβ42). The association between higher serum NFL and lower CTh retained statistical significance but that between higher plasma tau and lower CTh did not. As seen previously, WMHV was not associated significantly with any blood biomarker.

6.4 **Discussion**

6.4.1 Summary of results

These analyses addressed the predictions detailed in section 6.1 as follows:

- Higher plasma t-tau was associated with lower whole brain volume. Higher serum NFL, higher Simoa plasma Aβ42, higher LC-MS plasma Aβ1-42 and higher LC-MS plasma Aβ1-42/1-40 ratio were associated with higher ventricular volume.
- As predicted, higher plasma t-tau and higher serum NFL were associated with lower cortical thickness in an AD signature region.
- Higher serum NFL was associated with lower hippocampal volume only after excluding influential outliers, and there was no interaction of amyloid status on this association.
- None of the blood biomarkers was significantly associated with white matter hyperintensity volume.

6.4.2 Plasma tau and brain imaging

Using a different immunomagnetic reduction technique for measuring tau in plasma from that used in our study, Chiu *et al.* were unable to find any association between plasma tau and volumes of multiple brain structures or cortical thickness in cognitively normal Taiwanese individuals [223]. However, two much larger studies that are more comparable to Insight 46 (and also used Simoa assays) have reported significant associations. Mattsson *et al.* assessed plasma tau in ADNI samples and showed that elevated baseline plasma tau was significantly associated with increases in both baseline and longitudinal ventricular volumes across the whole mixed cohort (controls, MCI and AD-dementia) but subgroup analysis showed that the association was driven predominantly by individuals in the dementia phase of AD [220]. In the MCSA, Mielke *et al.* showed a cross-sectional association of higher plasma t-tau with lower AD signature cortical thickness, particularly in cognitively normal individuals and those with MCI [217]. In this same study, a homebrewed Simoa assay for plasma phospho-tau-181 (p-tau-181) did not show these associations in these subgroups, but did show an association with regional ^{18}F-flortaucipir binding that plasma t-tau did not. This implies that the t-tau assay may be functioning more as a marker of neurodegeneration, while the p-tau-181 assay is revealing a marker more reflective of cerebral tau deposition related to AD (the “T” of the ATN framework). As different plasma tau assays are devised, targeted at different post-translational modifications (including truncations – see Chapter 9 and [326]), they may reveal important differences in their associations with stage of disease, in a way that is already emerging for different tau phosphorylation sites in CSF [327].

6.4.3 Blood NFL and brain imaging

Plasma NFL was assessed in ADNI by Mattsson *et al.* using a homebrew Simoa assay [234] to show whole group (cognitively normal, MCI and AD-dementia) associations between higher NFL and higher ventricular volume, lower hippocampal volume and lower AD signature cortical thickness measured both at baseline and longitudinally; however, analyses by subgroup did not show statistically significant associations within the cognitively healthy individuals. Our study was able to demonstrate these associations in a larger sample of cognitively normal individuals at baseline, and the lack of interaction of amyloid status with these associations confirms the view that NFL is a non-specific biomarker of neurodegeneration. In contrast, there was no association between plasma NFL and WMHV in ADNI, and we replicate these findings.

Taking a similar approach to that adopted by the DIAN study [233], we predict that longitudinal assessment of serum NFL, in parallel with longitudinal brain imaging in Insight 46 phase 2, will reveal that rate of change of serum NFL will be a stronger predictor for atrophy than baseline serum NFL values. It will also be interesting to apply voxel-based morphometry, and/or regional cortical thickness estimations as done by Pereira *et al.* in ADNI [237], to derive an atrophy pattern that is particularly associated with serum NFL increase, as this regional information may give clues as to whether specific areas of the brain are generating the NFL signal seen in blood.

6.4.4 Plasma amyloid-β peptides and brain imaging

Reports of associations of plasma amyloid-β peptides with structural brain imaging measures have been inconsistent in the literature, and this is likely to at

least in part reflect technical differences in assays used. In ADNI, Toledo *et al.* utilised the INNO-BIA assay on the Luminex platform and did not demonstrate any association between either plasma Aβ42, Aβ40 or Aβ42/40 and white matter hyperintensity volume [328]. However, using the Simoa platform, Janelidze *et a*l. demonstrated associations between higher white matter lesion load, several other indicators of cardiovascular disease and higher values of both plasma Aβ42 and Aβ40 [202] but not CSF values of these peptides, which implied that the white matter lesion load associations with plasma Aβ42 and Aβ40 were unlikely to be influenced by cerebral amyloid deposition. Our study did confirm that cerebral PET-amyloid status did not itself associate with WMHV but we did not replicate the plasma findings of Janelidze *et al*., using either the Simoa platform or LC-MS. One possible reason for this is that Insight 46 participants had relatively good cardiovascular health (as compared to the whole NSHD birth cohort [274], so the WMHV variation across our cohort was narrow and the overall WMH burden was low.

In this study, a novel finding was of a cross-sectional association between increased ventricular volume (a global measure of atrophy) and both plasma Aβ1-42 (confirmed by two assay methods) and Aβ1-42/1-40 ratio as measured by the LC-MS method. This is intriguing as it suggests that these plasma values may be a proxy for a process that is associated with non-cerebral-amyloid-related neurodegeneration, as the association persisted despite adjustment for amyloid status. Longitudinal plasma amyloid and MRI data from Phase 2 of Insight 46 will be able to test this prediction.

7 Cross-sectional associations between blood biomarkers and cognition in Insight 46

7.1 Introduction

Insight 46 participants had detailed neuropsychological assessments as previously described in section 2.1.2.2.2 (page 156). Cross-sectional associations between blood biomarkers and cognition were probed in cognitively normal individuals from this cohort to test the following hypotheses:

- Higher plasma t-tau and serum NFL would associate with lower performance in the Preclinical Alzheimer's Cognitive Composite (PACC) and its constituents.
- Plasma amyloid markers would not associate cross-sectionally with cognitive scores, excepting potentially the 12-item Face-Name Associative Memory Examination (FNAME-12), as worse performance in the naming recall element of FNAME has been shown previously to associate cross-sectionally with higher cerebral amyloid deposition measured by PiB-PET [329].
- No blood biomarker would associate with changes in MMSE, as this test is largely at ceiling in the group of participants defined as "Cognitively Normal".

7.2 Methods

7.2.1 Outcome variable derivation

The list of neuropsychological tests applied in Insight 46 is given in Chapter 2 (section 2.1.2.2.2, page 156) and details of the tests are in the protocol publication [272]. The analyses of blood biomarker (BB) associations with cognition utilised only the previously validated test components of the battery.

They included Mini-Mental State Examination (MMSE), Delayed Logical Memory (LMD), Digit-Symbol Substitution (DSS), 12-item Face-Name Associative Memory Examination (FNAME-12) and Matrix Reasoning (MaR). A Preclinical Alzheimer's Cognitive Composite (PACC) was also generated by averaging the z-scores for MMSE, LMD, DSS and FNAME-12. This approach is similar to that detailed in publications describing longitudinal changes in PACC in preclinical Alzheimer's disease in the ADNI and AIBL studies [330] and in HABS [331], except for our substitution of the FNAME-12 for the Free and Selected Cued Reminding Test (FSCRT) that usually forms the fourth component of the PACC. This substitution was made so as to minimise overlap with another word-learning test that had been administered to the NSHD cohort at multiple time-points throughout adulthood [282]. FNAME-12, like FCSRT, assesses immediate and delayed episodic memory and is a challenging test for cognitively normal people.

For analyses of BB associations with individual tests, apart from MMSE, MaR and PACC, raw scores for all cognitive tests were standardised to z-scores (PACC is an average of z- scores; MMSE and MaR modelling employed a different approach to account for skew – see section 7.2.4, page 264).

7.2.2 Covariate derivation

The following life course data were obtained from the MRC Lifelong Health and Aging database for the NSHD and re-categorised as follows:

- Childhood cognition: Cognition had been assessed at age 8, 11 and 15 using four tests of verbal and non-verbal ability [332]. The sum of scores from the four tests at each age was standardised to a z-score relative to

the whole NSHD cohort. For these analyses, the value used for childhood cognition was the earliest available score.

- Educational attainment: The highest educational attainment or qualification obtained by age 26 had originally been classified by the Burnham scale [333]. For these analyses, education was further grouped into five categories: higher education (degree or equivalent), A-level or equivalent, O-level or equivalent, below O-level or vocational, and no qualification.
- Socioeconomic position: Adult socioeconomic position was derived from each participant's own occupation at age 53, or earlier if missing. For these analyses, it was binarized as manual or non-manual.

7.2.3 Inclusion and exclusion criteria

For all cognitive analyses, individuals with complete blood biomarker, *APOE,* and cognitive data were included.

Analyses were first performed in the Cognitively Normal group, which excluded those with prior neurological diagnoses and those fulfilling study criteria for MCI or dementia (see Box 2.1, page 155). Associations were then examined in the Cognitively Normal individuals who had known PET-amyloid status. Figure 7.1 (page 264) gives the details of numbers in these two groups.

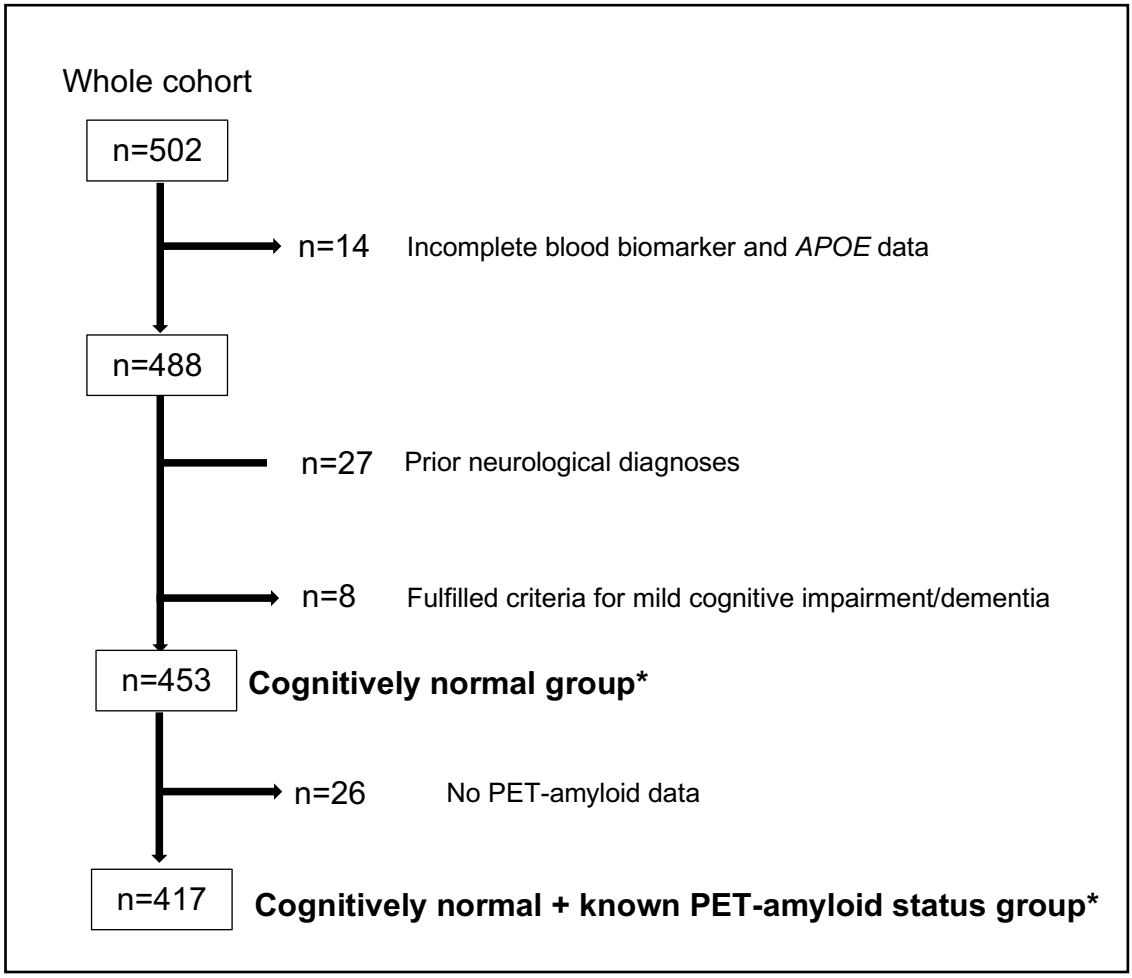


Figure 7.1: Inclusions and exclusions for analyses of associations between blood biomarkers and cognitive variables in phase 1 of Insight 46.

*For the FNAME-12 task, the numbers in these two groups were 452 and 416 respectively. For the single individual included in these analyses who did not complete FNAME-12, the PACC score was generated by averaging the z-scores of MMSE, LMD and DSS only.

7.2.4 Statistical analyses

7.2.4.1 Choice of covariates

Age at blood sampling (which was the same as age at neuropsychological assessment, because blood samples and neuropsychological data were acquired on the same day), sex, *APOE* ε4 carrier status, childhood cognitive ability, socioeconomic position and education were included as covariates in all models of cognitive outcomes. For modelling delayed logical memory, the time delay between immediate and delayed recall was included as an additional covariate. In analyses of the Cognitively normal individuals with known PET-amyloid status, amyloid status was included as an additional covariate.

Given the contributions of serum creatinine and BMI to blood biomarker variance (Chapter 5), the impact of introducing each or both of these variables into the base model without the other blood biomarkers was assessed. If there was a significant improvement in model fit (as evidenced by lowering of the Akaike Information Criterion by ≥ 10), and/or if there was a significant coefficient for creatinine or BMI, the adjusted coefficients derived from models incorporating blood biomarkers with and without creatinine or BMI were compared. Box 7.1 summarises the covariates included in the various models for each cognitive outcome.

Cognitively normal group

Model 1: Cognitive outcome ~ age + sex + *APOE* ε4 carrier status + childhood cognitive ability + socioeconomic position + educational attainment (+ time delay if appropriate)

Model 2: Cognitive outcome ~ (covariates of model 1) + serum creatinine

Model 3: Cognitive outcome ~ (covariates of model 1) + serum creatinine + BMI

Cognitively normal group with known PET-amyloid status

Model 4: Cognitive outcome ~ age + sex + *APOE* ε4 carrier status + amyloid status + childhood cognitive ability + socioeconomic position + educational attainment (+ time delay if appropriate)

Model 5: Cognitive outcome ~ (covariates of model 4) + serum creatinine

Model 6: Cognitive outcome ~ (covariates of model 5) + serum creatinine + BMI

Box 7.1: Covariates for statistical models of cognitive outcomes.

7.2.4.2 Choice of models: z-score based cognitive variables

Linear regressions of all cognitive variables except MMSE and MaR were undertaken against natural log-transformed BB variables, adjusting for covariates as described in section 7.2.4.1 above. For a 10% BB rise, the associated z-score-change (Δz) was given by the equation:

$$\Delta z = (\text{Coefficient}) * \ln(1.1)$$

Model assumptions for linear regressions were tested using plots of standardised residuals against the fitted values. Potentially influential outliers were considered by deriving Cook's distance for every individual for each model and re-running the models only in those individuals in whom the Cook's distance was less than 4/n.

7.2.4.3 Choice of models: raw score based cognitive variables

MMSE and MaR scores showed negative skew. Therefore, analyses of these variables against natural log-transformed BB variables, adjusting for the same covariates as in section 7.2.4.1 (page 264), employed generalised linear models with a binomial distribution, clustering by individual, with logit link. This approach converted raw scores to proportions out of the maximum possible scores, which were 30 for MMSE and 32 for MaR. Coefficients from these models were expressed as

$$OR = \left(\frac{p(\,score\ for\ specified\ BB\ value)}{1 - \ p\ (score\ for\ specified\ BB\ value}\right)$$

where OR is the odds ratio, and p(score) is the probability of a specific score at a specified value of the BB. This could then then be used to calculate the predicted raw MMSE or MaR score for specific values of the BB over the range present in the dataset. Where the odds ratios were significantly different from 1, the predicted scores were plotted over the BB range with 95% confidence intervals using a margins plot.

Model assumptions for these generalised linear models were tested using plots of Anscombe residuals against fitted values.

7.3 **Results**

7.3.1 Significance of serum creatinine and BMI as covariates

Appendix Table 12.7 (page 334) shows the “base models” of model type 3 before adding BB, in the Cognitively Normal group. Higher serum creatinine showed a significant association with lower MMSE, lower PACC, lower delayed Logical Memory (LMD) and lower 12-item Face-Name Association examination score (FNAME-12) but had no significant associations with MaR or Digit Symbol Substitution (DSS). Higher BMI showed a significant association with higher LMD but did not significantly improve model fit. Hence only the impact of serum creatinine on coefficients for BB was further assessed.

7.3.2 MMSE

No blood biomarker had a statistically significant association with MMSE across the two levels of analysis (Table 7.1, page 268).

Table 7.1: Associations between blood biomarkers and MMSE.
Generalised linear models, with a binomial distribution clustering by individual, were used. Coefficients were transformed and presented as odds ratio of MMSE score for 1 log unit increase in the respective blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at the level of p=0.05.
Model 1: MMSE ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; NFL, Simoa neurofilament light chain; ORΔMMSE, odds of change in Mini-Mental Status Examination score; t-tau, Simoa total (mid-region) tau.

	Cognitively normal (n=453)			Cognitively normal with known PET-amyloid status (n=417)		
	Model 1			Model 4		
BB	ORΔMMSE (95%CI)	*p*	AIC	ORΔMMSE (95%CI)	*p*	AIC
None	-	-	972	-	-	898
Aβ42	0.969 (0.939, 1.001)	0.060	971	0.975 (0.944,1.006)	0.110	898
Aβ40	0.972 (0.913, 1.034)	0.370	973	0.966 (0.909, 1.027)	0.268	898
Aβ42/40 ratio	0.980 (0.951, 1.010)	0.195	973	0.987 (0.957, 1.018)	0.406	899
t-tau	0.990 (0.961, 1.021)	0.523	974	0.987 (0.957, 1.017)	0.388	899
NFL	0.989 (0.963, 1.016)	0.420	974	0.995 (0.967, 1.024)	0.723	899
	Model 2			Model 5		
None	-	-	969	-	-	894
Aβ42	0.978 (0.947, 1.010)	0.180	970	0.983 (0.952, 1.016)	0.310	896
Aβ40	1.000 (0.933, 1.073)	0.993	971	0.999 (0.931, 1.072)	0.976	896
Aβ42/40 ratio	0.980 (0.951, 1.009)	0.172	970	0.985 (0.956, 1.015)	0.337	896
t-tau	0.997 (0.967, 1.027)	0.840	971	0.993 (0.964, 1.024)	0.661	896
NFL	1.000 (0.971, 1.029)	0.982	971	1.008 (0.977, 1.039)	0.626	896

7.3.3 Matrix Reasoning

Table 7.2 (page 270) shows that higher plasma Simoa Aβ42/40 ratio was associated with higher Matrix Reasoning (MaR) score. Including serum creatinine (models 2 and 5) did not improve model fit results, as would be expected given the lack of a significant association between serum creatinine and MaR (see Appendix Table 12.7, page 334), but it did demonstrate that the likely origin of the association between MaR and plasma Aβ42/40 ratio was that there was also an association between higher MaR and higher Simoa plasma Aβ42. Model 4 showed that this was in the context of lower MaR in PET-amyloid positive compared to negative individuals (ln odds ratio = - 0.282; 95% CI: -0.482, -0.083; p = 0.006). The predicted scores from model 4 across the range of values of plasma Aβ42/40 ratio in the cognitively normal group are shown in Figure 7.2.

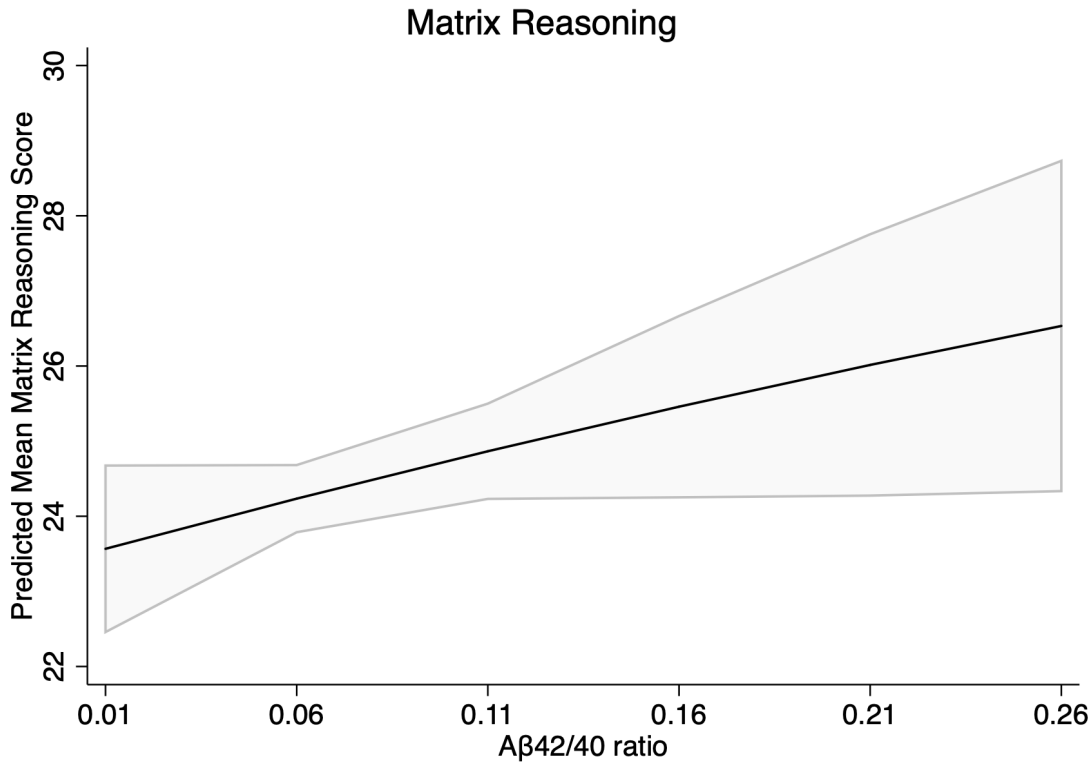


Figure 7.2: Model 4 prediction for Matrix Reasoning Scores over the range of plasma Aβ42/40 ratio values.
The black line shows predicted mean scores and the grey area indicates the 95% confidence interval.

Table 7.2: Associations between blood biomarkers and Matrix Reasoning score.
Generalised linear models were used, with a binomial distribution clustering by individual. Coefficients were transformed and presented as odds of change in MaR score for a 1 log unit increase in each blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at p=0.05.
Model 1: MaR ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; NFL, Simoa neurofilament light chain; ORΔMaR, odds ratio of change in Matrix reasoning score; t-tau, Simoa total (mid-region) tau.

	Cognitively normal (n=453)			Cognitively normal with known PET-amyloid status (n=417)		
	Model 1			Model 4		
BB	ORΔMaR (95%CI)	*p*	AIC	ORΔMaR (95%CI)	*p*	AIC
None	-	-	2884	-	-	2640
Aβ42	1.012 (0.995, 1.029)	0.177	2883	1.015 (0.997, 1.033)	0.103	2641
Aβ40	0.975 (0.943, 1.009)	0.146	2881	0.983 (0.947, 1.020)	0.356	2640
Aβ42/40 ratio	**1.020 (1.002, 1.039)**	**0.029**	2876	**1.020 (1.001, 1.039)**	**0.037**	2632
t-tau	0.994 (0.979, 1.011)	0.494	2885	0.996 (0.979, 1.012)	0.603	2641
NFL	0.992 (0.976, 1.007)	0.284	2883	0.994 (0.997, 1.011)	0.487	2640
	Model 2			Model 5		
None	-	-	2879	-	-	2637
Aβ42	1.016 (0.999, 3.370)	0.070	2874	**1.019 (1.001, 1.037)**	**0.043**	2631
Aβ40	0.984 (0.950, 1.019)	0.354	2878	0.991 (0.955, 1.029)	0.645	2638
Aβ42/40 ratio	**1.020 (1.002, 1.039)**	**0.029**	2870	**1.020 (1.001, 1.039)**	**0.039**	2629
t-tau	0.997 (0.981, 1.014)	0.764	2880	0.998 (0.981, 1.015)	0.823	2639
NFL	0.995 (0.980, 1.011)	0.519	2880	0.997 (0.981, 1.014)	0.725	2638

Notably, although the odds ratio (OR) for the association between Simoa Aβ42/40 ratio and MaR scores was similar to that for LC-MS Aβ1-42/1-40, the

latter did not achieve statistical significance (see Appendix Table 12.9, page 336).

7.3.4 Other cognitive variables

Table 7.3 (page 273) shows the associations between BB and PACC. Although model 1 showed an association of higher serum NFL and higher Simoa plasma Aβ40 with lower PACC, model 2 showed that these associations did not survive adjustment for serum creatinine, and model 2 provided an improved fit to the data than did model 1. After further adjustment for amyloid status (model 5) no significant associations between BB and PACC were found.

Table 7.4 (page 274) shows the associations between BB and DSS. All four models demonstrated that a 10% increase in Simoa plasma Aβ40 was associated with a DSS *z*-score drop of approximately 0.07. Models 2 and 5 did not fit the data significantly better than models 1 and 4 respectively, as was expected given that serum creatinine did not itself significantly associate with DSS (see Appendix Table 12.7, page 334).

Table 7.5 (page 275) shows the associations between BB and LMD. Although model 1 showed an association of higher Simoa plasma Aβ40 with lower LMD, and this survived adjustment for amyloid status (model 4), models 2 and 5 showed that this association did not survive adjustment for serum creatinine. Models 2 and 5 did not fit the data significantly better than models 1 and 4 respectively, despite the fact that higher serum creatinine was itself associated with lower LMD (see Appendix Table 12.7, page 334).

Table 7.6 shows the associations between BB and FNAME-12. Although Models 2 and 5 fit the data significantly better than models 1 and 4 respectively, all four models consistently demonstrated an association of higher plasma t-tau and higher serum NFL with lower FNAME-12. A 10% increase in plasma tau or in serum NFL was associated with a drop of 0.02-0.03 in FNAME-12 *z*-score. Sub-score analyses according to name recall and occupation recall demonstrated that these associations with plasma tau and serum NFL persisted for both these elements of the FNAME-12 (not shown).

A graphical summary of the Simoa BB coefficients from model 1 for each of these cognitive variables is shown in Figure 7.3 (page 277).

Notably, associations between Simoa plasma Aβ40 and PACC, DSS and LMD were not replicated by LC-MS plasma Aβ1-40 (see Appendix Table 12.8, page 335).

Table 7.3: Associations between blood biomarkers and PACC.
Linear regression coefficients were transformed and presented as z-score change (Δz) for a 10% rise in the respective blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at the level of p=0.05.
Model 1: PACC ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; NFL, Simoa neurofilament light chain; PACC, Preclinical Alzheimer's Cognitive Composite; t-tau, Simoa total (mid-region) tau, Δ*z,* z-score change

	PACC							
	Cognitively normal (n=453)				Cognitively normal with known PET-amyloid status (n=417)			
	Model 1				Model 4			
BB	Δ*z* (95% CI)	*p*	AIC	r^2	Δ*z* (95% CI)	*p*	AIC	r^2
None	-	-	747	0.333	-	-	682	0.334
Aβ42	-0.013 (-0.029, 0.003)	0.100	746	0.337	-0.015 (-0.032, 0.002)	0.091	681	0.339
Aβ40	**-0.032 (-0.060, -0.004)**	**0.026**	743	0.341	**-0.036 (-0.065, -0.007)**	**0.014**	678	0.344
Aβ42/40 ratio	-0.003 (-0.019, 0.013)	0.707	748	0.333	-0.003 (-0.020, 0.014)	0.709	685	0.344
t-tau	-0.011 (-0.023, 0.001)	0.065+	745	0.337	-0.012 (-0.024, 0.001)	0.068	681	0.339
NFL	**-0.016 (-0.030, -0.003)**	**0.018**	741	0.343	-0.012 (-0.026, 0.002)	0.091	680	0.340
	Model 2				Model 5			
None	-	-	733	0.354	-	-	671	0.356
Aβ42	-0.008 (-0.024, 0.008)	0.333	735	0.356	-0.101 (-0.027, 0.007)	0.268	671	0.358
Aβ40	-0.015 (-0.045, 0.014)	0.307	735	0.356	-0.020 (-0.051, 0.011)	0.213	671	0.358
Aβ42/40 ratio	-0.003 (-0.019, 0.012)	0.694	736	0.354	-0.004 (-0.020, 0.013)	0.656	673	0.356
t-tau	-0.006 (-0.019, 0.006)	0.311	735	0.356	-0.006 (-0.020, 0.006)	0.276	672	0.357
NFL	-0.011 (-0.025, 0.003)	0.119	732	0.359	-0.006 (-0.021, 0.008)	0.374	672	0.357

+ After excluding potentially influential outliers, this achieved statistical significance: Δ*z* = -0.117 (-0.229, - 0.006), *p* = 0.038 (n = 428).

Table 7.4: Associations between blood biomarkers and DSS.
Linear regression coefficients were transformed and presented as z-score change (Δ*z)* for a 10% rise in the respective blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at the level of p=0.05.
Model 1: DSS ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; DSS, Digit Symbol Substitution z-score; NFL, Simoa neurofilament light chain; t-tau, Simoa total (mid-region) tau, Δ*z,* z-score change

	DSS							
	Cognitively normal (n=453)				Cognitively normal with known PET-amyloid status (n=417)			
	Model 1				Model 4			
BB	Δ*z* (95% CI)	*p*	AIC	r^2	Δ*z* (95% CI)	*p*	AIC	r^2
None	-	-	1188	0.181	-	-	1098	0.178
Aβ42	-0.003 (-0.028, 0.023)	0.834	1190	0.181	-0.006 (-0.034, 0.022)	0.679	1099	0.179
Aβ40	**-0.060 (-0.102, -0.018)**	**0.005**	1183	0.194	**-0.073 (-0.117, -0.029)**	**0.001**	1091	0.197
Aβ42/40 ratio	0.016 (-0.009, 0.042)	0.203	1189	0.184	0.017 (-0.010, 0.044)	0.218	1098	0.182
t-tau	-0.011 (-0.031, 0.008)	0.183	1189	0.183	-0.012 (-0.033, 0.009)	0.264	1099	0.181
NFL	-0.007 (-0.030, 0.015)	0.524	1190	0.182	0.001 (-0.023, 0.025)	0.912	1100	0.178
	Model 2				Model 5			
None	-	-	1189	0.184	-	-	1099	0.181
Aβ42	0 (-0.026, 0.026)	0.977	1191	0.184	-0.003 (-0.031, 0.025)	0.822	1101	0.181
Aβ40	**-0.067 (-0.102, -0.012)**	**0.013**	1185	0.194	**-0.073 (-0.119, -0.027)**	**0.002**	1093	0.197
Aβ42/40 ratio	0.163 (-0.009, 0.042)	0.206	1189	0.187	0.017 (-0.010, 0.043)	0.227	1099	0.184
t-tau	-0.009 (-0.029, 0.012)	0.397	1190	0.185	-0.010 (-0.032, 0.012)	0.375	1100	0.182
NFL	-0.005 (-0.028, 0.019)	0.705	1190	0.185	0.005 (-0.019, 0.028)	0.700	1101	0.181

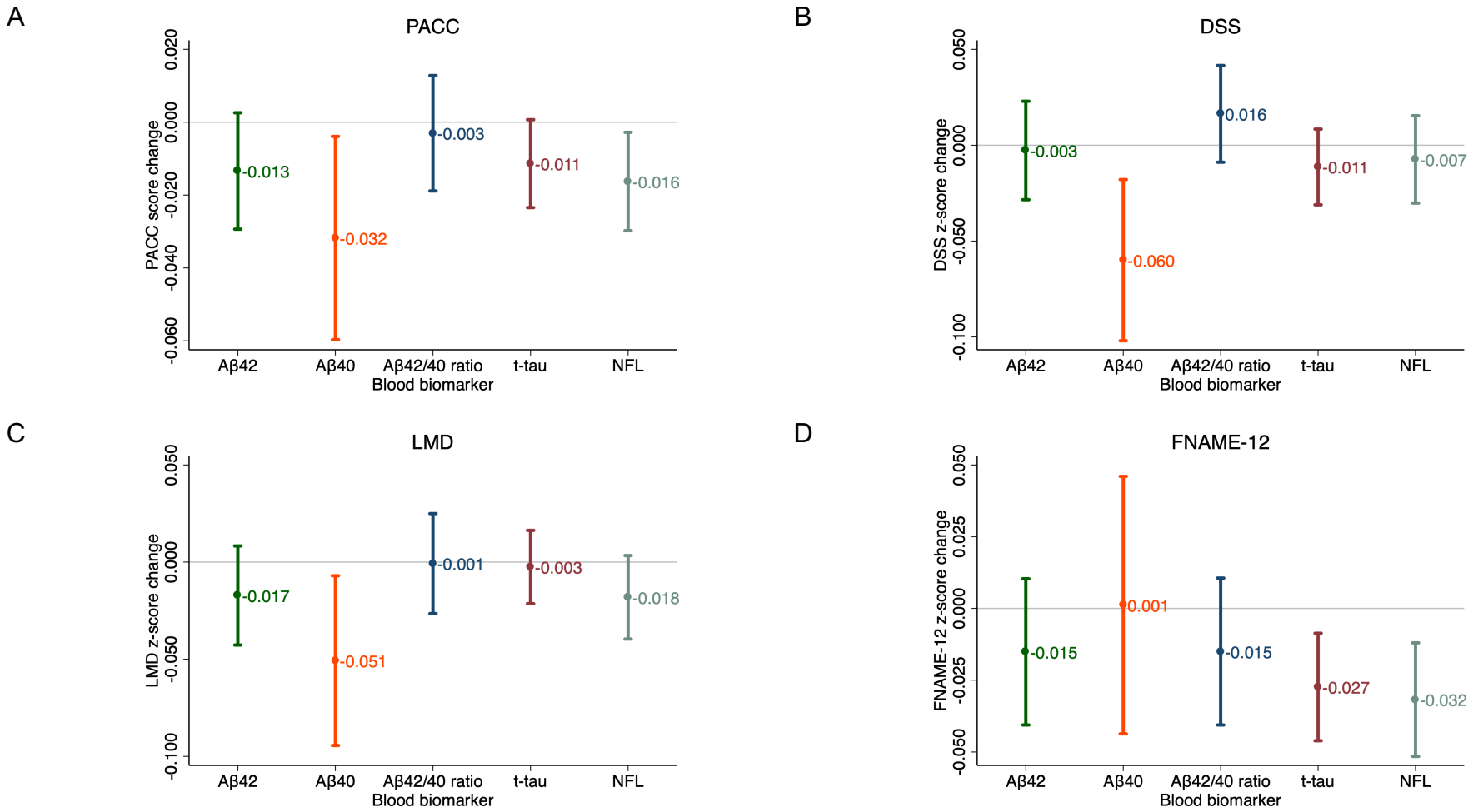
Table 7.5: Associations between blood biomarkers and LMD.
Linear regression coefficients were transformed and presented as z-score change (Δz) for a 10% rise in the respective blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at the level of p=0.05.
Model 1: LMD ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + time delay + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; LMD, Delayed Logical Memory z-score; NFL, Simoa neurofilament light chain; t-tau, Simoa total (mid-region) tau, Δ*z,* z-score change

	LMD							
	Cognitively normal (n=453)				Cognitively normal with known PET-amyloid status (n=417)			
	Model 1				Model 4			
BB	Δ*z* (95% CI)	*p*	AIC	r^2	Δ*z* (95% CI)	*p*	AIC	r^2
None	-	-	1191	0.156	-	-	1098	0.164
Aβ42	-0.017 (-0.043, 0.008)	0.185	1192	0.159	-0.019 (-0.046, 0.008)	0.166	1098	0.168
Aβ40	**-0.051 (-0.094, -0.007)**	**0.023**	1188	0.165	**-0.049 (-0.097, -0.002)**	**0.042**	1096	0.173
Aβ42/40 ratio	-0.001 (-0.027, 0.025)	0.952	1193	0.156	-0.003 (-0.030, 0.023)	0.810	1100	0.164
t-tau	-0.003 (-0.021, 0.016)	0.791	1193	0.156	0.001 (-0.019, 0.021)	0.898	1100	0.164
NFL	-0.018 (-0.040, 0.003)	0.098	1190	0.162	-0.015 (-0.037, 0.008)	0.200	1098	0.168
	Model 2				Model 5			
None	-	-	1186	0.169	-	-	1093	0.178
Aβ42	-0.011 (-0.038, 0.015)	0.410	1188	0.170	-0.013 (-0.041, 0.015)	0.354	1094	0.180
Aβ40	-0.034 (-0.081, 0.012)	0.146	1186	0.173	-0.031 (-0.082, 0.019)	0.226	1094	0.181
Aβ42/40 ratio	-0.001 (-0.027, 0.025)	0.945	1188	0.169	-0.004 (-0.031, 0.023)	0.774	1095	0.178
t-tau	0.004 (-0.016, 0.023)	0.716	1188	0.169	0.007 (-0.013, 0.028)	0.484	1095	0.179
NFL	-0.012 (-0.035, 0.011)	0.291	1187	0.171	-0.008 (-0.032, 0.016)	0.498	1095	0.179

Table 7.6: Associations between blood biomarkers and FNAME-12.
Linear regression coefficients were transformed and presented as z-score change (Δz) for a 10% rise in the respective blood biomarker. Robust standard errors were used to calculate the 95% confidence intervals of the coefficients (brackets). Values in bold are statistically significant at the level of p=0.05.
Model 1: FNAME-12 ~ age + sex + *APOE* ε4 status + childhood cognition + socioeconomic position + educational attainment + time delay + natural log-transformed blood biomarker
Model 2: As per model 1 + serum creatinine
Model 4: As per model 1 + amyloid status
Model 5: As per model 4 + serum creatinine
Aβ42, Simoa amyloid-β-42; Aβ40, Simoa amyloid-β-40; AIC, Akaike Information Criterion; BB, Blood biomarker; FNAME-12, 12-point Face-Name Associate Memory Examination z-score; NFL, Simoa neurofilament light chain; t-tau, Simoa total (mid-region) tau, Δ*z,* z-score change

	FNAME-12							
	Cognitively normal (n=452)				Cognitively normal with known PET-amyloid status (n=416)			
	Model 1				Model 4			
BB	Δ*z* (95% CI)	*p*	AIC	r^2	Δ*z* (95% CI)	*p*	AIC	r^2
None	-	-	1124	0.246	-	-	1032	0.241
Aβ42	-0.013 (-0.037, 0.011)	0.299	1126	0.248	-0.016 (-0.041, 0.010)	0.233	1033	0.244
Aβ40	0.004 (-0.039, 0.047)	0.849	1127	0.247	0.002 (-0.043, 0.047)	0.930	1034	0.241
Aβ42/40 ratio	-0.014 (-0.038, 0.010)	0.263	1125	0.249	-0.016 (-0.041, 0.010)	0.230	1033	0.244
t-tau	**-0.026 (-0.044, -0.007)**	**0.007**	1120	0.258	**-0.027 (-0.046, -0.008)**	**0.005**	1027	0.254
NFL	**-0.032 (-0.051, -0.013)**	**0.001**	1115	0.266	**-0.031 (-0.051, -0.012)**	**0.002**	1024	0.261
	Model 2				Model 5			
None	-	-	1024	0.256	-	-	1025	0.257
Aβ42	-0.009 (-0.033, 0.016)	0.492	1026	0.257	-0.009 (-0.034, 0.016)	0.472	1027	0.258
Aβ40	0.028 (-0.021, 0.077)	0.255	1025	0.259	0.029 (-0.020, 0.078)	0.241	1026	0.260
Aβ42/40 ratio	-0.016 (-0.041, 0.009)	0.212	1024	0.259	-0.016 (-0.041, 0.008)	0.194	1027	0.261
t-tau	**-0.022 (-0.041, -0.003)**	**0.021**	1021	0.265	**-0.022 (-0.041, -0.003)**	**0.023**	1023	0.265
NFL	**-0.026 (-0.047, -0.005)**	**0.014**	1019	0.269	**-0.026 (-0.046, -0.005)**	**0.015**	1021	0.270

Figure 7.3: Cognitive *z*-score changes associated with a 10% increase in each BB.
As per model 1; grey lines indicate 0. A: preclinical Alzheimer's cognitive composite (PACC), B: digit symbol substitution (DSS), C: delayed logical memory LMD), D: 12-item face-name associative memory examination (FNAME-12).



7.4 **Discussion**

7.4.1 Summary of results

These analyses addressed the hypotheses detailed in section 7.1 (page 261) as follows:

- No blood biomarker showed a statistically significant association with MMSE.
- Lower Simoa plasma Aβ42/40 ratio was associated with lower MaR; this survived adjustment for serum creatinine and PET-amyloid status.
- Higher Simoa plasma Aβ40, t-tau and serum NFL were associated with lower PACC but these associations were significantly attenuated by adjustment for serum creatinine.
- Higher plasma t-tau and serum NFL were associated with lower FNAME-12, but higher Simoa plasma Aβ40 was associated with lower DSS. All these associations survived adjustment for serum creatinine and PET-amyloid status.
- Higher Simoa plasma Aβ40 was associated with lower LMD but this association was significantly attenuated by adjustment for serum creatinine.
- None of the associations detailed above between Simoa measures of Aβ40 or Aβ42/40 ratio and cognitive variables were replicated when measured by LC-MS.

7.4.2 Blood biomarkers and PACC or its constituents

Few studies have analysed cognitive performance in relation to blood biomarkers of amyloid, tau and neurofilament light chain in cognitively normal individuals including those at risk of sporadic Alzheimer's disease; fewer still

have incorporated cognitive tests aimed at detecting subtle preclinical changes. For example, although Mattsson *et al*. reported on cross-sectional associations between higher Simoa plasma t-tau and worse Alzheimer's Disease Assessment Scale–Cognitive subscale (ADAS-cog) performance in ADNI and BIOFINDER, analysis within the cognitively normal subgroups did not show significant associations [220]. Similarly, in ADNI, plasma NFL had no significant association with either cross-sectional or longitudinal cognitive measures including ADAS-cog, LMD, DSS and MMSE in cognitively normal individuals, even though such associations were present in the MCI and AD groups [234]. Dage *et al.* assessed a mixed group from the MCSA (cognitively normal and MCI) and showed a cross-sectional association between increased Simoa plasma t-tau and reduced memory *z*-score (comprising combined elements including LMD and other verbal and visual recall tests) [221] but did not present analyses restricted to the cognitively normal sub-group. The larger number of cognitively normal individuals included in our study may have enabled us to detect cross-sectional associations between plasma t-tau and serum NFL, and PACC and FNAME-12, that did not achieve statistical significance in the aforementioned studies. Our cross-sectional findings confirm that longitudinal assessment of these biomarkers will be worthwhile in relation to phase 2 of Insight 46 and if serum NFL rate of change were to associate with longitudinal changes in PACC or its constituents, this would echo reports from the domain of familial AD, in which associations between both baseline levels and rates of change of serum NFL have been found with MMSE and logical memory [233, 247].

FNAME-12 was included in the cognitive testing battery for Insight 46 as a constituent of the PACC but also because of a previous report that FNAME

performance inversely correlates with cerebral PET-amyloid in cognitively normal individuals [329]. However, in our analyses, neither PET-amyloid status nor plasma Aβ42/40 ratio associated significantly with FNAME-12 scores. Instead, associations were found between both higher plasma t-tau and serum NFL and lower FNAME-12 scores, which are consistent with the aforementioned results of Dage *et al*. for plasma t-tau, albeit using a different delayed recall test.

To our knowledge this study is the first to describe an association between higher Simoa plasma Aβ40 and lower PACC, DSS and LMD scores in cognitively normal individuals. Given that the relationship with DSS survived adjustment for creatinine, it is unlikely that the association is related to plasma Aβ40 acting merely as a surrogate marker of renal function. DSS is a measure of executive function and speed, which classically are attributable to frontal and subcortical networks that might differentially be affected by cerebrovascular disease. There are conflicting reports of plasma Aβ40 being a marker of cerebrovascular disease, which may at least in part be due to differing methods of measurement. In ADNI, Toledo *et al.* utilised the INNO-BIA assay on the Luminex platform and did not demonstrate any association between either plasma Aβ42, Aβ40 or Aβ42/40 and cross-sectional cognitive scores but showed an association between *low* baseline plasma Aβ40 and longitudinal cognitive decline in a composite score comprising (among other tests) ADAS-cog, MMSE and DSS. In BIOFINDER (a mixed group including cognitively normal, subjective cognitive decline, MCI and AD), Janelidze *et al.* used Simoa assays and did not show any cross-sectional association between the three plasma amyloid tests and MMSE or ADAS-cog in any diagnostic group. Our

own data using the LC-MS assay values for the amyloid peptides did not show any significant associations. Our findings regarding Simoa plasma Aβ40 should therefore be interpreted with caution and will be informed by longitudinal cognitive assessment in our cohort as well as external replication.

7.4.3 Blood biomarkers and Matrix Reasoning

Of all the cognitive variables examined here, only MaR showed a significant relationship with Simoa plasma Aβ42/40. This is particularly interesting because MaR was also the test showing the clearest difference between PET-amyloid positive and negative individuals. As a measure of non-verbal reasoning ability, MaR may depend particularly upon function of parietal and posterior association areas. As these are regions in which amyloid-β deposition is known to occur early in the disease trajectory, it is intriguing to speculate that MaR may provide evidence of a deleterious effect of amyloid-β deposition. A widely accepted view of the relative contributions of brain pathologies in symptomatic AD is that tau more than amyloid-β correlates with cognitive deficits as evidenced by *post-mortem* neuropathological studies [334] and CSF biomarker assessment [335] but there is some evidence that subtle deficits in the visuospatial domain more closely associate with amyloid-β rather than tau deposition patterns as assessed cross-sectionally by PET [336] and that longitudinal cognitive profiles in cognitively normal individuals can be predicted more by amyloid PET than by CSF tau measures [337]. These seemingly contradictory cognitive correlations of the more “established” biomarkers of AD may arise from the fact that CSF and PET measures of a given molecular hallmark of AD reflect different cellular processes, but may also result from the heterogeneity of the cognitive testing across all the aforementioned studies, which are generally weighted toward

assessing verbal and memory functions rather than visuospatial ability. It will therefore be important, when considering more exploratory biomarkers such as Simoa plasma Aβ42/40, to have independent replication of our findings regarding MaR, in diverse cohorts. Longitudinal assessment utilising Insight 46 phase 2 data will also allow us to test the prediction that individuals showing the greatest increment in PET-amyloid-β deposition between phases 1 and 2 will have the greatest drop in Simoa plasma Aβ42/40 and in MaR.

8 N-terminal tau in CSF and plasma from two independent cohorts as a biomarker for Alzheimer's disease

8.1 Introduction

8.1.1 Publication statement

The work included in this chapter has been published previously [326] and is included here as per the publisher's policy with regards to thesis publications.

8.1.2 Background

Tau assays typically use mid-region-directed monoclonal antibodies and often are referred to as "total tau" assays. However, such assays (including the widely used INNOTEST® ELISAs and the Simoa tau 2.0 assay) cannot detect fragments of tau that lack all or part of the mid-region domain. This is important given that the primary structure of extracellular tau is heterogeneous and that such assays do not show a good correlation between CSF and plasma measurements of tau [338].

In this study, we sought to answer the following questions:

- Is there a tau fragment population that correlates well between CSF and plasma?
- Does/do the assay(s) for this tau fragment population provide good separation of AD and healthy controls in plasma?

8.2 **Methods**

8.2.1 Specimens

Initial experiments developing the assays used CSF and plasma specimens from healthy volunteers and from discarded clinic samples at Harvard (IRB approval Walsh-2016P000291/BWH).

After the assays had been developed, samples from the UCL DRC and HABS cohorts were used to form the "Discovery Cohort" and samples from UCSD formed the "Validation Cohort". Details of both cohorts are given in section 2.1.3, page 163).

8.2.2 CSF and clinical case definition

For the HABS and UCSD samples, all CSF was first characterised at UCL by the author, using INNOTEST® Aβ42 and t-tau ELISAs (as described in section 2.2.1, page 165) using the same batch of reagents in each case for all samples. The UCL DRC samples had previously been characterised by testing by the Neuroimmunology Clinical Laboratory at the National Hospital for Neurology and Neurosurgery, using the same assays according to the manufacturer's instructions.

AD-dementia, AD-MCI and control (NC) diagnoses were defined by combined CSF and clinical criteria as shown in Box 8.1 (page 285). The CSF cut-points were chosen according to a prior study by Weston *et al*. examining CSF concordance with amyloid PET [339].

AD-dementia:
MMSE 15-24, CSF tau/Aβ1–42 ratio > 0.88 and CSF Aβ1–42 ≤ 630 pg/ml

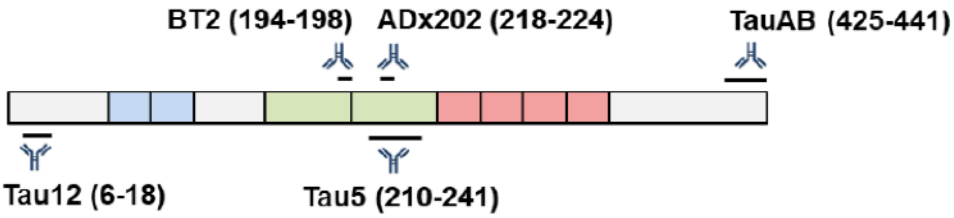
AD-MCI:
MMSE 25-29, CSF tau/Aβ1–42 ratio > 0.88 and CSF Aβ1–42 ≤ 630 pg/ml

NC:
MMSE 28-30, CSF tau/Aβ1–42 ratio < 0.5 and CSF Aβ1–42 > 630 pg/ml

Box 8.1: Combined clinical and CSF case definitions for both the discovery and validation cohorts.
MMSE, mini mental state examination score; NC, control

8.2.3 Tau assay development

Four assays were applied by Dr Zhicheng Chen to the Discovery Cohort CSF: a mid-region assay as previously published by Professor Walsh and colleagues [119] and three novel assays (directed against NT1, NT2 and FL tau) which were developed by Dr Chen. Figure 8.1 gives the target sequences of the antibody pairs used for each assay and Table 8.1 gives the sources of the antibodies and concentrations used (page 286). Consumables and reagents other than the antibodies and standards were obtained from Quanterix Corporation (Lexington, MA). The NT1 and NT2 assays were first developed as ELISAs for CSF and then migrated to the Simoa platform for testing in plasma. Initial attempts at developing the FL assay as an ELISA were unsuccessful, due to the inability of the assay to detect the low concentrations of FL tau in CSF, so the FL assay was developed directly on the Simoa platform for testing in CSF and plasma.



Assay	Capture antibody	Biotinylated detector antibody	ELISA LLoQ (pg/ml)	Simoa LLoQ (pg/ml)
Mid-region	BT2	Tau5	51.6-31.3	-
NT1	Tau12	BT2	15.6-62.5	0.2-0.7
NT2	Tau12	ADx202	15.6-31.3	0.2-0.7
FL	Tau12	TauAB	15.6-31	0.2-0.7

Figure 8.1: Details of the antibody pairs used in the four tau assays.
The lower limits of quantification (LLoQ) of the assays on the ELISA and Simoa platforms are indicated.

Table 8.1: Antibodies, sources and concentrations used for the four tau assays.

Antibody	Tau Epitope	Source	Concentration for ELISA (µg/ml)	Concentration for Simoa assay (µg/ml)
Tau12	6-18	EMD Millipore	2.5 (capture)	0.6 (detection)
BT2	194-198	Thermo Scientific	2.5 (capture) 1.7 (detection)	2000 (conjugation to beads)
Tau5	210-241	Biolegend	1.7 (detection)	-
ADx202	218-224	ADx	1.7 (detection)	2000 (conjugation to beads
TauAB	425-441	MedImmune	1.7 (detection)	1000 (conjugation to beads)

All assays had been validated by Dr Zhicheng Chen against three recombinant tau constructs prepared in Professor Walsh's laboratory – NT (amino acids 2-230, lacking 44-103), CT (231-441) and FL (1-441). Validation procedures included dilution linearity and spike recovery.

8.2.4 Assay procedures in the participant cohorts

8.2.4.1 Standards

When testing the participant samples of the Discovery Cohort, in order to be able to compare the recovered concentrations from each assay, the same recombinant human tau 381 standard (Sigma Aldrich, St Louis, MO) was used for the mid-region, NT1 and NT2 assays. The FL (tau441) standard was used for the FL assay.

In the Validation cohort, only the NT1 assay was tested in CSF and plasma, and the tau381 standard was used.

8.2.4.2 ELISAs

The mid-region, NT1 and NT2 ELISAs were performed in the Discovery Cohort by Dr Zhicheng Chen, following the procedures previously published [119], with modifications relevant to the antibody pairs used in each of the four ELISAs as detailed in Figure 8.1 and Table 8.1 (page 286). Samples were assayed after two freeze-thaw cycles.

8.2.4.3 Simoa assays

The FL, NT1 and NT2 Simoa assays were performed in the Discovery and Validation cohorts by Dr Zhicheng Chen and Dr David Mengel. Capture antibodies were first conjugated to paramagnetic beads as detailed in Table 8.1. Plasma and CSF specimens were thawed to room temperature and centrifuged at 14 000 g for 4 minutes, then diluted 1:4 in Tau 2.0 sample diluent. Tau381 standard was diluted linearly with Tau 2.0 sample diluent to yield a calibration concentration range spanning 0.02 to 540 pg/ml. Samples,

standards and blanks were presented in 1.5 ml low binding Eppendorf tubes and were analysed in triplicate. For the Discovery Cohort, samples were assayed after two freeze-thaw cycles by Dr Chen. For the Validation Cohort, samples were assayed after one freeze thaw cycle, separately by Dr Chen and Dr Mengel, and samples were included in the analysis if they had a CV of <20% across the two runs.

The assays utilised a “three-step” protocol at room temperature on a Simoa HD-1 analyser (Quanterix Corporation, Lexington, MA). Assay step details are given in Table 8.2.

Table 8.2: Simoa assay steps for the NT1, NT2 and FL assays.

Step	
1	100 µL of standard/blank/1:4 diluted sample added to capture beads and mixed for 30 minutes
Wash	Beads harvested and washed with Simoa wash buffer
2	Biotinylated detection antibody (0.6 µg/mL) added and incubated for 10.5 minutes
Wash	Simoa wash buffer – beads washed three times
3	150 pM streptavidin-β-galactosidase added
Wash	Simoa wash buffer
Read	Resorufin β-D-galactopyranoside substrate added, beads resuspended and loaded on to Simoa disc arrays; concentrations calculated from 5 point logistic regression standard curves

8.2.5 Statistical analysis

8.2.5.1 Attribution and approach

For the original publication [326], statistical analyses were undertaken by Dr David Mengel using SPSS statistics version 24.0 (IBM, Armonk, NY, USA). However, the author repeated the analyses with Stata version 14.2 using the original data as listed in the supplementary information of the publication, for

presentation in this chapter. The analytical approach was similar to that in the publication, except for the author further adding aspects to the CSF vs plasma correlations and the receiver operating characteristics analyses as detailed below, as well as performing a power calculation based on the discovery cohort data, to ascertain the number required in the validation cohort to obtain an equivalent difference at 80% power and a significance level of $p = 0.05$.

8.2.5.2 Analyses similar to those in the publication

Differences in CSF and blood biomarker values between diagnostic groups were examined using Kruskal-Wallis H test with *post hoc* Dunn's test. Bonferroni-corrected 2-tailed $p < 0.05$ was deemed statistically significant.

In each cohort, receiver operating characteristics (ROC) analysis was undertaken for the plasma assays using logistic regression for three binary outcomes: NC vs all AD, NC vs AD-MCI and NC vs AD-dementia.

Cut point transfer was examined by using a Youden's index cut-point (the point at which the sum of sensitivity and specificity was maximised) from the discovery cohort and applying this to the validation cohort to ascertain the resulting sensitivity and specificity.

8.2.5.3 Analyses added by the author

For Simoa assays performed in both CSF and plasma (NT1 and FL), correlation between values obtained across the two biofluids was ascertained by Pearson's correlation coefficient and the slope of the line of best fit, which was deemed to be statistically significant if its 95% confidence interval did not overlap zero.

Comparative ROC analyses were undertaken in the discovery cohort using plasma NT1, NT2 and FL as predictors of the NC vs all-AD outcome. In the validation cohort, additional data on APOE ε4 genotype were available, and ε4 heterozygotes and homozygotes were both classified as carriers. Further ROC analysis was undertaken incorporating age, sex, *APOE* ε4 carrier status and plasma NT1 into a logistic regression model, and comparing the result to a base model incorporating these predictor variables without plasma NT1. Areas under the curves (AUC) were compared using χ^2 tests.

Retrospective minimum sample number calculations for the Validation Cohort, based on the plasma NT1 level difference seen in the discovery cohort between all NC and all AD, are presented in section 8.3.3.1 (page 299), first assuming similar mean values, standard deviations and differences between groups as the Discovery Cohort, and secondly assuming lower mean values in the AD groups in the Validation Cohort than in the Discovery Cohort, in proportion to the CSF t-tau differences.

8.3 **Results**

8.3.1 CSF characterisation of samples

Demographic characteristics and INNOTEST® CSF ELISA values are shown for the Discovery and Validation cohorts in Table 8.3. Within each cohort the diagnostic groups were well-matched by age and sex. Participants in the Validation cohort were on average 10 years older than those in the Discovery cohort. For those with AD-MCI and AD-dementia, the INNOEST CSF t-tau values were also lower in the Validation than in the Discovery cohort. As expected, the percentage of APOE ε4 carriers (*i.e.* those who carried one or two alleles) was higher in the AD-dementia and AD-MCI groups compared to the NC group in the validation cohort.

Table 8.3: Characteristics of samples in the Discovery and Validation cohorts.

	N (%) or mean ± SD						
	Discovery cohort (HABS + UCL), n = 65				**Validation cohort (UCSD), n = 86**		
	NC (HABS) n = 10	NC (UCL) n = 9	AD-MCI n = 21	AD-dementia n = 25	NC n = 41	AD-MCI n = 22	AD-dementia n = 23
Sex (female)	7 (70.0)	7 (77.8)	16 (76.2)	13 (52.0)	27 (56.3)	10 (45.5)	12 (52.2)
Age (years)	69.8 ± 9.8	59.8 ± 6.5	65.3 ± 6.7	61.0 ± 6.3	71.8 ± 6.0	73.2 ± 8.1	72.4 ± 8.3
MMSE	N/A	29.4 ± 0.5	26.1 ± 1.2	20.0 ± 2.9	29.3 ± 0.8	26.9 ± 1.4	19.8 ± 2.4
CSF Aβ1-42 (pg/ml)	972.7 ±199.7	950.4 ± 212.0	405.2 ± 83.7	384.0 ± 130.3	947.0 ± 193.3	497.3 ± 72.0	433.3 ± 84.7
CSF t-tau (pg/ml)	236.1 ± 72.2	282.1 ± 78.5	861.3 ± 373.3	921.2 ± 480.4	245.6 ± 104.4	687.3 ± 214.7	641.8 ± 222.3
APOE ε4 carrier (1 or 2 alleles)*	N/A	N/A	N/A	N/A	9/39 (23.1)	16/22 (72.7)	19/22 (86.4)

**APOE* ε4 carrier status was available for 83 of 86 individuals in the validation cohort so carrier percentages are indicated in each column relative to the number available.

8.3.2 Discovery cohort: DRC and HABS samples

8.3.2.1 CSF

The mid-region, NT1 and NT2 ELISAs (Figure 8.2, page 293) and NT1 Simoa assay (Figure 8.3A, page 294) detected higher levels of tau in AD-dementia and AD-MCI compared to NC ($p < 0.001$ on Kruskal-Wallis H test with *post hoc* Dunn's test). However, the FL Simoa assay did not distinguish between diagnostic groups as clearly, primarily because of differences within the control groups (Figure 8.3B, page 294).

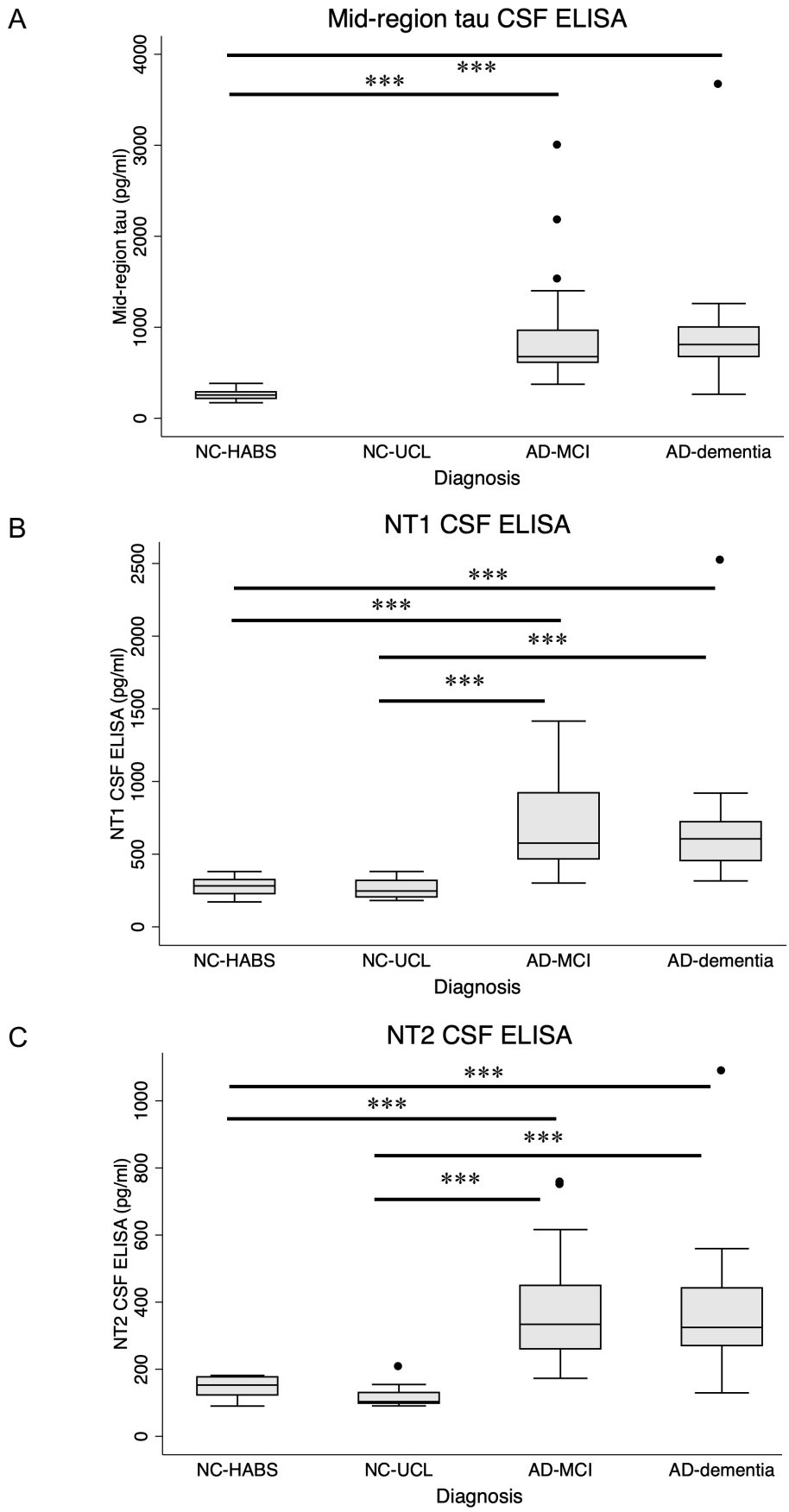


Figure 8.2: Discovery cohort CSF ELISA results (n = 65).
Boxes show medians and interquartile ranges; whiskers show 95% ranges. Mid-region tau was not quantified in the NC-UCL samples. A: Mid-region tau, B: NT1, C: NT2
*** Bonferroni-corrected $p < 0.001$ on Kruskal-Wallis H test with Dunn's *post hoc* test).

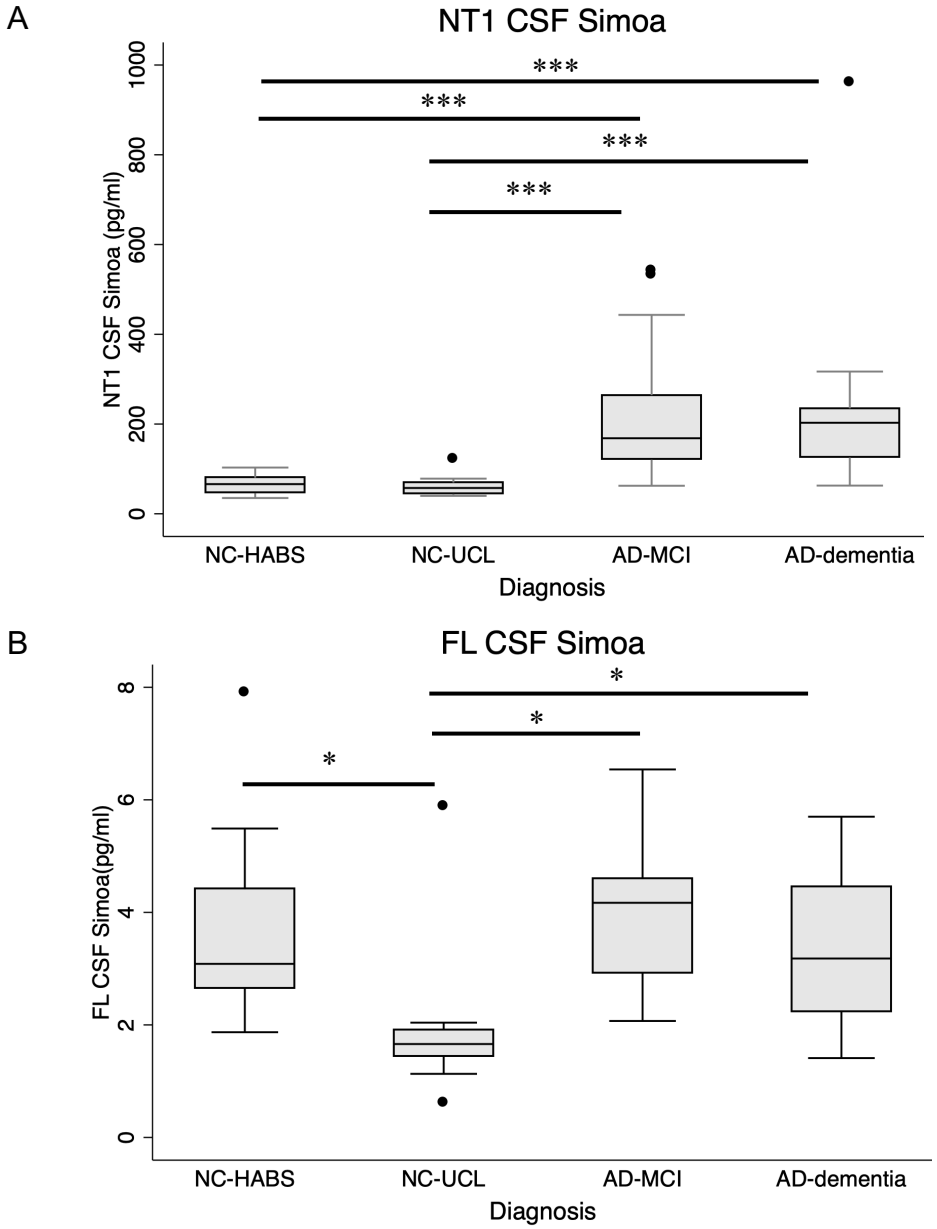


Figure 8.3: Discovery cohort CSF Simoa results (n = 65).
Boxes show medians and interquartile ranges; whiskers show 95% ranges. A: NT1, B: FL
Kruskal-Wallis test with Dunn's *post hoc* test: * Bonferroni-corrected p <0.05; *** p < 0.001

8.3.2.2 Plasma

The NT1 assay gave good separation between NC and AD groups ($p < 0.001$ for the NC vs AD-MCI and NC vs AD-dementia comparisons, Kruskal-Wallis H test with *post hoc* Dunn's test) but the NT2 and FL assays did not (Figure 8.4, page 295).

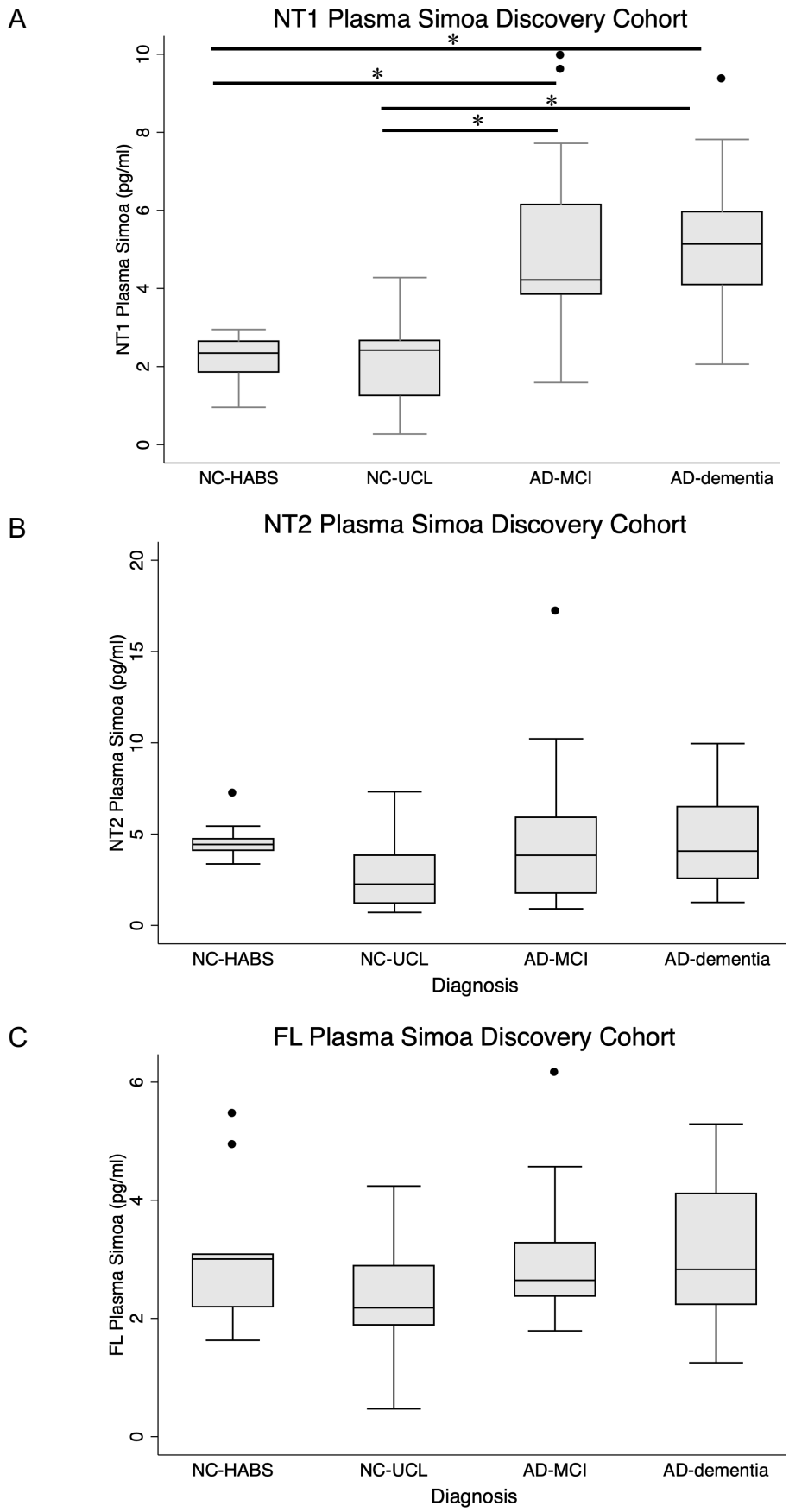


Figure 8.4: Discovery cohort plasma Simoa results (n = 65).
Boxes show medians and interquartile ranges; whiskers show 95% ranges. Mid-region tau was not quantified in the NC-UCL samples. A: NT1 B: NT2, C: FL
* Bonferroni-corrected $p < 0.05$ on Kruskal-Wallis H test with Dunn's *post hoc* test).

8.3.2.3 Simoa assay comparisons of CSF and plasma

As shown in Figure 8.5 below, there was a modest correlation between values obtained from plasma and CSF using the NT1 assay (r = 0.371, p = 0.002) but no significant correlation between values obtained using the FL assay (r = 0.215, p = 0.089). NT1 values obtained in plasma were two orders of magnitude lower than those obtained in CSF, whereas values were similar for FL in both plasma and CSF.

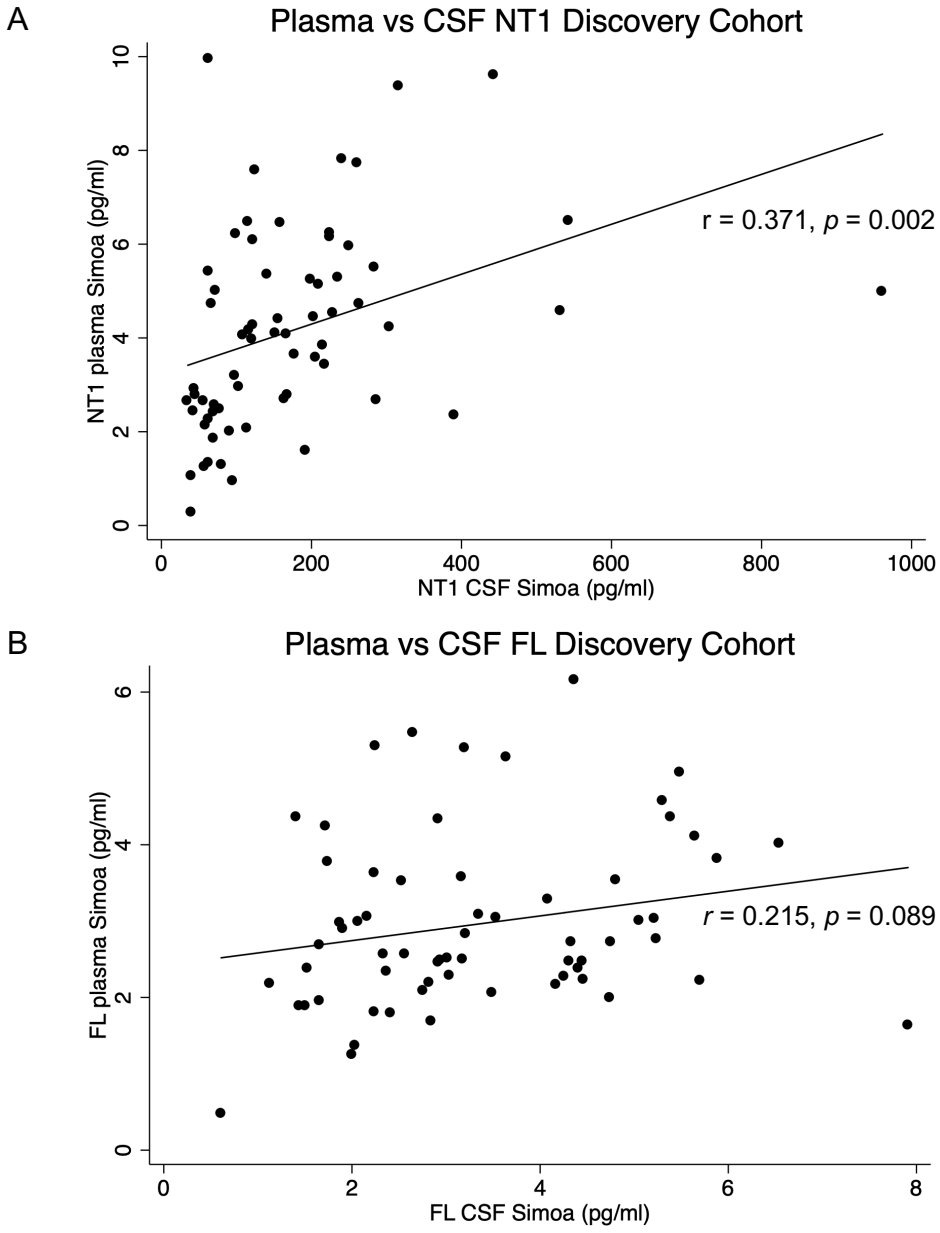
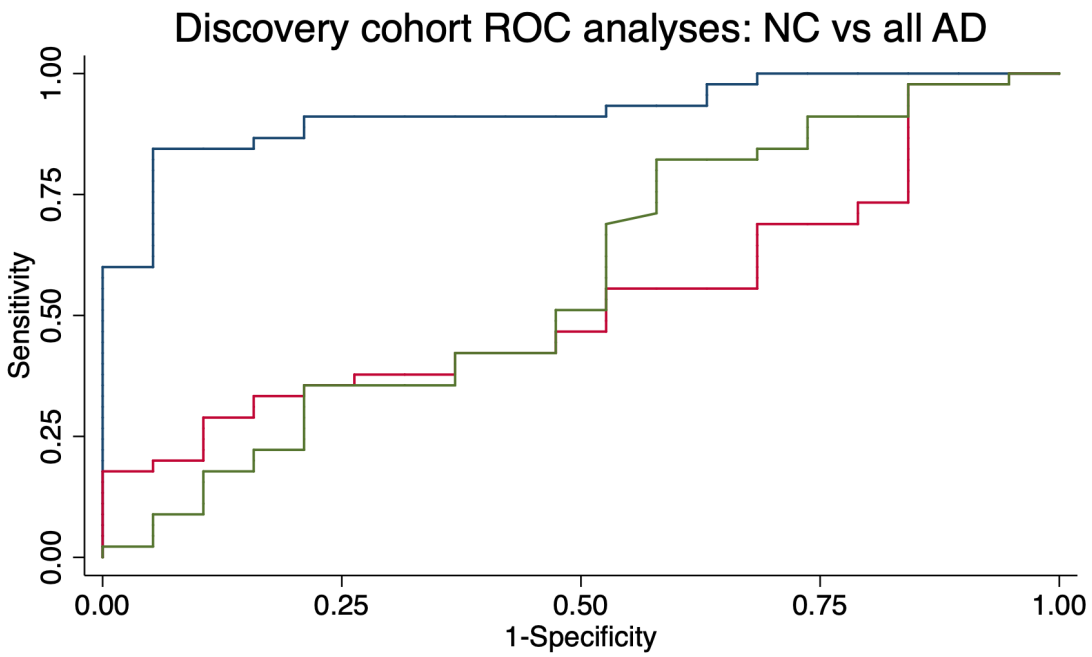


Figure 8.5: Plasma vs CSF values obtained from NT1 (A) and FL (B) assays in the Discovery cohort (n = 65).
Lines of best fit with Pearson correlation coefficients and p values are shown.

8.3.2.4 Receiver operating characteristics analyses

Comparative ROC analyses using the Simoa plasma assays to predict NC vs all AD (Figure 8.6 below) showed that the NT1 assay had the best performance and the other two assays performed no better than chance. Therefore, only NT1 was tested in the validation cohort.



Assay	AUC	95% CI of AUC	
NT1	0.919	0.852, 0.986	χ^2 (equality of AUC): p < 0.0001)
NT2	0.535	0.384, 0.685	
FL	0.574	0.408, 0.739	

Figure 8.6: Comparative ROC analyses for the discovery cohort (n = 65), for classification of NC vs all AD.
ROC curves are shown for NT1 in blue, NT2 in red and FL in green.
AD, Alzheimer's disease; AUC, area under the curve; NC, healthy control; ROC, receiver operating characteristics

The ROC analyses for NT1 for the NC vs AD-MCI and NC vs AD-dementia contrasts are shown in Figure 8.7 (page 298). NT1 retained a good discriminant ability for both comparisons (AUC 0.880 and 0.956 respectively).

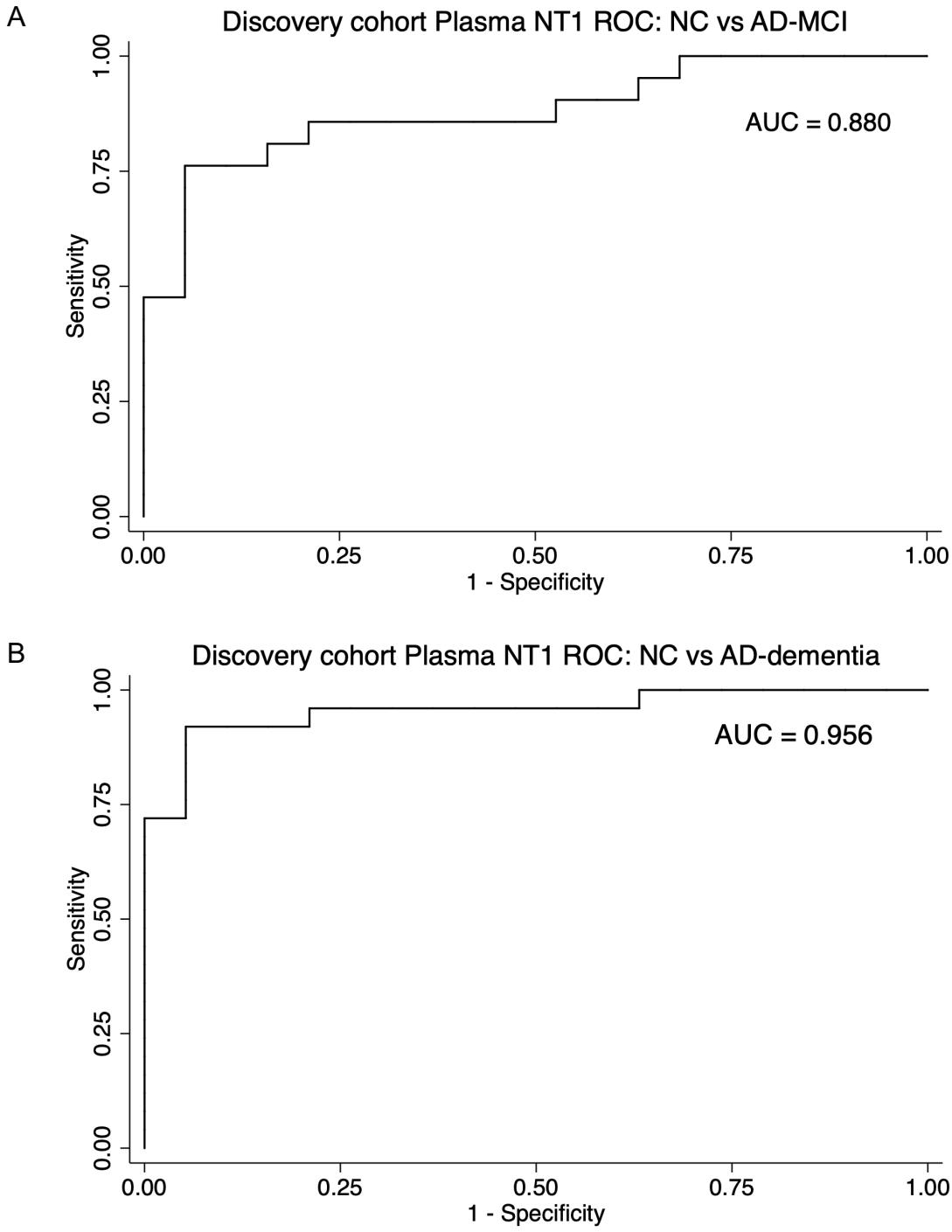


Figure 8.7: ROC analyses for NC vs AD-MCI and NC vs AD-dementia in the discovery cohort.
A: NC vs AD-MCI (n = 40) B: NC vs AD-dementia (n = 44)
AD, Alzheimer's disease; AUC, area under the curve; MCI, mild cognitive impairment; NC, healthy control; ROC, receiver operating characteristics

8.3.3 Validation cohort: UCSD plasma

8.3.3.1 Retrospective calculation to ascertain minimum sample size for group comparisons of plasma NT1

Assuming similar means and standard deviations for plasma NT1 values as in the Discovery cohort, but a roughly 1:1 ratio of NC to AD in the Validation cohort, the minimum number of samples required to detect the same difference, at 95% power and a two-tailed significance level of 0.05 would be 12. However, given that the CSF-t-tau values were lower in individuals with AD pathology the Validation cohort (mean: 664 pg/ml) than in the Discovery cohort (mean: 894 pg/ml), assuming the means for plasma NT1 would also be lower in the Validation cohort by a similar weighting, the minimum number of samples required to detect a significant difference, at 95% power and a two-tailed significance level of 0.05, would be 40. Hence the number of individuals analysed in the Validation cohort (n = 86) would be more than adequate.

8.3.3.2 Group comparisons of plasma NT1

The NT1 assay was applied to plasma samples from UCSD and showed good separation of NC from both AD groups ($p < 0.001$ for the NC vs AD-MCI and NC vs AD-dementia comparisons, Kruskal-Wallis H test with *post hoc* Dunn's test, Figure 8.8).

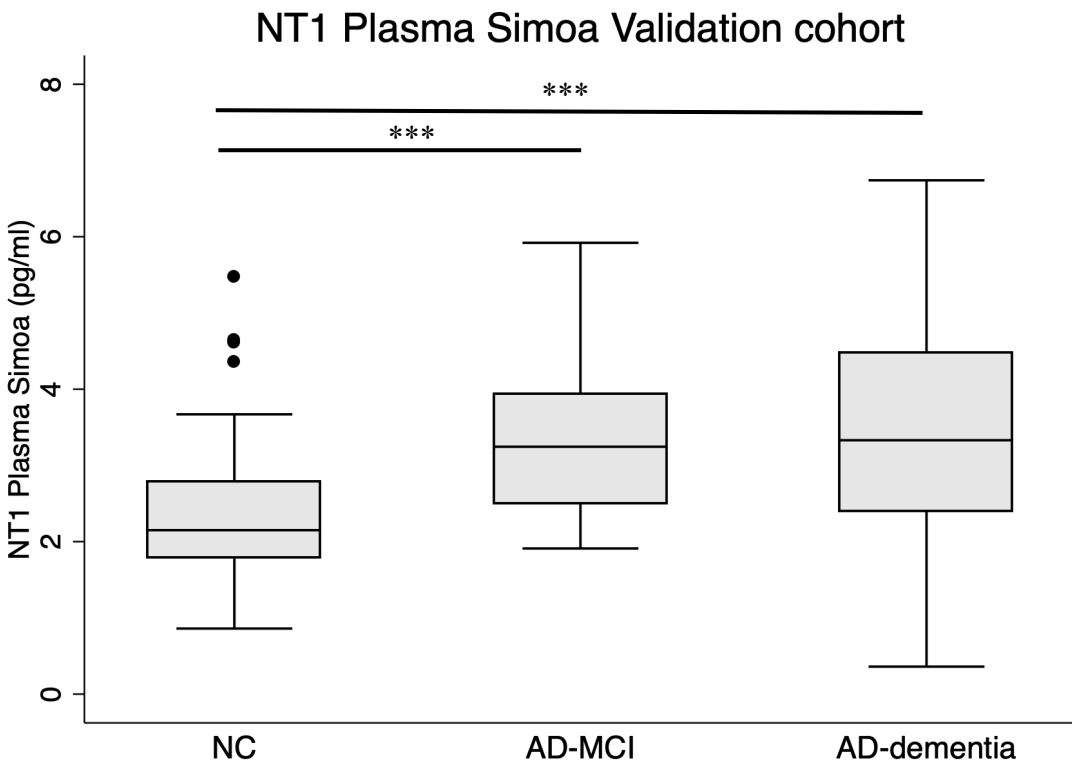


Figure 8.8: Validation cohort NT1 plasma Simoa results (n = 86). Boxes show medians and interquartile ranges; whiskers show 95% ranges.
*** Bonferroni-corrected $p < 0.001$ on Kruskal-Wallis H test with Dunn's post hoc test).

8.3.3.3 Receiver operating characteristics analysis

The ROC analyses for the NC vs AD-MCI and NC vs AD-dementia comparisons in the validation cohort are shown in Figure 8.9 (page 301).

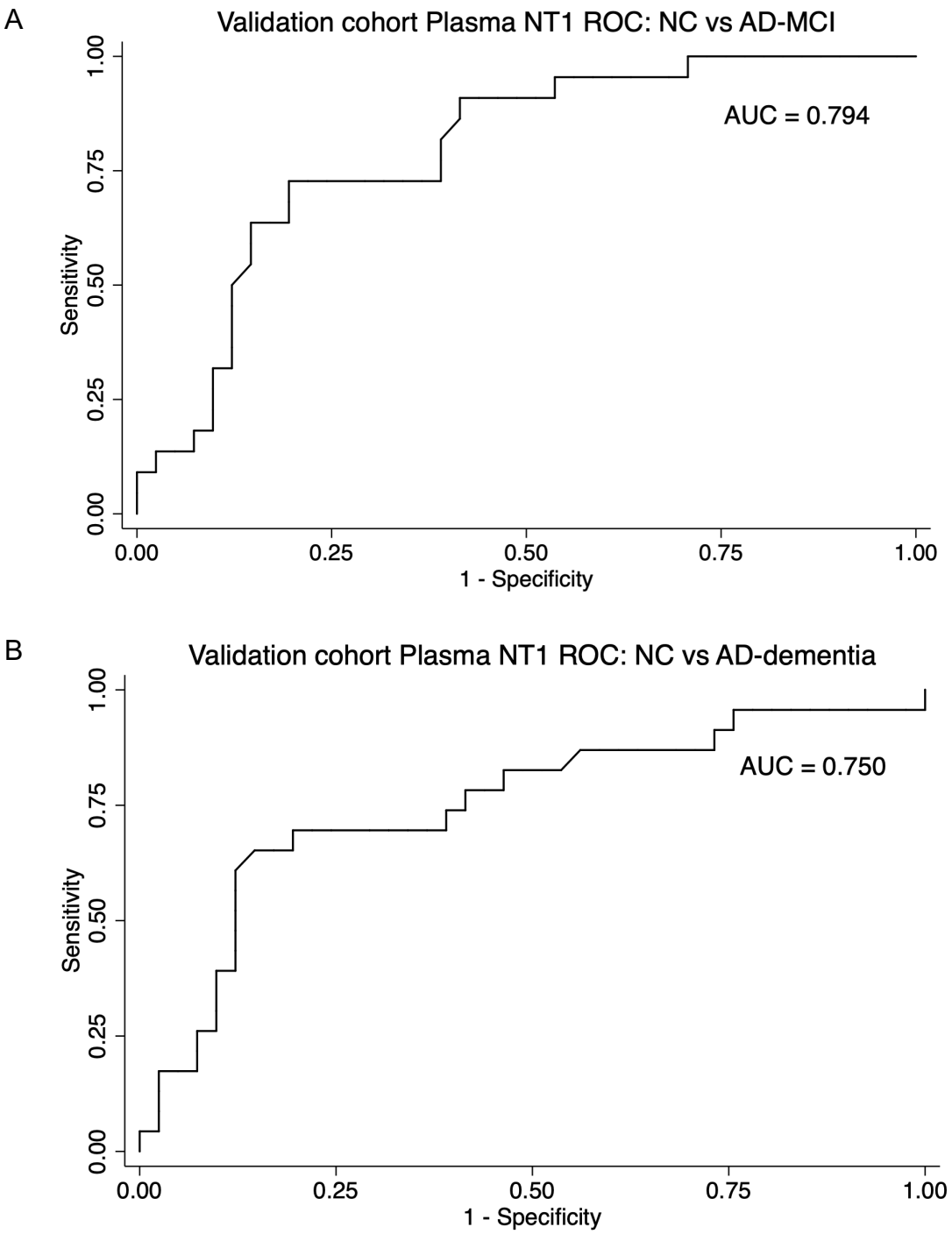
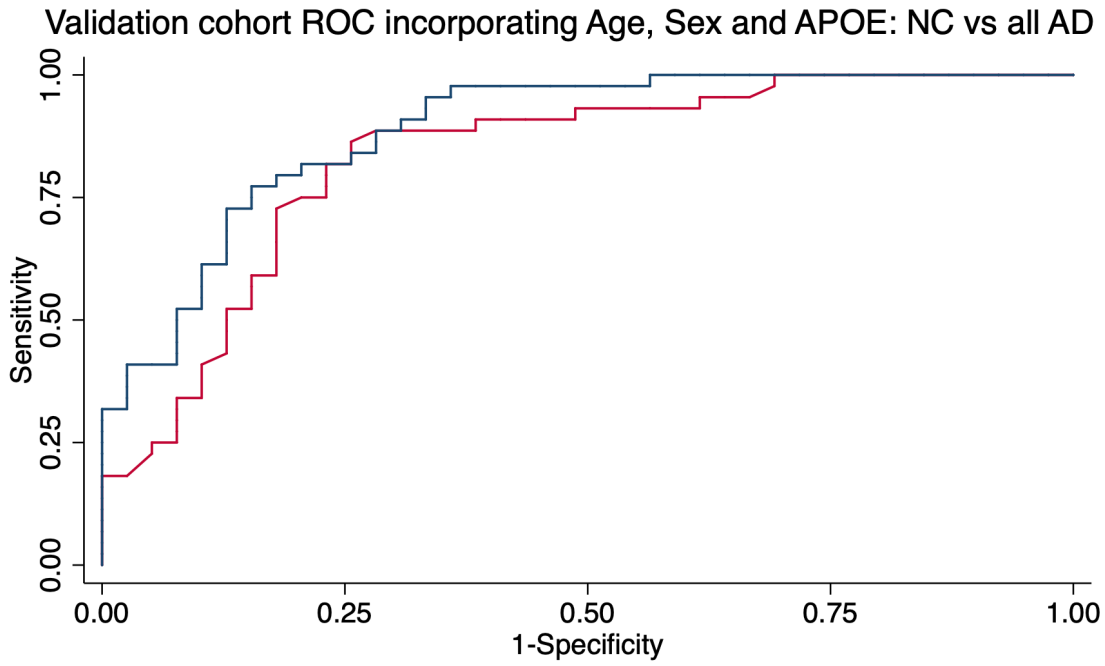


Figure 8.9: ROC analyses for NC vs AD-MCI and NC vs AD-dementia in the validation cohort.
A: NC vs AD-MCI (n = 63) B: NC vs AD-dementia (n = 64)
AD, Alzheimer's disease; AUC, area under the curve; MCI, mild cognitive impairment; NC, healthy control; ROC, receiver operating characteristics

The ROC analyses for the individuals from the validation cohort with known age, sex and *APOE* ε4 carrier status (n = 83) are shown in Figure 8.10 (page 302). There was no significant improvement in the AUC when plasma NT1 was added to the model.



Model	AUC	95% CI of AUC	
Age + Sex + *APOE*	0.831	0.740, 0.922	χ^2 (equality of AUC): p = 0.115
Age + Sex + *APOE* + Plasma NT1	0.888	0.818, 0.958	

Figure 8.10: Comparative ROC analyses for the NT1 assay (blue) relative to a base model incorporating age, sex and APOE ε4 carrier status in the validation cohort (red) for classification of NC vs all AD (n = 83).
AD, Alzheimer's disease; APOE, apolipoprotein E; AUC, area under the curve; NC, healthy control; ROC, receiver operating characteristics

8.3.4 ROC comparisons between the cohorts and cut point transfer

Table 8.4 (page 303) summarises the unadjusted AUC for plasma NT1 for both cohorts for the NC vs all AD comparison. The AUC overall were lower in the validation than in the test cohort. The Youden's index cut-point for the Discovery cohort was 2.95 pg/ml, yielding a sensitivity of 84.8% and a specificity of 94.7%. When the same cut-point was applied to the validation cohort, the sensitivity was 66.7% and the specificity was 80.5%. However, when the Youden's index cut-point from the Validation cohort was used, the sensitivity improved slightly to 71.1%

Table 8.4: Comparisons of unadjusted plasma NT1 AUC and cut-points for the Discovery and Validation cohorts.
A: Area under the curve (AUC) and Youden's index value of plasma NT1 (pg/ml) in each cohort
B: Sensitivity and specificity yielded by choice of different cut-points in the two cohorts

A

Cohort	AUC (NC vs all AD)	Youden's index value of plasma NT1 (pg/ml)
Discovery	0.919	2.95
Validation	0.771	2.83

B

Cohort	Plasma NT1 cut-point chosen (pg/ml)	Sensitivity (%)	Specificity (%)
Discovery	2.95	84.8	94.7
Discovery	2.83	84.8 – 87.0	84.2
Validation	2.95	66.7	80.5
Validation	2.83	71.1	80.5

8.4 **Discussion**

8.4.1 Summary of results

This study addressed the two main questions as follows:

- Is there a tau fragment population that correlates well between CSF and plasma?

This work demonstrated that tau measured by the NT1 Simoa assay correlated moderately between CSF and plasma, unlike published comparisons of mid-region tau measurements in CSF and plasma, which showed a poor correlation [220, 338], and unlike tau measured by the FL Simoa assay in this study, which showed no significant correlation. Absolute values obtained by the FL assay in CSF and plasma were similar, whereas published mid-region assays and the NT1 assay yielded plasma values that were at least two orders of magnitude lower than those in CSF. This suggests that plasma is likely to contain FL tau from a peripheral source.

- Does this tau fragment population, measured in blood, provide good separation between those with AD pathology and controls?

The plasma NT1 assay provided very good separation between all AD (both AD-dementia and AD-MCI) and NC in the Discovery cohort but moderately good separation in the Validation cohort. The cut-point yielding the maximum accuracy in the Discovery cohort gave fair sensitivity and moderate specificity in the Validation cohort.

8.4.2 Relative strengths and limitations of this study

This exploratory study benefited from use of combined clinical and biomarker-based diagnostic categorisation of patient samples, and rigorous validation of

the novel assays using both spike recovery of specific peptide constructs, as well as use of a single recombinant tau standard to allow direct comparison of concentrations of the different tau fragment populations. By using an independent validation cohort, we were able to test and confirm the ability of the plasma NT1 Simoa assay to separate those with AD pathology from controls.

The interpretation of the results of the study is limited by certain key observations. Firstly, the plasma NT1 assay did not perform as well in the Validation cohort as it did in the Discovery cohort, even when using a cut-point optimised for the former. A clue to the possible reasons for this arises from examining the differences in demographics between the two cohorts; individuals from the Validation cohort were on average ten years older and had lower CSF t-tau (mid-region tau) than those from the Discovery cohort. This might imply that those with AD pathology in the Validation cohort had a less aggressive form of AD, or that the controls in the Validation cohort might exhibit an age-related increase in NT1-measured tau that reduced the discriminant value of the assay. Although an exploratory analysis was undertaken to examine the utility of adding plasma NT1 to a base model of age, sex and *APOE* ε4 carrier status to predict diagnosis in the Validation cohort, the AUC for the two models showed no statistically significant difference. A similar analysis could not be undertaken in the Discovery cohort as information on *APOE* ε4 carrier status was not available. Therefore, replication in a larger cohort of samples would be required to provide sufficient statistical power to demonstrate a significant contribution of NT1 above the base model. Finally, given the case-control design of the study, we cannot confirm whether the elevation in NT1 seen in the individuals with AD pathology relative to controls is a specific feature of AD

pathology itself, or a more general feature of neurodegeneration. In order to probe this further, we plan to continue our collaboration to examine CSF and plasma from individuals with a wider range of pathologies (including various forms of tauopathy such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD), and dementias which are not characterised by tauopathy as the main feature, like svPPA and DLB) to compare to AD cases. As tau PET continues to develop, it will also be important to examine the extent of association between plasma NT1 and AD-associated cerebral tau deposition, in a similar approach to that utilised recently for assays of plasma mid-region tau and phospho-tau-181 [217]. Such studies will also allow for estimation of the relative predictive abilities of these and the NT1 assay for cerebral tau deposition in asymptomatic individuals from cohorts such as Insight 46.

8.4.3 Relevant literature published after this work

Cicognola *et al.* [340] recently published details of two novel N antibodies directed at a.a. 123 and a.a. 224, which they used to develop immunoassays showing that the N-224 but not N-123 fragments are present in *post mortem* immunohistochemistry for neurofibrillary tangles, are elevated in cerebrospinal fluid from patients with AD but not those with PSP or CBD relative to controls, and are enriched in neuronally derived (compared to peripherally derived) extracellular vesicles in serum from patients with AD and controls. Their CSF N-224 assay replicated our CSF NT2 assay findings, in which we used the ADx202 antibody targeted at a.a. 218-224. They also extended the potential utility of the CSF N-224 assay by demonstrating AD-specificity relative to other 4R tauopathies. However, as regards testing in blood, they only tested four AD

and control serum samples each and there were no obvious differences between groups in N-224 in neuronally derived extracellular vesicles. While this is consistent with our NT2 assay not showing a difference between plasma in AD vs controls, their N-224 assay has not yet been applied to untreated plasma of the type we used in our experiments, which is likely to require a highly sensitive method such as Simoa technology to ensure accurate quantification.

9 Discussion

9.1 Summary of key results

The work presented here on blood biomarkers of core AD pathologies (amyloid, tau and neurodegeneration) has yielded several important results as follows, to answer the questions posed in section 1.17 (page 148):

- Chapter 3: Up to four freeze-thaw cycles have no effect on Simoa blood biomarkers of NFL, t-tau or Aβ42, but plasma Aβ40 levels reduce after the third cycle. Paired plasma and serum measured in the same individuals indicate that t-tau levels are much lower in serum than in plasma; Aβ40 and Aβ42 levels are slightly lower in serum than in plasma, but NFL levels are slightly higher in serum than in plasma.

- Chapter 4: In Insight 46 phase 1, females had higher levels than males of serum NFL, and plasma t-tau, Aβ42 and Aβ40, and this survived adjustment for important covariates. Higher serum creatinine was also associated with higher values of each of these biomarkers, but not with the plasma Aβ42/40 ratio. Higher plasma t-tau was associated with higher BMI but higher serum NFL was associated with lower BMI. Lower values of plasma Aβ42 and Aβ42/40 ratio were both associated with carriage of one or two *APOE* ε4 alleles, but also with higher cerebral amyloid PET SUVR independently of *APOE* ε4 carrier status. Over the very narrow age range of this cohort, higher plasma Aβ42 and Aβ42/40 ratio were associated with higher age.

- Chapter 5: For detection of PET-amyloid-positivity, the LC-MS plasma Aβ1-42/1-40 ratio performed better than the Simoa plasma Aβ42/40 ratio measured in the same cognitively normal individuals in Insight 46 phase 1. The LC-MS assay performed better than a predictive model incorporating age, sex and APOE ε4 carrier status. The relative scan number reduction and cost savings to a clinical trial that would result from introduction of the LC-MS assay as a pre-screening test would be greater if the test were applied to a population/age range in which the prevalence of PET-amyloid-positivity is low (e.g. younger individuals). In those individuals who were discordant for the PET scan and LC-MS-assigned amyloid status, the majority were “blood positive, PET negative”.

- Chapter 6: In analyses of structural brain imaging variables of cognitively normal individuals in Insight 46 phase 1, after adjustment for relevant covariates, higher plasma t-tau was associated with lower whole brain volume and lower AD signature region cortical thickness. Higher serum NFL was associated with higher ventricular volume, lower AD signature region cortical thickness and lower hippocampal volume. Higher plasma Aβ42 (measured on both the Simoa and LC-MS platforms) and higher LC-MS Aβ1-42/1-40 ratio were also associated with higher ventricular volume; however none of these biomarkers was associated with white matter hyperintensity volume.

- Chapter 7: In phase 1 of Insight 46, neuropsychological analyses of cognitively normal individuals showed that after adjustment for relevant

covariates (including importantly a measure of childhood cognition which is a unique feature using data from a birth cohort), higher plasma t-tau, higher serum NFL and higher Simoa plasma Aβ40 were associated with lower PACC scores, however all these associations were attenuated by further adjustment for serum creatinine. Analyses of components of the PACC showed that higher plasma t-tau and serum NFL were both associated with lower FNAME-12 scores, and higher Simoa plasma Aβ40 was associated with lower DSS scores, and these associations remained robust to adjustment for all relevant covariates including serum creatinine and PET-amyloid-status. The only biomarker to associate with worse performance in matrix reasoning was lower Simoa plasma Aβ42/40, but the significance of this association was diminished when measuring LC-MS plasma Aβ1-42/1-40. No blood biomarker was significantly associated with MMSE (a test that was at ceiling in this cohort).

- Chapter 8: The NT1 assay correlated moderately well between plasma and CSF from the same individuals in a Discovery cohort of individuals from the HABS and DRC studies, but the FL assay showed no significant correlation. Plasma NT1 was able to distinguish very well in the Discovery cohort between individuals with AD pathology compared to controls (as defined by combined CSF and clinical criteria) but performed moderately well in a Validation cohort of individuals from UCSD who were similarly defined but on average 10 years older than those in the Discovery cohort.

9.2 Potential future research avenues related to this body of work

The analyses presented so far of phase 1 blood biomarker data from Insight 46 have confirmed that several important cross-sectional associations exist between blood, imaging and cognitive measures. Phase 2 of data collection, which commenced in January 2018, is due to complete in 2020, and will provide longitudinal measures of all these variables, as well as a CSF resource to probe for novel biomarkers. Some of the hypotheses which will be tested include

- Higher baseline plasma t-tau and/or serum NFL will be associated with higher global brain atrophy (as evidenced by greater reduction in WBV and greater increase in VV) and greater reductions in PACC, LMD, FNAME-12 particularly in amyloid positive individuals
- Higher baseline and/or greater increase in serum NFL will be associated with greater reduction in HV, particularly in amyloid positive individuals
- The greatest decrease in LC-MS plasma Aβ42/40 ratio from phase 1 to 2 will occur in those amyloid negative individuals who are APOE ε4 carriers or who are near the cut-point for PET-amyloid positivity.
- LC-MS plasma Aβ42/40 ratio in phase 2 will show better concordance with CSF Aβ42/40 ratio (assessed by LC-MS or immunoassay methods) than with amyloid PET
- Synaptic markers, such as neurogranin, and microglial activation markers, such as sTREM2, will show selective elevation in the CSF of cognitively normal amyloid positive individuals compared to amyloid negative individuals

The unique aspect of life-course data may also be integrated into blood biomarker analyses, for example by examining the effects of trajectories of

variables such as BMI on plasma t-tau and serum NFL (given the difference observed in associations with BMI at the cross-sectional level for these two biomarkers).

Continuation into a potential "phase 3" of Insight 46 will also benefit from assessment by tau PET imaging, against which both the commercially available Simoa mid-region tau assay and novel tau-based blood biomarkers, such as the plasma N-terminal assays described in Chapter 9 [326] or phospho-tau-181 [217], may be validated.

9.3 **Relative strengths and limitations of the Insight 46 study**

Insight 46 benefits from uniquely deep phenotyping of participants, including detailed neuroimaging and neuropsychology protocols and integration of life course data, allowing analyses such as those presented in Chapter 7 to control for factors such as childhood cognition. Most large biomarker studies do not have access to such direct measures and are only able to control for education level. As its participants are drawn from the world's longest surviving birth cohort, they have demonstrated motivation to continue in the overall NSHD and retention for phase 2 is currently in excess of 95%. We therefore anticipate that collection of data relating to overall cognitive impairment/dementia outcomes will occur either through NSHD or through further phases of the sub-study. Consent is being sought for *post-mortem* brain donation, which may provide the "gold standard" pathological confirmation that is lacking in many other biomarker studies.

Some of the study's strengths may also be interpreted as limitations. By examining participants of similar age, we controlled for the influence of age itself

on variation in blood biomarkers, but our findings cannot strictly be generalised to individuals of all ages, and require replication in other studies with wider age ranges. While the NSHD cohort was representative of mainland Britain at the start of the study in 1946, Insight 46 itself is no longer representative of the NSHD sample, in that Insight 46 is enriched for higher cognitive function and better health [274]. This is likely to result in the associations we have seen being under-estimations of true effects.

Both NSHD and Insight 46 are non-representative of today's multi-ethnic Britain. Western world-centric and Caucasian-biased studies dominate both the AD and biomarker literature (see Figure 9.1, page 315). This is likely to be related both to inequalities in AD research funding and in broader health infrastructures between developed and developing countries, as well as to lack of diversity in research participation. Extrapolation of most research findings to non-Caucasians and populations in the developing world is therefore difficult, and it is likely that uptake of research findings into clinical practice will be affected further by the impact of cultural and socioeconomic factors on access to healthcare by relatively disadvantaged groups, and differing priorities in public health policy (shaped ultimately by financial constraints) between countries.

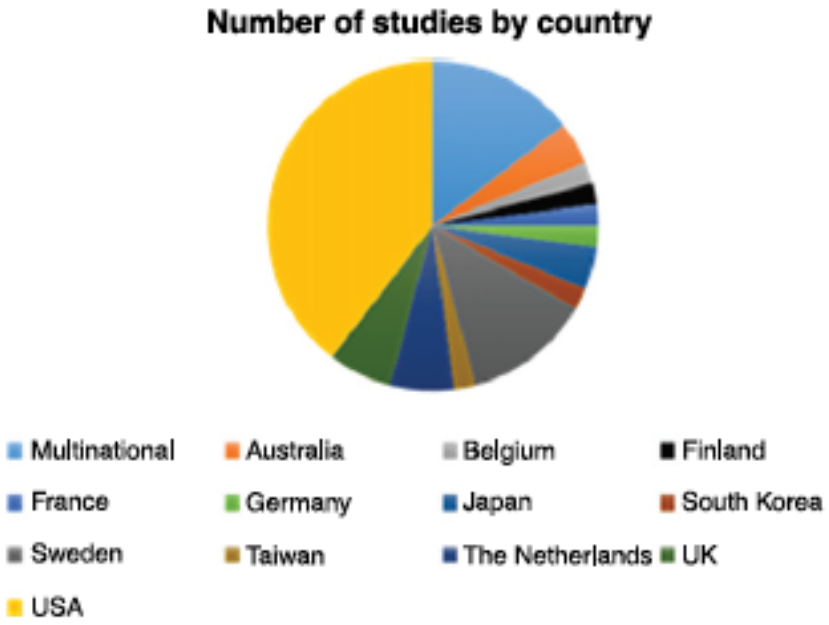


Figure 9.1: Locations of longitudinal biomarker studies.
As reviewed in 2017 by Lawrence et al. [341]; reproduced with permission. The proportion of studies in each country is indicated out of a total of 48. Countries specifically named in multinational studies included: France, Germany, Switzerland, the United Kingdom. One article did not specify in which countries the study took place. One article specified multinational European study sites, 2 articles specified North American, and 1 study featured USA, Australia, Europe, and Argentina.

Increasing diversity in research participation is one of the aims of the Diversity and Disparities Professional Interest Area of the Alzheimer's Association [342]; it is an important step in ensuring that research retains relevance to ethnically diverse populations, and to developing world populations, in which dementia prevalence is rising even faster than in the developed world. In the fluid biomarker domain, very few studies have been designed to specifically test ethno-racial differences. For example, a small study of plasma biomarkers in women with amnestic MCI versus cognitively normal controls, using multiplexed ELISAs, showed that Hispanic women with amnestic MCI had higher plasma Aβ42 than matched controls, but this difference was not found in African Americans or in non-Hispanic White individuals, and conversely White and Hispanic women with amnestic MCI had higher plasma Aβ40 than their respective control groups, but this was not found in African Americans [343].

Studies of CSF biomarkers comparing African Americans and non-Hispanic Whites have also been undertaken in small numbers of participants and have shown lower CSF t-tau and p-tau levels in African Americans with cognitive impairment compared to Whites, despite comparable Aβ42 and Aβ42/Aβ40 [344, 345]. While such studies add a layer of complexity to the problem of cut-point determination, they also have been criticized for implying that race is easily categorised as a biological construct, and for possible residual confounds of socioeconomic and cultural factors that influence participation in biomarker sampling in the first place. As polygenetic risk factors are probed further, differences between individuals in non-genetic fluid biomarkers will likely have to be reframed in this context, rather than that of ethnic differences *per se*.

9.4 **Ethical issues raised by biomarker research and screening**

Even after addressing inequalities in research participation and generalisability, core criteria for population screening tests (as described by Wilson and Jungner in their seminal World Health Organisation publication [346]) state that the disease for which screening is being undertaken should have an available treatment for people who are clearly symptomatic, before consideration of identifying asymptomatic individuals. It is therefore clear that the state of development of AD biomarkers as screening tools does not currently support their application to the population at large, but they may be particularly applicable to individuals who are to be recruited to clinical trials. The ethical implications of biomarker-based diagnosis and screening for AD studies and trials have recently been reviewed [347]. Among the key areas of concern are disclosure of biomarker status and/or genetic risk to participants. In Insight 46 a decision was taken not to disclose amyloid PET findings or CSF biomarker data to participants, in the context of this being an observational rather than

interventional study, and a current lack of interventional studies/clinical trials in the UK in the pre-symptomatic phase of sporadic AD. However, if such trials were implemented and/or a disease modifying treatment for symptomatic disease became clinically available, it is likely that the risk-benefit ratio would be in favour of disclosure, at least in interventional studies. The impact of disclosure of *APOE* genotype to asymptomatic adult children of patients with Alzheimer's disease has previously been assessed in the REVEAL (Risk Evaluation and Education for Alzheimer's Disease) study [348]. This showed that regardless of *APOE* ε4 carriership, cognitively normal individuals randomised to the disclosure and non-disclosure groups did not have any statistically significant difference in time-averaged anxiety, depression or test-related distress over a 12-month follow-up period, but this was in the context of a study design that incorporated extensive genetic counselling before and after the disclosure (or non-disclosure), and excluded people with high pre-existing anxiety levels from participation. The Anti-Amyloid Treatment in Asymptomatic Alzheimer's disease (A4) study published a disclosure protocol for amyloid PET scan results [349], which also highlighted the need to assess baseline anxiety and depression, and to frame the risks in the context of the background population prevalence of amyloid positivity and of AD. It is therefore likely that if a pre-screening blood test were employed in a clinical trial, individuals taking such a blood test would need similar assessment and counselling before and after the blood test itself, to understand the relationship between the blood test result and the potential onward testing with a more definitive modality such as PET or CSF.

The work detailed here has shown that while blood biomarkers of amyloid, tau and NFL are promising, they do not associate with more direct measures of preclinical AD-related brain pathology (such as amyloid PET, brain volumes or cortical thickness) to a degree that would allow these blood biomarkers to replace the more direct measures, either in screening for amyloid-β pathology (as in the case of plasma amyloid-β measures) or in tracking atrophy or cognitive change (as in the case of t-tau or NFL). However, plasma amyloid-β testing might at least contribute toward reduced costs and radiation exposure associated with PET scan as a screen for a clinical trial. In a similar way, associations between tau and NFL and longitudinal brain measures, which we plan to assess as the study continues, may provide methods of stratifying individuals recruited to such trials. For example, they might be incorporated into the AT(N) research framework, or allow for assessing treatment response in cognitively normal individuals. Nevertheless, it is clear that blood biomarkers outside of AT(N) need to be assessed in large well-characterised cohorts of cognitively normal people, and may yet provide the additional information that integrates these core pathologies of AD with vascular, inflammatory and synaptic pathologies that are also likely to be evolving in the pre-symptomatic phase.

10 Publications and presentations arising from this work

10.1 First author publications

Chen Z*, Mengel D*, Keshavan A*, Rissman RA, Billinton A, Perkinton M, Percival-Alwyn J, Schultz A, Properzi M, Johnson K, Selkoe DJ, Sperling RA, Patel P, Zetterberg H, Galasko D, Schott JM and Walsh DM. Learnings about the complexity of extracellular tau aid development of a blood-based screen for Alzheimer's disease. Alzheimer's and Dementia 2019; 15(3): 487-496. *Joint first authors

Keshavan A, Heslegrave A, Zetterberg H and Schott JM. Stability of blood-based biomarkers of Alzheimer's disease over multiple freeze-thaw cycles. Alzheimer's and Dementia (Amst) 2018; 10: 448-451.

Keshavan A, Heslegrave A, Zetterberg H and Schott JM. Blood Biomarkers for Alzheimer's Disease: Much Promise, Cautious Progress. Molecular Diagnosis and Therapy 2017;21(1):13-22.

10.2 Co-author publications

Lane CA, Barnes J, Nicholas JM, Sudre C, Cash DM, Parker TD, Malone I, Lu K, Collins J, James S, Keshavan A, Murray-Smith H, Wong A, Buchanan SM, Keuss SE, Gordon E, Coath W, Barnes A, Dickson J, Modat M, Thomas D, Crutch SJ, Kuh D, Hardy R, Richards M, Fox NC and Schott JM. Early midlife blood pressure influences late life cerebrovascular disease and brain volume but not β-amyloid load – results from the 1946 British Birth cohort. Lancet Neurology (accepted).

Lu K, Nicholas JM, Collins J, James S, Parker TD, Lane CA, Keshavan A, Keuss SE, Buchanan SM, Murray-Smith H, Cash DM, Sudre C, Malone I, Coath W, Wong A, Henley S, Crutch SJ, Fox NC, Richards M and Schott JM. Cognition at age 70: life course predictors and associations with brain pathologies. Neurology (accepted).

James S, Lane CA, Parker TD, Lu K, Collins JD, Murray-Smith H, Byford M, Wong A, Keshavan A, Buchanan S, Keuss S, Kuh D, Fox NC, Schott JM and Richards M. Using a birth cohort to study brain health and preclinical dementia: recruitment and participation rates in Insight 46. BMC Research Notes 2018, 11(1):885

10.3 **First author oral presentations**

Keshavan A, Lane CA, Parker TD, Lu K, Cash DM, Sudre CH, Nicholas JM, Heslegrave AJ, James S-N, Murray-Smith H, Buchanan SM, Keuss SE, Thomas D, Malone IB, Wong A, Richards M, Zetterberg H, Fox NC and Schott JM. Blood biomarkers of amyloid, tau and neurofilament light chain in the 1946 British birth cohort – relationships with cerebral amyloid and brain imaging. Alzheimer's Research UK Conference 2019 PhD student day.

10.4 **First author poster presentations**

Keshavan A, Lane CA, Parker TD, Lu K, Cash DM, Sudre CH, Nicholas JM, Heslegrave AJ, James S-N, Murray-Smith H, Buchanan SM, Keuss SE, Thomas D, Malone IB, Wong A, Richards M, Zetterberg H, Fox NC and Schott JM. Plasma amyloid, tau and serum neurofilament light chain in Insight 46 –

associations with cognition and brain imaging. Alzheimer's Association International Conference 2019.

Keshavan A, Paterson RW, Nicholas JM, Heslegrave A, Fox NC, Mummery CJ, Rohrer JD, Rossor MN, Warren JD, Zetterberg H and Schott JM. ATN classification: utility and pitfalls in fluid biomarker-based stratification. Association of British Neurologists and Society for British Neurosurgeons Joint Meeting 2018.

11 Statement of contribution

Experimental work:

The author performed the assays for all experiments involving CSF or blood analysis, except for

- CSF Aβ42 and t-tau values for the DRC cohort (Chapter 8): these were obtained from the Neuroimmunology Clinical Laboratory at NHNN
- Simoa novel tau assays in CSF and blood (Chapter 8): these were devised and performed by Dr Zhicheng Chen with initial statistical analysis for the publication performed by Dr David Mengel (Brigham and Women's Hospital/Harvard Medical School)
- LC-MS quantification of plasma Aβ peptides in phase 1 of Insight 46: this was performed by Dr Josef Pannee (University of Gothenburg/Sahlgrenska University Hospital)

Statistical analysis:

The author had access to all primary data and performed all statistical analyses presented here. Dr Jennifer Nicholas provided methodological advice.

Funders:

- Wolfson Foundation – Clinical Research Fellowship grant
- Weston Brain Institute and Selfridges Group Foundation – "Developing blood-based biomarkers to detect preclinical Alzheimer's disease and predict progression" award 176724
- Leveraged funding was also obtained from multiple other sources for Insight 46 as a whole but not specifically for the blood biomarker work included in this thesis; these included Alzheimer's Research UK, the

Wellcome Trust, the Drake Foundation, Brain Research UK and the British Heart Foundation. Avid Radiopharmaceuticals provided the ^{18}F-florbetapir tracer (Amyvid™) free of cost but had no role in the design, conduct, analysis or reporting of Insight 46 study findings.

Wolfson PhD programme:

- Ms Elizabeth Halton: administration and guidance
- Prof Nick Wood and the Wolfson PhD board

DRC prospective cohort:

- Dr Ross Paterson – set up the study and performed most of the sampling between August 2013 and October 2016
- DRC clinical fellows and advanced nurse practitioner Ms Frankie O'Shea - participation in the sampling rota

Insight 46:

- DRC academic staff: Prof Jonathan Schott, Prof Nick Fox, Dr Josephine Barnes, Dr David Cash
- MRC NSHD team including Prof Diana Kuh, Prof Nishi Chaturvedi, Prof Marcus Richards, Dr Andrew Wong
- Insight 46 administrative team: Study coordinator Ms Heidi Murray-Smith, Ms Molly Cooper
- Clinical fellows:
- Dr Christopher Lane and Dr Thomas Parker set up the study and performed most of the Phase 1 clinical assessments and blood sampling between August 2016 and October 2017; they also provided the cortical

thickness and white matter lesion volume data respectively which were used in modelling serum NFL

- Dr Sarah Keuss and Dr Sarah Buchanan helped with writing protocols for Phase 2 and with performing most of the clinical assessments and blood sampling from October 2017 till present

- Neuropsychologists: Mrs Kirsty Lu, Ms Jessica Collins, Dr Sarah James, Ms Ivanna Pavisic
- DRC Imaging team: Mr Will Coath, Ms Jana Klimova, Dr Ian Malone, Dr Marc Modat, Dr Carole Sudre, Dr David Thomas
- Neuroradiologists: Dr Chandrashekar Hoskote and Dr Sachit Shah
- Radiographers and Nuclear Medicine staff at University College London Hospital

Leonard Wolfson Biomarker Laboratory/UK Dementia Research Insitute:

- Laboratory leads: Prof Henrik Zetterberg and Dr Amanda Heslegrave
- Research technicians: Ms Martha Foiani, Ms Carolin Heller, Dr Jamie Toombs, Ms Elena Veleva: sample pre-processing for both the DRC prospective and Insight 46 cohorts

Neuroimmunology Clinical Laboratory:

- Dr Miles Chapman, Ms Neghat Lakdawala: teaching on INNOTEST® ELISAs

Provision of samples for CSF validation study and novel tau fragment Simoa study:

- Prof Reisa Sperling (HABS)

- Prof Douglas Galasko (UCSD)

Collaboration on novel tau fragment Simoa study:

- Prof Dominic Walsh, Dr Zhicheng Chen and Dr David Mengel (Brigham and Women's Hospital/Harvard Medical School)

Collaboration on 1946 phase 1 plasma amyloid study:

- Dr Josef Pannee, Dr Ulf Andreasson, Prof Kaj Blennow (University of Gothenburg/ Sahlgrenska University Hospital)

12 Appendix: Insight 46 supplementary analyses

12.1 Binary amyloid status

Table 12.1: Receiver operating characteristics analyses for cerebral PET-amyloid status in all individuals with complete plasma amyloid-β, APOE and PET-amyloid data (n = 449).
Areas under the curve (AUC) are shown with their 95% confidence intervals.
* χ^2 p = 0.091 compared to model 1 $^{+}$ χ^2 p <0.0001 compared to model 1

	Simoa		**LC-MS**	
	AUC	95% CI	AUC	95% CI
Model 1 (Base): Age + Sex + *APOE* ε4 carrier status	0.691	0.625, 0.757	0.691	0.625, 0.757
Model 2: Base + plasma Aβ42	0.703	0.637, 0.768	0.773	0.716, 0.830
Model 3: Base + plasma Aβ40	0.714	0.656, 0.773	0.694	0.629, 0.758
Model 4: Base + plasma Aβ42/40 ratio	0.736*	0.675, 0.795	0.839^{+}	0.795, 0.883

12.2 **MRI measures**

Table 12.2: Base models of structural brain imaging outcomes incorporating BMI and serum creatinine as covariates (base model type 4). Analyses were undertaken in the Cognitively Normal group. Values in bold are statistically significant at the level of p = 0.05

	WBV[a]		VV[a]		HV[a]		WMHV[b]		CTh[c]	
n	415		415		415		407		417	
	β	*p*	*β*	*p*	*β*	*p*	Exponentiated coefficient	*p*	*β*	*p*
Age	**-0.008**	**0.003**	0.054	0.093	**-0.016**	**0.018**	1.139	0.073	0.001	0.811
Male Sex (reference: female)	**-0.021**	**<0.001**	-0.043	0.462	0.007	0.571	**0.607**	**0.001**	**0.008**	**0.021**
APOE ε4 Carrier (reference: non-carrier)	0.006	0.190	-0.035	0.450	0.006	0.506	1.103	0.434	0.001	0.755
PET-amyloid positive (reference: negative)	0	0.991	0.069	0.251	**-0.023**	**0.049**	0.999	0.996	0.001	0.745
Total intracranial volume*	**0.934**	**<0.001**	**2.619**	**<0.001**	**0.528**	**<0.001**	1.001	0.118	-	-
Serum creatinine	**0.000**	**0.037**	0.002	0.251	0.000	0.168	**1.010**	**0.027**	**0.000**	**0.010**
BMI	0.000	0.752	-0.005	0.374	**0.002**	**0.045**	1.008	0.507	0.000	0.420
Constant	**0.792**	**0.003**	**-19.51**	**<0.001**	**-1.626**	**0.011**	0.079	0.623	**1.043**	**<0.001**

[a] Linear regression models for natural log-transformed brain volumes were used. [b] As WMHV was negatively skewed, generalised linear models using gamma distributions with log link were used. [c] Linear regression models for natural log transformed CTh were used.
* natural log transformed for linear regressions of type a; not included in type c

Table 12.3: Models exploring potential interactive effects of amyloid status and plasma tau on structural brain imaging outcomes (model type 5). Analyses were undertaken in the Cognitively Normal group. Values in bold are statistically significant at the level of p = 0.05

	WBV[a]		VV[a]		HV[a]		WMHV[b]		CTh[c]	
n	415		415		415		407		417	
Model fit statistics	AIC	r^2	AIC	r^2	AIC	r^2	AIC		AIC	r^2
Model 1 (without interaction term)	-1506	0.809	492	0.236	-800	0.262	7756		-1713	0.019
Model 5 (with interaction term)	-1504	0.809	494	0.236	-798	0.262	7757		-1716	0.021
Model 5 coefficients	β	*p*	β	*p*	β	*p*	Exponentiated coefficient	*p*	β	*p*
Age	**-0.008**	**0.003**	0.053	0.106	**-0.016**	**0.022**	1.12	0.123	0.000	0.842
Male Sex (reference: female)	**-0.019**	**<0.001**	-0.017	0.761	0.014	0.239	**0.722**	**0.019**	0.003	0.325
Total intracranial volume*	**0.927**	**<0.001**	2.603	<0.001	**0.521**	**<0.001**	1.001	0.152	-	-
APOE ε4 Carrier (reference: non-carrier)	0.005	0.241	-0.036	0.436	0.006	0.524	1.059	0.638	0.001	0.765
PET-amyloid positive (reference: negative)	0.002	0.878	-0.007	0.958	-0.016	0.593	1.452	0.326	-0.007	0.525
Plasma tau[+]	**-0.011**	**0.049**	0.017	0.763	-0.007	0.606	1.002	0.967	**-0.012**	**0.007**
PET-amyloid positive x plasma tau[+] (reference: negative)	-0.002	0.892	0.083	0.531	-0.008	0.775	0.880	0.252	0.009	0.386
Constant	**0.855**	**0.001**	**-19.33**	**<0.001**	**-1.515**	**0.019**	0.431	0.879	**1.040**	**<0.001**

[a] Linear regression models for natural log-transformed brain volumes were used. [b] As WMHV was negatively skewed, generalised linear models using gamma distributions with log link were used. [c] Linear regression models for natural log transformed CTh were used. * natural log transformed for linear regressions of type a; not included in type c. [+] natural log transformed for linear regressions of type a and c.

Table 12.4: Models exploring potential interactive effects of amyloid status and serum NFL on structural brain imaging outcomes (model type 5). Analyses were undertaken in the Cognitively Normal group. Values in bold are statistically significant at the level of p = 0.05

	WBV[a]		VV[a]		HV[a]		WMHV[b]		CTh[c]	
n	415		415		415		407		417	
Model fit statistics	AIC	r^2	AIC	r^2	AIC	r^2	AIC		AIC	r^2
Model 1 (without interaction term)	-1501	0.807	488	0.245	-803	0.267	7754		-1713	0.020
Model 5 (with interaction term)	-1499	0.807	490	0.245	-802	0.268	7754		-1711	0.021
Model 5 coefficients	β	*p*	β	*p*	β	*p*	Exponentiated coefficient	*p*	β	*p*
Age	**-0.008**	**0.003**	0.051	0.110	**-0.016**	**0.022**	1.117	0.152	0.001	0.723
Male Sex (reference: female)	**-0.018**	**<0.001**	-0.007	0.906	0.011	0.316	**0.701**	**0.012**	0.004	0.231
Total intracranial volume*	**0.931**	**<0.001**	**2.562**	**<0.001**	**0.532**	**<0.001**	1.001	0.119	-	-
APOE ε4 Carrier (reference: non-carrier)	0.006	0.210	-0.042	0.365	0.006	0.532	1.026	0.828	0.001	0.678
PET-amyloid positive (reference: negative)	-0.011	0.784	-0.089	0.839	0.065	0.403	1.661	0.100	0.015	0.646
Serum NFL$^+$	0.000	0.958	**0.113**	**0.071**	-0.015	0.300	1.006	0.112	**-0.009**	**0.034**
PET-amyloid positive x serum NFL$^+$ (reference: negative)	0.003	0.785	0.052	0.720	-0.029	0.259	0.979	0.069	-0.004	0.686
Constant	**0.833**	**0.002**	**-19.22**	**<0.001**	**1.571**	**0.014**	0.673	0.942	**1.030**	**<0.001**

[a] Linear regression models for natural log-transformed brain volumes were used. [b] As WMHV was negatively skewed, generalised linear models using gamma distributions with log link were used. [c] Linear regression models for natural log transformed CTh were used. * natural log transformed for linear regressions of type a; not included in type c. $^+$ natural log transformed for linear regressions of type a and c.

Table 12.5: Associations between Simoa plasma Aβ42, Aβ40, Aβ42/40 ratio, tau, serum NFL and MRI measures in the Full Data group as determined by model type 1.
Coefficients are presented as ratio change for a 10% increase in each blood biomarker. Robust standard errors were used to calculate the confidence intervals. Coefficients in bold are significant at the p=0.05 level.

	WBV[a]			VV[a]			HV[a]			WMHV[b]			CTh[c]		
n	449			449			449			436			451		
	Ratio change	95% CI	p	Ratio change	95% CI	p	Ratio change	95% CI	p	Ratio change	95% CI	p	Ratio change	95% CI	p
Aβ42	1.000	0.999, 1.001	0.871	**1.015**	**1.001, 1.029**	**0.038**	0.999	0.996, 1.002	0.507	0.999	0.980, 1.018	0.939	1.000	0.999, 1.001	0.376
Aβ40	1.000	0.998, 1.002	0.884	1.010	0.986, 1.035	0.408	0.999	0.993, 1.004	0.689	1.001	0.999, 1.003	0.518	0.998	0.997, 1.000	0.046
Aβ42/40	1.000	0.999, 1.001	0.786	1.011	0.998, 1.024	0.099	1.000	0.997, 1.002	0.707	0.526	0.004, 69.395	0.796	1.000	0.999, 1.001	0.778
Tau	**0.999**	**0.998, 1.000**	**0.024**	1.003	0.994, 1.012	0.508	1.000	0.997, 1.002	0.827	0.980	0.899, 1.068	0.645	0.999	0.998, 1.000	0.077
NFL	1.000	0.999, 1.001	0.745	**1.013**	**1.003, 1.024**	**0.013**	**0.997**	**0.995, 0.999**	**0.014**	1.005	0.997, 1.012	0.247	**0.999**	**0.998, 1.000**	**0.009**

[a] Linear regression models for natural log-transformed brain volumes incorporated each natural log-transformed blood biomarker singly while adjusting for age, sex, total intracranial volume, *APOE* $\varepsilon 4$ status and PET-amyloid-status.
[b] As WMHV was negatively skewed, generalised linear models using gamma distributions with log link were employed, incorporating each blood biomarker singly while adjusting for age, sex, total intracranial volume, *APOE* $\varepsilon 4$ status and PET-amyloid-status.
[c] Linear regression models for natural log transformed CTh incorporated each natural log-transformed blood biomarker singly while adjusting for age, sex, APOE $\varepsilon 4$ status and PET-amyloid-status.

Table 12.6: Associations between Simoa and LC-MS blood biomarkers and MRI measures in the Cognitively normal group with a full set of Simoa and LC-MS data, as determined by model type 1.
Coefficients are presented as ratio change for a 10% increase in each blood biomarker. Robust standard errors were used to calculate the confidence intervals. Coefficients in bold are significant at the p=0.05 level.

	WBV[a]			VV[a]			HV[a]			WMHV[b]			CTh[c]		
n	411			411			411			403			413		
	Ratio change	95% CI	*p*	Ratio change	95% CI	*p*	Ratio change	95% CI	*p*	Ratio change	95% CI	*p*	Ratio change	95% CI	*p*
Simoa															
Aβ42	1.000	0.999, 1.002	0.601	1.009	0.995, 1.024	0.200	1.000	0.997, 1.003	0.879	1.003	0.982, 1.025	0.754	1.000	0.999, 1.001	0.627
Aβ40	1.001	0.999, 1.003	0.583	1.010	0.985, 1.036	0.434	1.000	0.995, 1.006	0.875	1.001	0.999, 1.003	0.429	0.999	0.997, 1.000	0.175
Aβ42/40	1.000	0.999, 1.001	0.813	1.006	0.992, 1.019	0.406	1.000	0.997, 1.002	0.809	1.246	0.007, 232.98	0.934	1.000	0.999, 1.001	0.822
t-tau	**0.999**	**0.998, 1.000**	**0.020***	1.004	0.995, 1.014	0.321	0.999	0.997, 1.001	0.333	0.963	0.873, 1.062	0.451	**0.999**	**0.998, 1.000**	**0.010**
NFL	1.000	0.999, 1.001	0.950	**1.012**	**1.001, 1.023**	**0.032**	0.998	0.996, 1.000	0.119	1.004	0.996, 1.012	0.290	**0.999**	**0.998, 1.000**	**0.012**
LC-MS															
Aβ1-42	1.000	0.998, 1.001	0.786	**1.020**	**1.004, 1.037**	**0.017**	0.999	0.996, 1.003	0.691	1.011	0.992, 1.030	0.253	0.999	0.998, 1.000	0.090
Aβ1-40	0.999	0.997, 1.001	0.232	1.008	0.988, 1.030	0.413	1.000	0.995, 1.004	0.872	1.001	0.999, 1.003	0.093	0.998	0.995, 1.000	0.064
Aβ1-42/ 1-40	1.001	0.999, 1.002	0.555	**1.021**	**1.001, 1.042**	**0.036**	0.999	0.995, 1.003	0.715	0.571	0.001, 245.41	0.856	1.000	0.998, 1.003	0.724

[a] Linear regression models for natural log-transformed brain volumes incorporated each natural log-transformed blood biomarker singly while adjusting for age, sex, total intracranial volume, APOE $\varepsilon 4$ status and PET-amyloid-status.

Cont'd
[b] As WMHV was negatively skewed, generalised linear models using gamma distributions with log link were employed, incorporating each blood biomarker singly while adjusting for age, sex, total intracranial volume, APOE $\varepsilon 4$ status and PET-amyloid-status.
[c] Linear regression models for natural log transformed CTh incorporated each natural log-transformed blood biomarker singly while adjusting for age, sex, APOE $\varepsilon 4$ status and PET-amyloid-status.
*After removal of potentially influential outliers, the result was attenuated and lost statistical significance (n=389, p = 0.200)

12.3 **Cognition**

Table 12.7: Base models of cognitive variables incorporating BMI and serum creatinine as covariates (model type 3).
Analyses were undertaken in the Cognitively Normal group (n=453). Values in bold are statistically significant at the level of $p = 0.05$. [a] Generalised linear model (binomial family), logit link. [b] Linear regression

	MMSE[a]		MaR[a]		PACC[b]		LMD[b]		DSS[b]		FNAME-12[b]	
Age	-0.119	0.167	**-0.126**	**0.016**	-0.036	0.354	0.033	0.591	-0.030	0.628	-0.062	0.288
Male Sex (reference: female)	**-0.258**	**0.038**	-0.021	0.788	**-0.283**	**<0.001**	**-0.381**	**<0.001**	**-0.251**	**0.008**	**-0.371**	**<0.001**
APOE ε4 Carrier (reference: non-carrier)	-0.045	0.705	0.042	0.587	0.010	0.855	0.102	0.26	-0.025	0.793	-0.007	0.937
Non-manual socioeconomic position (reference: manual)	0.048	0.754	**0.211**	**0.039**	**0.184**	**0.01**	0.137	0.196	**0.209**	**0.083**	**0.328**	**0.005**
Childhood cognition	**0.263**	**0.006**	**0.122**	**0.018**	**0.260**	**<0.001**	**0.273**	**<0.001**	**0.253**	**<0.001**	**0.338**	**<0.001**
Educational attainment (reference: none)												
Below O-level/vocational	-0.359	0.102	0.111	0.587	-0.205	0.128	0.051	0.740	-0.205	0.244	-0.329	0.097
O-level/equivalent	0.282	0.097	**0.494**	**<0.001**	0.172	0.058	0.096	0.511	**0.387**	**0.004**	-0.067	0.639
A-level/equivalent	**0.577**	**0.002**	**0.584**	**<0.001**	**0.310**	**0.001**	0.160	0.259	**0.501**	**<0.001**	0.154	0.253
Degree/equivalent	**0.466**	**0.038**	**0.767**	**<0.001**	**0.365**	**0.001**	0.302	0.096	**0.551**	**0.001**	0.254	0.132
Serum creatinine	**-0.006**	**0.011**	-0.003	0.107	**-0.006**	**<0.001**	**-0.007**	**0.044**	-0.003	0.194	**-0.008**	**0.001**
BMI	-0.003	0.835	-0.009	0.286	0.004	0.386	**0.016**	**0.048**	0	0.964	0.002	0.836
Time delay	-	-	-	-	-	-	-0.005	0.536	-	-	-	
Constant	**12.455**	**0.041**	**9.791**	**0.009**	2.612	0.343	-2.276	0.603	1.904	0.668	4.708	0.259

Table 12.8: Associations between Simoa and LC-MS blood biomarkers and z-score based cognitive measures in the Cognitively normal group with a full set of Simoa and LC-MS data, as determined by model type 1 (n = 449).
Coefficients are presented as z-score change (Δ*z*) for a 10% increase in each blood biomarker, adjusted for age, sex, APOE ε4 status, socioeconomic position, childhood cognition and educational attainment. Robust standard errors were used to calculate the 95% confidence intervals for Δ*z*. Coefficients in bold are significant at the p=0.05 level.

	PACC			LMD			DSS			FNAME-12		
n	449			449			449			448		
	Δ*z*	95% CI	*p*	Δ*z*	95% CI	*p*	Δ*z*	95% CI	*p*	Δ*z*	95% CI	*p*
Simoa												
Aβ42	-0.015	-0.032, 0.001	0.065*	-0.020	-0.046, 0.005	0.119	-0.003	-0.029, 0.023	0.814	-0.016	-0.040, 0.008	0.201
Aβ40	**-0.034**	**-0.062, -0.006**	**0.017**	**-0.054**	**-0.098, -0.010**	**0.016**	**-0.058**	**-0.100, -0.016**	**0.007**	-0.002	-0.045, 0.041	0.923
Aβ42/40	-0.004	-0.020, 0.012	0.635	-0.002	-0.029, 0.024	0.854	0.016	-0.101, 0.041	0.229	-0.015	-0.039, 0.010	0.238
t-tau	-0.011	-0.024, 0.001	0.064	-0.003	-0.022, 0.016	0.787	-0.011	-0.030, 0.009	0.288	**-0.026**	**-0.045, -0.008**	**0.006**
NFL	**-0.017**	**-0.030, -0.003**	**0.017**	-0.018	-0.039, 0.004	0.110	-0.007	-0.030, 0.017	0.579	**-0.033**	**-0.051, -0.014**	**0.001**
LC-MS												
Aβ1-42	-0.010	-0.030, 0.010	0.334	-0.017	-0.047, 0.013	0.259	-0.007	-0.039, 0.025	0.672	-0.013	-0.045, 0.019	0.426
Aβ1-40	-0.017	-0.045, 0.011	0.237	-0.025	-0.069, 0.020	0.283	-0.036	-0.086, 0.013	0.151	-0.015	-0.061, 0.031	0.533
Aβ1-42/ 1-40	-0.003	-0.025, 0.019	0.782	-0.008	-0.045, 0.028	0.653	0.015	-0.027, 0.056	0.489	-0.009	-0.044, 0.026	0.624

*After removal of potentially influential outliers, this result achieved statistical significance (n = 422, p = 0.028)

Table 12.9: Associations between Simoa and LC-MS blood biomarkers and raw score based cognitive measures (MMSE and MaR) in the Cognitively normal group with a full set of Simoa and LC-MS data, as determined by model type 1 (n = 449).
Coefficients are presented as odds of change of score (ORΔ) for a 1 log unit increase in each blood biomarker, adjusted for age, sex, APOE ε4 status, socioeconomic position, childhood cognition and educational attainment. Robust standard errors were used to calculate the 95% confidence intervals for ORΔ. Coefficients in bold are significant at the p=0.05 level.

	MMSE			MaR		
n	449			449		
	ORΔ	95% CI	*p*	ORΔ	95% CI	*p*
Simoa						
Aβ42	0.708	0.496, 1.010	0.057	1.101	0.922, 1.315	0.287
Aβ40	0.714	0.368, 1.387	0.321	0.769	0.538, 1.099	0.150
Aβ42/40	0.810	0.584, 1.112	0.206	1.201	0.995, 1.450	0.056
t-tau	0.896	0.651, 1.232	0.498	0.953	0.805, 1.127	0.572
NFL	0.857	0.648, 1.136	0.285	0.932	0.783	1.089
LC-MS						
Aβ1-42	0.956	0.600, 1.523	0.849	1.117	0.867, 1.439	0.391
Aβ1-40	1.136	0.589, 2.189	0.703	0.974	0.670, 1.416	0.889
Aβ1-42/1-40	0.860	0.510, 1.452	0.574	1.186	0.853, 1.648	0.309

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