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Cerebrospinal fluid biomarkers in Alzheimer's Disease: from bedside to bench and back

A thesis submitted for the Degree of Doctor of Philosophy

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In memory of Margaret Mackinnon

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease that results in cognitive impairment and death. The pathological hallmarks are extracellular cortical amyloid plaques and intraneuronal tangles composed of hyperphosphorylated tau. Although environmental and genetic factors contribute to the development of AD, the sequence of pathophysiological events that lead to Alzheimer's dementia is not yet completely clear. The clinical diagnosis of AD during life can be challenging and factors that explain clinical phenotypic heterogeneity and variability in rates of disease progression are not well understood. Biomarkers, objective measures of biological function, can be employed to support a clinical diagnosis of AD and may be abnormal before the onset of clinical symptoms. Imaging and cerebrospinal fluid biomarkers (CSF) are now incorporated into clinical and research diagnostic criteria. CSF, which is in direct contact with the brain, is a promising source of biomarkers and has the potential to differentiate AD from other neurodegenerative dementias, explain clinical heterogeneity within AD and elucidate the role of other pathobiological pathways. Ultimately CSF biomarkers might facilitate diagnosis of AD in its pre-clinical phase and allow for treatment responses to be measured.

In this thesis CSF samples from clinical cohorts of individuals with AD, other neurodegenerative diseases and healthy controls are analysed using an extended panel of enzyme-linked immunosorbent assays (ELISA) and a novel mass spectrometry based assay. For the established CSF biomarkers, the practical issues related to collection, transportation and storage of CSF are investigated. Amyloid positron emission tomography (PET) imaging is investigated as a means of validating clinical cutpoints. An extended panel of established and emerging ELISAs is used to determine the diagnostic utility of biomarkers for differentiating AD from other neurodegenerative dementias and for explaining phenotypic heterogeneity within AD. The role of CSF biomarkers as predictors of disease progression is investigated employing robust measures of brain atrophy as surrogate measures of rates of neurodegeneration. Finally CSF samples are probed for new AD biomarkers using a novel mass spectrometry based assay.

A number of practical conclusions are drawn from this work: aliquot storage volume is identified as an important confounder in measured CSF b-Amyloid concentration. CSF laboratory transportation methods are shown not to have a significant impact

on measured biomarker concentration. Amyloid PET is a valuable means of validating clinical diagnostic cutpoints of core CSF biomarkers. Tau/Ab1-42 ratio, Ab40/42 ratio, P-tau and NFL emerge as having diagnostic utility for differentiating AD from other neurodegenerative diseases, and have high sensitivity and specificity for distinguishing AD from bvFTD, SD and healthy controls. Important differences in T-tau, P-tau and neurofilament light distinguish different AD atypical phenotypes and may help to elucidate underlying biological differences between these syndromes: individuals with the visual variant of AD (posterior cortical atrophy) have the lowest levels of CSF Tau and lowest rates of cognitive decline while the frontal executive cases have highest levels of NFL and highest rates of cognitive decline indicating more rapid neurodegeneration. Several novel biomarkers including trefoil factor 3 and several markers involved in vascular remodeling, amyloid processing and neuroinflammation are identified as predictors of increased atrophy rates in amyloid positive individuals suggesting possible independent mechanisms driving differing rates of neurodegeneration between individuals. Other novel AD biomarkers including malate dehydrogenase are identified as distinguishing AD from controls using a novel mass spectrometry based assay. Moreover, this assay demonstrates how mass spectrometry might be used for biomarker discovery and rapid development of a high throughput multiplexed clinical CSF assay.

Taken together these results address some of the unanswered questions about how CSF should be collected, handled and stored to optimize analytical standardization, and how clinical results might be validated using amyloid PET. This work establishes the clinical utility of established biomarkers for differentiating AD from other neurodegenerative diseases and identifies established and novel biomarkers that might explain clinical heterogeneity and rates of progression between individuals. Finally a method for rapidly developing new biomarkers is tested and validated.

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Abbreviations

Aβ	beta amyloid
AD	Alzheimer's disease
ADNI	Alzheimer Disease Neuroimaging Initiative
ADRDA	Alzheimer's Disease and Related Disorders Association
AIBL	Australian Imaging Biomarkers and Lifestyle
ALS	amyotrophic lateral sclerosis
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APOE	apolipoprotein E
APP	amyloid precursor protein
BACE	beta-APP cleaving enzyme
BOLD	blood–oxygen-level-dependent
BSI	Boundary Shift Integral
BvFTD	behavioural variant frontotemporal dementia
DLB	dementia with Lewy bodies
CBS	corticobasal syndrome
CDR	clinical dementia rating scale
CERAD	Consortium to Establish a Registry for Alzheimer's disease
CJD	Creutzfeldt-Jakob disease
CNS	central nervous system
CSF	cerebrospinal fluid
CT	computerised tomography
CV	co-efficient of variation
DIAN	Dominant Inherited Alzheimer's Disease Network
DTE	dithiothreitol
ELISA	enzyme-linked immunosorbent assays
FAD	familial Alzheimer's disease
FDG	fludeoxyglucose
FDR	false discovery rate
fMRI	functional magnetic resonance imaging
FTD	frontotemporal dementia
FTLD	frontotemporal lobar degeneration
fvAD	frontal variant Alzheimer's disease
GABA	gamma-aminobutyric acid
GNT	graded naming test
GWAS	genome wide association study
HC	healthy control
HFABP	heart fatty acid binding protein
HIV	human immunodeficiency virus
HMPAO	hexamethylpropyleneamine
HSV	herpes simplex virus
IAA	Iodoacetic Acid
IQR	interquartile range
IWG	International Working Group
LC-MS	liquid chromatography mass spectrometry

LPA	logopenic aphasia
MAPT	microtubule-associated protein tau
MCI	mild cognitive impairment
MSD	Mesoscale discovery
MMSE	mini mental state examination
MRI	magnetic resonance imaging
MRM	multiple reaction monitoring
MS	multiple sclerosis
NFL	neurofilament light
NHNN	National Hospital for Neurology and Neurosurgery
NINCDS	National Institute of Neurological and Communicative Disorders and Stroke
NMDA	N-methyl-D-aspartate receptor
NSE	neuron-specific enolase
OPLS-DA	orthogonal projection to discriminant analysis
PCA	posterior cortical atrophy
PDD	Parkinson's disease dementia
PET	positron emission tomography
PIB	Pittsburgh compound
PNFA	progressive non-fluent aphasia
PPA	primary progressive aphasia
PSEN	presenilin
PSP	Progressive Supranuclear Palsy
P-tau	phosphoryated tau
REM	rapid eye movement
RMT	Recognition Memory Tests
ROC	Receiver operating characteristic
RT-QUIC	real-time quaking-induced conversion
SD	semantic dementia
SD	standard deviation
SILK	stable isotope-labelling kinetic
SPECT	single photon emission tomography
SOP	standard operating procedure
SUVR	standardised uptake value ratio
TDP	TAR DNA-binding protein
TFF	trefoil factor
TREM	Triggering receptor expressed on myeloid cells
T-tau	total tau
VAD	vascular dementia
VEGF	vascular endothelial growth factor
VGKC	voltage gate potassium channel
VLP	visinin-like protein
VOSP	visual object and space perception
YOAD	Young onset Alzheimer's disease

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Chapter 1. Introduction

1.1 Alzheimer's Disease

The Problem

Alzheimer's disease (AD) is an inevitably progressive neurodegenerative condition that results in cognitive impairment and premature death, emotional distress for individuals, families and carers, and significant economic burden for governments and society.

Alzheimer's disease is caused by abnormal accumulation of the proteins β-amyloid and tau in the brain and to make a definitive diagnosis examination of brain tissue is required. Making a clinical diagnosis of Alzheimer's disease during life can be challenging and still rely primarily on a clinical assessment which is not 100% accurate even in specialist centres. Clinicians and researchers currently lack the tools that allow them to estimate an individual patient's prognosis or to monitor disease progression in response to potential disease modifying therapies. The pathobiological pathways leading to sporadic AD are incompletely understood, but a number of genetic and environmental factors have been identified as playing a role[1] and a hypothetical model of AD pathophysiology has been proposed[2] which will be expanded on later in this chapter. To improve clinical diagnosis, facilitate prognostication, measure response to treatment and to further advance knowledge of AD pathobiology *in vivo*, objective markers of biological function termed "*biomarkers*", are required.

A number of biomarkers are available clinically, including measures of atrophy from brain imaging using MRI or CT and measurement of cerebrospinal fluid (CSF) proteins relevant to AD. These are now both incorporated into diagnostic criteria for AD[3, 4]. CSF is in close proximity to the brain and as a result is a promising source of novel biomarkers. However, our understanding of the CSF biomarkers, both established and novel remains incomplete, and a number of outstanding issues in their application in the clinical and research setting exist.

Critical knowledge about the practicalities of how CSF should be collected, stored and analysed remains incomplete. There is significant uncertainty about how reproducible and reliable results of existing assays are, since a number of potential confounding variables in biomarker measurement have not been explored. There are also challenges relating to the clinical application and validation of biomarker assays. However, the role of existing biomarkers for differentiating AD from other neurodegenerative dementias or for explaining the clinical phenotypic heterogeneity or rates of clinical progression within AD has still to be fully investigated. Biomarkers that help to provide individuals and their families with reliable diagnostic and prognostic information are desperately required.

It is well recognised that a preclinical phase of AD occurs years, possibly decades before the onset of clinical symptoms[1]. Biomarkers will be essential for detecting and tracking AD in its earliest stages, with the eventual aim of treatment before irreversible neurodegeneration has occurred. Distinguishing AD from other forms of dementia at this early stage is vital to facilitate recruitment to clinical trials, and establishing biomarkers that track disease progression is critical for monitoring drug target engagement.

Finally, the range of currently available biomarkers does not reflect the complexity and heterogeneity of AD pathobiology. The discovery of novel biomarkers, which reflect dysfunction in multiple disease pathways, will facilitate better understanding of AD pathophysiology.

This introductory chapter sets out the basic clinical, pathological and epidemiological features of AD and AD mimics. An overview of the potential role of biomarkers, biomarkers currently available in clinical practice, and promising new biomarkers in development is provided with particular focus on cerebrospinal fluid (CSF). The clinical utility and limitations of existing biomarkers is discussed and the pressing case for new AD biomarkers made.

1.1.2 History and Epidemiology

The German neuropathologist Alois Alzheimer described plaques, neurofibrillary tangles and arteriosclerotic changes in the neocortex of a 56 year old woman in 1906 and subsequently Arnold Pick defined 'Alzheimer's disease' as a form of early onset dementia. This concept held sway until the 1960s when it became apparent that the pathological changes underlying early and late onset AD were similar, and since then Alzheimer's disease pathology has been recognised as the most common cause of early onset and late onset cognitive impairment[5]. The single greatest risk factor for sporadic AD is age: incidence at the age of 60-69 is under 1%[6-9] but this increases to 6-10% at age 80-89. Prevalence of dementia in the UK increases steadily with age. At age 65 the prevalence is approximately 2%, increasing to 20% by age 85. Approximately 65% of all those with dementia have AD[10]. Data for those age >90 is

limited[1] but it appears that whilst the prevalence continues to increase, the incidence of AD might fall in the very oldest people[1]. Evidence suggests AD is more prevalent in women than men[10, 11]. A number of environmental risk factors are now recognised for sporadic AD: risk factors for cardiovascular health such as obesity[12, 13], hypertension[14, 15], smoking[16, 17] and high cholesterol[18 13] contribute to the risk of symptomatic AD while physical exercise[19, 20] and diet low in saturated fat may be protective[5]. Being socially and cognitively active may protect against dementia[21, 22], while low educational attainment is a risk factor[23]. There is, however, a lack of lifelong epidemiological studies to determine the true impact of lifestyle factors in early life. And until recently owing to the lack of good accurate biomarkers for AD and limited pathology proven studies, many epidemiological studies struggle to differentiate AD from other causes of cognitive impairment which may confound results[1].

1.1.3 Clinical features

Memory impairment is the commonest clinical presentation of AD. Less common AD syndromes can lead with visual, language, behavioural or dysexecutive features.

Typical memory led AD, by far the commonest clinical presentation is characterized by early insidious impairment of episodic memory followed by spatial memory deficits. Patients often lack insight, but relatives may report repeated questioning, forgotten messages or errands or misplaced objects[24]. In early to moderate disease, social façade is typically preserved[25]. As the disease progresses, other cortical areas become involved and eventually there is involvement of multiple cognitive domains,

sufficient to impair activities of daily living and for the individual to fulfill criteria for dementia[3].

The visual variant of AD, sometimes referred to as 'biparietal AD' or posterior cortical atrophy (PCA) is a relatively rare form characterized by impairments of visuoperceptual, visuospatial function, literacy, numeracy and praxic skills[26, 27]. Affected individuals may have features of Balint's syndrome (simultanagnosia, oculomotor apraxia, optic apraxia, environmental agnosia) or Gerstmann's syndrome (acalculia, agraphia, left/right disorientation, finger agnosia)[28] and may report positive visual phenomena such as prolonged colour after images[29] or perception that static objects are moving[30]. On examination, visual field defects, dyspraxia, myoclonus, extrapyramidal features or the motor signs of corticobasal syndrome may be seen[31].

The language led variant of AD, or 'logopenic aphasia' is characterised by early and progressive impairment of language, with a distinctive linguistic profile. Speech is typically slower, characterized by word retrieval difficulties, frequent pauses and difficulty with repetition and comprehension[32] and phonological dyslexia[33].

Frontal AD is characterized by progressive behavioural changes or a dysexecutive syndrome[34, 35]. This is the rarest and least well studied AD variant and there are currently no consensus diagnostic criteria making diagnosis during life a challenge; this syndrome can be indistinguishable from behavioural variant frontotemporal dementia[35].

In clinical practice there is often significant overlap between these syndromes and they often merge as the disease progresses. Atypical presentations are more likely to occur in young onset disease: approximately 1/3rd individuals presenting at age <65 years have an atypical presentation, compared to ~5% in later onset disease[36, 37]. These different clinical phenotypes are underpinned by imaging differences, such as atrophy patterns and patterns of cortical tau deposition[34]. There may also be genetic differences, for example APOE4 allele expression may be less frequent in posterior cortical atrophy[27]. However, the major causes of clinical heterogeneity are, as yet, largely unexplained.

1.1.4 Differential Diagnosis of AD

Making a definitive diagnosis of AD during life, based on clinical features alone is not 100% reliable as a number of other neurodegenerative and non-neurodegenerative diseases can present similarly. Ultimately pathological confirmation is required and even then diagnosis is not always clear-cut and features of more than one disease can be present. In this section the mimics of 'typical' memory led AD and of the 'atypical' clinical syndromes are discussed.

Individuals commonly present to doctors with concerns about their memory in midlife. Such patients are frequently concerned about their memory but report 'normal' phenomena such as forgetting why one has entered a room[25] or where one has placed an object. Such cases may not necessarily be underpinned by a neurodegenerative process and can be a feature of anxiety or normal ageing. However the situation is somewhat complicated by the fact that conversion to AD is higher in this group[38]. "Red flags" for AD are early topographical memory problems,

and loss of social or occupational abilities as corroborated by an informant who knows the individual well[25]. Depression may also contribute to poor memory registration and careful questioning may elicit a history of the biological features of depression. However, it is increasingly recognised that depression may also accompany the early stages of AD[39].

Reversible metabolic conditions such as vitamin B12 deficiency, hypothyroidism and thiamine deficiency may result in cognitive impairment or worsen existing cognitive problems and can be excluded with simple blood tests. Autoimmune conditions such as voltage gate potassium channel (VGKC) complex encephalitis may also cause an amnestic syndrome; these syndromes are typically much more rapid, and accompanied by other factors, including seizures[40]. The clinical phenotype of VGKC-complex encephalitis continues to expand and it is possible that some forms might mimic more typical amnestic AD. Antibodies against AMPA, GABA, mGluR5 and NMDA receptors can also result in cognitive syndromes and in some cases this may broadly resemble the progressive cognitive decline of amnestic AD[41].

Infectious conditions such as herpes simplex encephalitis, syphilis or HIV can all cause dementia but rarely closely mimic AD[25]. Individuals with temporal lobe epilepsy may present with an amnestic syndrome, however the course is unlikely to follow the insidiously progressive course as seen in AD. Mass lesions of the mesial temporal lobes can cause a progressive amnestic syndrome although this is usually easily resolved with suitable brain imaging.

Vascular cognitive impairment can resemble AD clinically resulting in a progressive memory led syndrome. Although imaging can be useful to assess the burden of

vascular disease, concomitant vascular disease is commonly seen in, and can compound AD. Microhaemorrhages can be a marker of both amyloid pathology and vascular disease although their distribution can be helpful in differential diagnosis[42].

The major mimics of AD are other neurodegenerative diseases including dementia with Lewy bodies (DLB), frontotemporal lobar degeneration (FTLD) and a range of rarer diseases including argyrophilic grain disease.

DLB is the 2nd commonest neurodegenerative disease and at least in its classical form is characterized by distinct clinical features that are rarely seen in AD[43]. Thus core clinical features of DLB are: (1) prominent fluctuations in cognition and alertness; (2) well-formed recurrent visual hallucinations; and (3) features of motor Parkinsonism[44]. Other supportive features include hallucinations in other modalities, dysautonomia, recurrent falls and rapid eye movement (REM) sleep disorder. However the most common presenting feature of DLB is still impairment of episodic memory[43], and the prevalence of the core and supportive features, in pathologically confirmed DLB cases is still relatively low[43, 45]. Moreover a large proportion of pathologically confirmed DLB cases also have co-existing amyloid plaque pathology at autopsy[45] potentially confusing the use of amyloid specific biomarkers.

Frontotemporal dementia (FTD) is a clinically and pathologically heterogeneous group of neurodegenerative diseases. FTD refers to the clinical syndrome that is underpinned by the pathological entity of frontotemporal lobar degeneration (FTLD). A significant proportion of FTD cases are caused by autosomal dominant genetic mutations (MAPT, GRN, C9ORF72) but the majority are sporadic diseases[46] due to a range of proteinopathies. Clinically, FTD broadly presents with

either a behavioural syndrome (behavioural variant FTD; BvFTLD) or a progressive language syndrome (primary progressive aphasia; PPA). PPA can in turn be divided into progressive non-fluent aphasia (PNFA), semantic dementia (SD) and logopenic aphasia (LPA) which each have distinct but often overlapping linguistic profiles [32]. However, some individuals can present with an insidious decline of episodic memory, for example those with mutations of the tau gene[47] who also have prominent hippocampal atrophy on brain imaging. Individuals with mutations in the C9ORF72 gene can have prominent episodic and topographical memory impairment[48].

The differential diagnosis of posterior cortical atrophy offers its own challenges. Individuals frequently present to opticians and ophthalmologists reporting visual problems and it can take some time to recognise that the anterior visual pathways are blameless. While most cases of PCA are caused by Alzheimer's disease, DLB, corticobasal degeneration, and prion diseases can present with an insidiously progressive visual syndrome[27].

1.1.5 Neuropathology of AD

1.1.5.1 Macroscopic pathology

At autopsy the AD brain typically weighs ~10% less than in healthy age matched controls[49] with the temporal lobes weighing approximately 50% less. Symmetrical cortical atrophy is seen, with greatest involvement of the medial temporal lobes (Figure 1.1), beginning in the entorhinal cortex and spreading to the hippocampus[50].

Figure 1.1

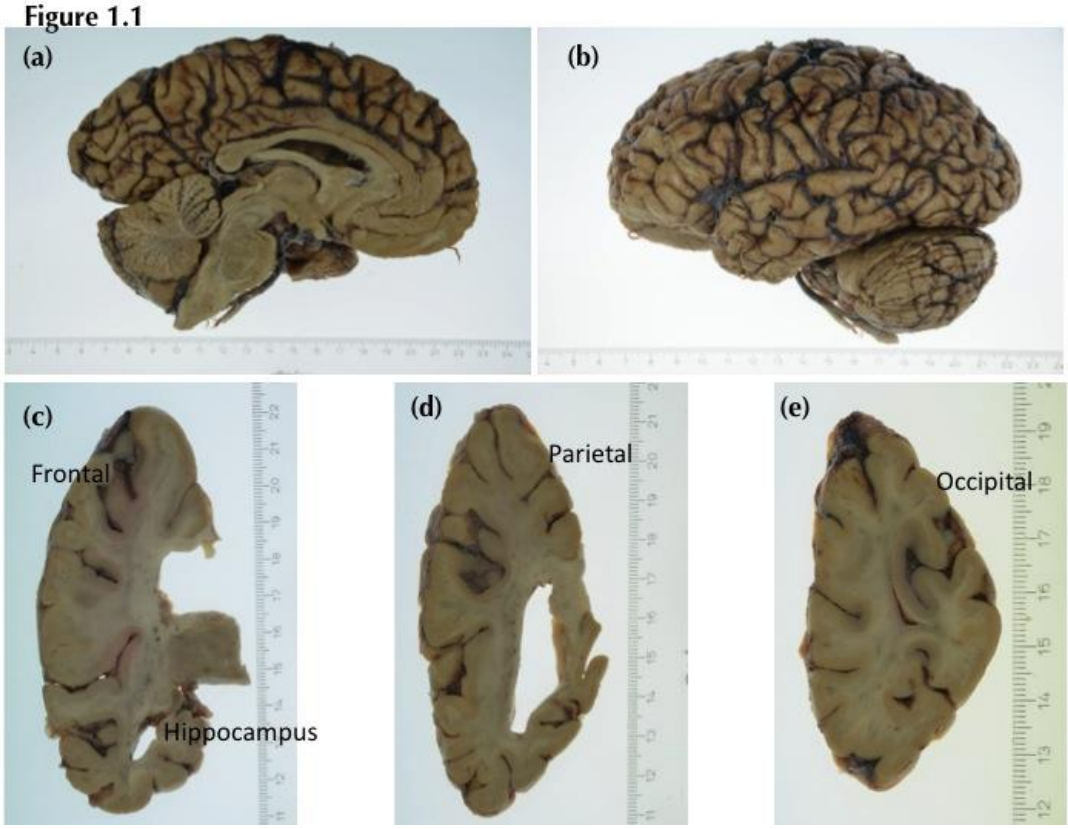

Figure 1.1 Macroscopic pathological appearance in advanced amnestic AD showing marked temporal parietal and occipital atrophy of the left cerebral hemisphere. Figure 1.1(a) left hemisphere, midline sagittal section; (b) left hemisphere sagittal section; (c) left hemisphere demonstrating hippocampal atrophy and frontal lobe atrophy; (d) left hemisphere coronal section parietal lobe; (e) left hemisphere sagittal section occipital lobe (images courtesy of Dr Tammaryn Lashley, Queen Square Brain Bank).

Figure 1.2

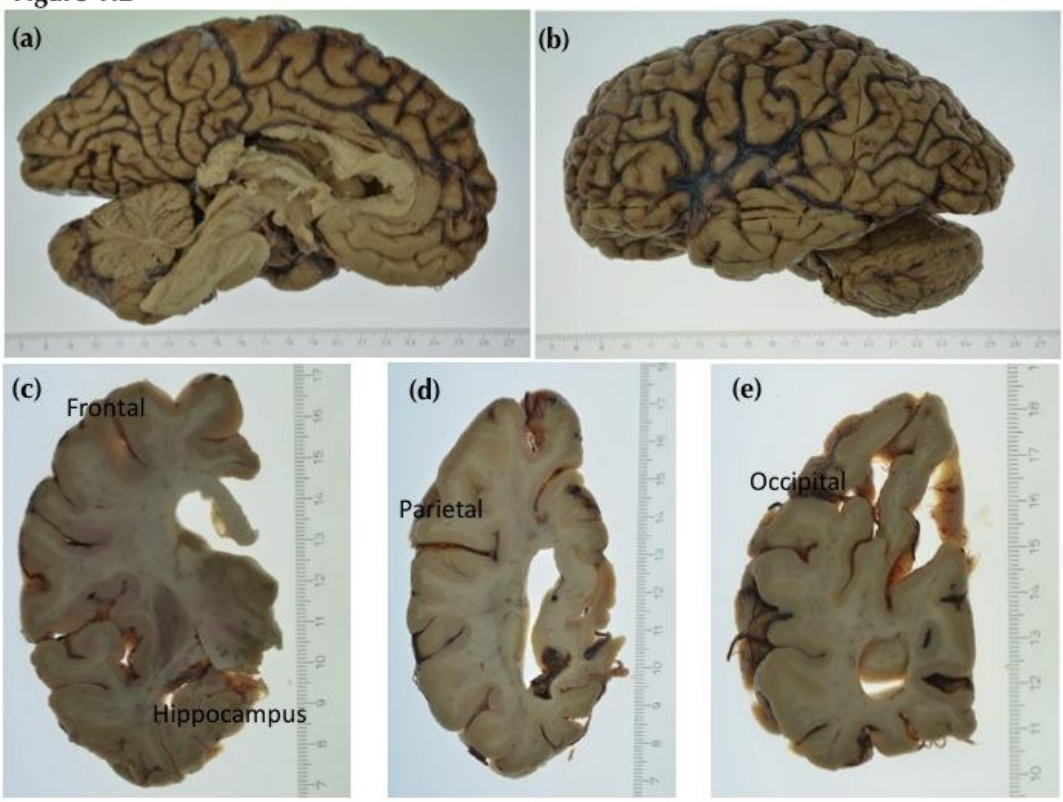

Figure 1.2 Macroscopic pathological appearance in advanced posterior cortical atrophy showing marked temporal parietal and occipital atrophy of the left cerebral hemisphere with atrophy most marked in the occipital lobe. Figure 1.2(a) left hemisphere, midline sagittal section; (b) left hemisphere sagittal section; (c) left hemisphere demonstrating hippocampal atrophy and frontal lobe atrophy; (d) left hemisphere coronal section parietal lobe; (e) left hemisphere sagittal section occipital lobe (images courtesy of Dr Tammaryn Lashley, Queen Square Brain Bank).

1.1.5.2 Microscopic Pathology

"In the centre of an otherwise almost normal cell there stands out one or several fibrils due to their characteristic thickness and peculiar impregnability …numerous small miliary foci are found in the superior layers. They are determined by the storage of a peculiar material in the cortex"
A. Alzheimer, 1907

The pathological hallmarks of AD are: extracellular amyloid plaques (Figure 1.3a); intracellular neurofibrillary tangles containing hyperphosphorylated tau (Figure 1.3b)[51] as depicted by Alzheimer (Figure 1.4) and neuropil threads, which are dendritic and axonal deposits of tau and phosphorylated tau. There is often associated neuroinflammation with microglial activation[52] and amyloid deposition within blood vessels (cerebral amyloid angiopathy) and evidence of neuronal and synaptic loss. Amyloid and Tau containing lesions have a broadly predictable pattern of distribution, with amyloid plaques found throughout the cortex but tau containing lesions are typically seen in the limbic and association cortices and follow a predictable pattern of spread[50, 53, 54] which is poorly correlated with amyloid deposition. As will be discussed in section 1.3, CSF measures of Aβ1-42 (reduced in AD), and elevation of total tau (T-tau) and phosphorylated tau (P-tau) are core biomarkers of AD that are thought to reflect these core pathologies.

A **B** **C**

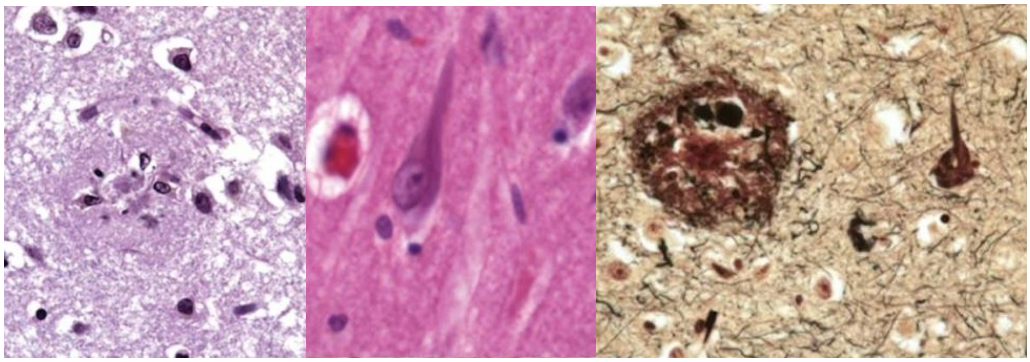

Figure 1.3 A Amyloid plaque from an H&E stained section of frontal cortex; **B** Tau tangle from an H&E stained hippocampal pyramidal neurone; **C** Silver stained slide showing both plaque and tangle pathology. Reproduced from Serrano-pozo *et al*[50].

Other pathological findings such as vascular disease, TDP-43 pathology, hippocampal sclerosis and Lewy Body Disease often co-exist, however their relationship with AD pathology is unclear and their relative contribution to cognitive impairment in any given individual is virtually unknown[55].

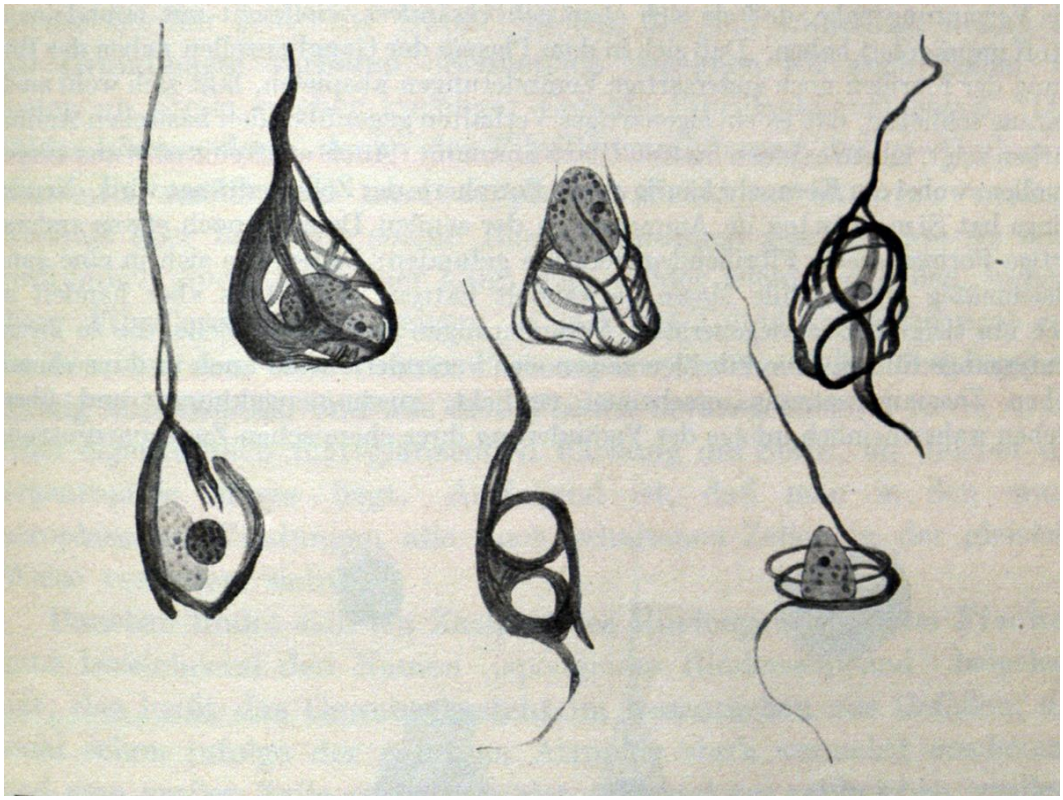

Figure 1.4 Sketch of neurofibrillary tangles in the advanced stages of AD, by A Alzheimer, published in Zeitschrift für die gesamte Neurologie und Psychiatrie: Originalen, 1911.

1.1.5.3 Neuropathological Criteria

Pathological examination remains the 'gold standard' for AD diagnosis and a number of published neuropathological staging methods are in use. Braak criteria[54] and Consortium to Establish a Registry for Alzheimer's disease (CERAD) criteria[56] were designed to quantify the burden of amyloid containing neuritic plaques. Subsequent Thal criteria[53] recognised that amyloid exists in a number of different forms, not exclusively the neuritic plaques seen in advanced disease and that deposition occurs in a hierarchy of brain regions according to a distinct sequence (Figure 1.5). Braak criteria for neurofibrillary pathology[57] are used to stage Tau pathology (Figure 1.6). These systems are now combined in the National Institute of Aging NIA consensus neuropathological criteria for AD[55].

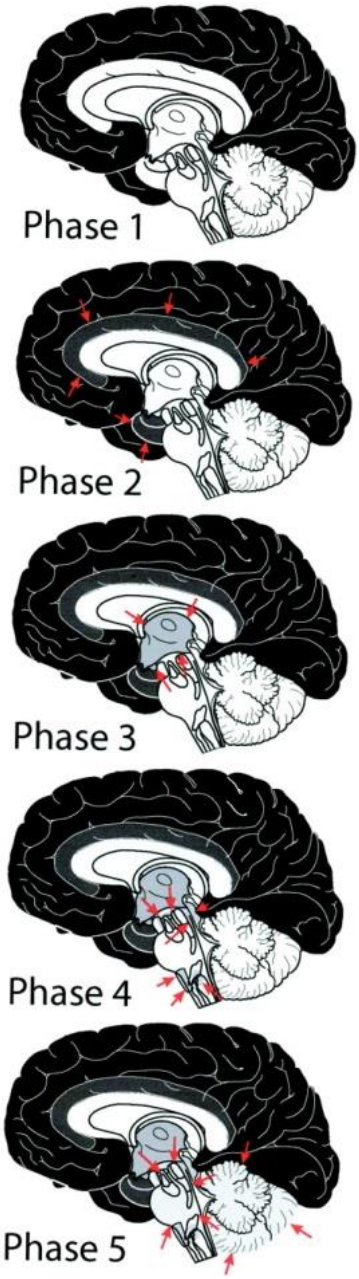

Figure 1.5 Thal Amyloid beta staging in Alzheimer's disease. Phases of β-amyloidosis: Phase 1 is characterized by neocortical Aβ deposition only (Neocortex is black); Phase 2 demonstrates additional allocortical Aβ deposition; Phase 3 shows additional Aβ deposits in diencephalic nuclei (red arrows) and the striatum; phase 4 shows additional Aβ deposits in distinct brainstem nuclei (red arrows), and phase 5 in the cerebellum and additional brainstem nuclei (red arrows)[53].

Figure not included due to copyright restrictions

Figure 1.6 Braak and Braak tau stages of cortical neurofibrillary pathology.

Not included for copyright reasons

It is not clear whether the pattern and distribution of microscopic AD pathology reliably distinguishes typical and atypical forms of AD since any case series have been small, and have provided conflicting conclusions[27].

1.1.6 AD pathophysiology: The Amyloid Cascade Hypothesis

The amyloid cascade hypothesis is the preeminent hypothesis for AD pathobiology, placing amyloid precursor protein (APP) processing and production of toxic β-amyloid (Aβ) species as a key stage in the pathogenic process. Accumulation of amyloid moieties—initially in the form of toxic, soluble oligomers and latterly deposited in the cortex as plaques—is arguably the priming event that subsequently leads to a cascade of events including synaptic dysfunction, microglial and astrocytic activation, abnormal tau deposition and neuronal death, atrophy and ultimately to cognitive symptoms and dementia[60]. The hypothesised mechanisms for amyloid misprocessing are depicted in Figure 1.7.

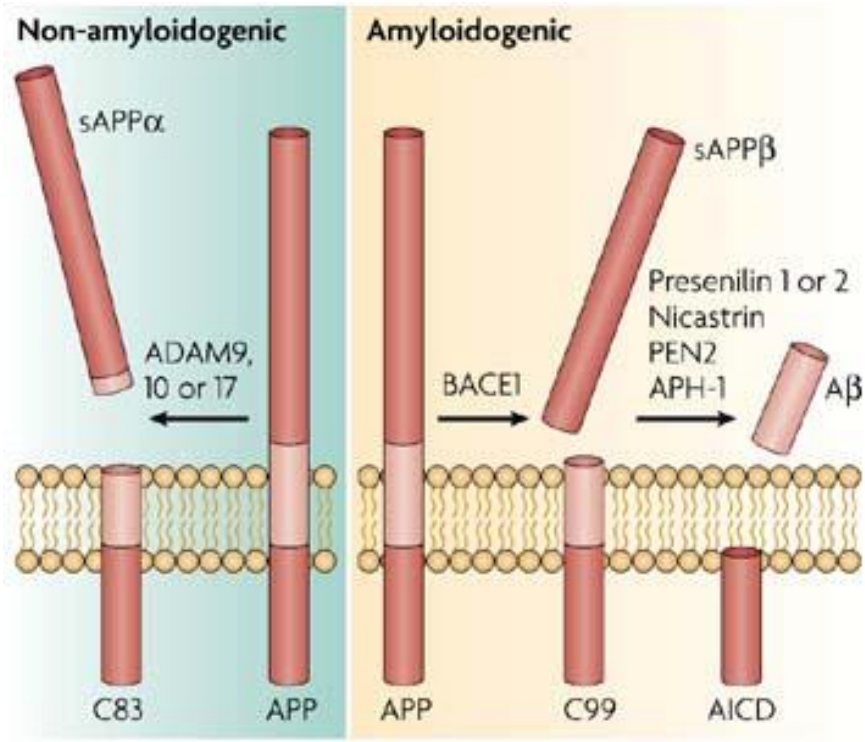

Figure 1.7 The two major pathways of APP (amyloid precursor protein) processing: (1) The non-amyloidogenic pathway. APP can be cleaved by the enzyme alpha secretase (consisting of the a disintegrin and metalloprotease (ADAM) enzymes) to produce sAPP-alpha (sAPPα) and the shorter C83 fragment. APP not cleaved by this route can be processed by the (2) amyloidogenic pathway. APP is cleaved by beta secretase (BACE1) to produce sAPP-beta (sAPPβ), leaving behind the C99 fragment which become the subject of cleavage by the gamma-secretase complex, composed of presenilin 1 or 2, nicastrin, anterior pharynx defective (APH-1) and presenilin enhancer 2 (PEN2). This process can produce fragments of varying length, but mainly Aβ1-40, but also the highly amyloidogenic Aβ1-42. Image reproduced from LaFerla *et al* [61].

The amyloid cascade hypothesis drew heavily on emerging knowledge of AD genetics and in particular instructive cases of familial AD (FAD), discussed later in

this chapter. The discovery that a mutation in the gene coding for the protein APP, the protein which is sequentially cleaved by the β-APP cleaving enzyme (BACE) and gamma secretase was a seminal observation that led Hardy and Higgins to describe the amyloid cascade hypothesis. This was further supported by the observation that mutations in the presenilin genes, which code for proteins involved in the active catalytic sites of gamma secretase led to AD. Since then another mutation in the APP gene which results in reduced BACE cleavage has been shown to be protective against developing AD[62]. The stable isotope-labeling kinetic (SILK) experiments, which allow for measurement of synthesis and clearance of amyloid, have demonstrated a modest reduction in amyloid clearance in sporadic late onset AD[63] providing *in vivo* evidence for altered amyloid metabolism. Whilst this hypothesis explains many aspects of current knowledge of AD pathology, it is incomplete. For example, it is not yet clear how amyloid deposition and tau accumulation are related, exactly how neuronal death occurs, whether inflammation in AD is protective, or harmful or why therapies altering amyloid production or clearance have failed[64].

1.1.7 Genetic determinants and risk factors

1.1.7.1 Familial Alzheimer's disease

A minority of individuals develop AD as a result of inheriting dominant pathogenic mutations of the presenilin 1 or 2 (components of γ-secretase) or APP genes[65]. As mentioned previously, these mutations result in alterations in amyloid processing either through altered γ-secretase function or because of mutations in APP that do not allow normal γ-secretase cleavage[66]. Numerous mutations in these genes have been recognised to cause AD with a penetrance of 100%, with affected individuals generally presenting in their 30s to 50s[67]. These account for less than 1% of cases of

AD overall[66], but are over-represented in young-onset cases. In individuals presenting with symptoms at <50 years of age, sporadic AD is extremely rare[68]. Similarly individuals with Down's syndrome (Trisomy 21) develop AD, typically in their 30-50s because of over-expression of gene products located on chromosome 21. APP is a gene product of chromosome 21[69], but so too are a number of pro-inflammatory mediators such as S100β and a number of cytokines known to cause microglial activation which may explain why the disease course is often more aggressive in this group[70].

1.1.7.2 Genetic risk factors for sporadic Alzheimer's disease

In individuals who develop AD without a dominantly inherited familial mutation, a number of susceptibility genes have been detected. The apolipoprotein E 4 allele (APOE4) is a major risk factor for AD[71] and although its role remains incompletely understood, the protein APOE is a major cholesterol transporter which facilitates lipid transport and supports cell repair within the brain[72]. APOE influences CSF Aβ1-42 in asymptomatic carriers and is dependent on the dose of APOE4 allele[73 74, 75]. Furthermore, it is associated with neural network connectivity disruption on fMRI in individuals without preclinical fibrillar amyloid deposition on PIB PET[76] and with altered patterns of cerebral blood flow on $H_2{}^{15}O$ PET scans in healthy college-aged individuals[77], indicating that it may have a biological role in AD pathology that predates amyloid deposition, widely regarded as one of the earliest events in AD pathophysiology[78].

Genome wide association studies (GWAS) allow for a very large number of genes to be tested in an unbiased way and several large studies have explored genetic risk factors in AD. Nine genes (ABCA7, BIN1,CD33, CLU, CR1, CD2AP, EPHA1,

MS4A4, PICALM) account for about 35% of genetic risk of sporadic AD[79], and a recent multicentre meta-analysis of GWAS data for over 70,000 individuals has identified more than a dozen others. These genetic risk factors strongly implicate four major pathways in AD pathogenesis: the innate immune system; endosomal vesicle recycling; cholesterol metabolism and protein ubiquitination.

Recently, the gene coding for TREM2, which is expressed widely in the brain, particularly the white matter, has been identified using exome sequencing as a susceptibility gene in sporadic AD[80]. TREM2 has a role modulating immune responses in macrophages and dendritic cells—further evidence supporting involvement of the innate immune system.

These pathways may help to explain some of the phenotypic diversity in AD and may in turn lead to new biomarker discoveries and provide new drug targets. Whilst the amyloid cascade hypothesis may be correct, AD pathophysiology is clearly much more than just accumulation of amyloid alone and involves multiple biological pathways[81, 82].

1.1.8 Alzheimer's disease progression

The temporal relationship of the pathological and clinical events that occur in AD, and how they relate to each other has been studied from a number perspectives: using information gleaned from neuropathological studies, familial studies and longitudinal ageing studies. It is now widely acknowledged that the pathophysiological events in AD occur in a specific sequence with the earliest event, probably amyloid deposition, occurring years and possibly decades before the onset

of clinical symptoms. This hypothetical model, summarised by Jack and colleagues[83], makes predictions about how biomarker changes might reflect these pathophysiological changes and so these are also considered briefly here but discussed fully in the second part of this introduction.

1.1.8.1 Current evidence for earliest changes in AD

1.1.8.1.1 Neuropathological studies

As most neuropathological studies in AD are inevitably carried out in the latest stages of the disease, there are limitations to what AD neuropathological evidence can say about the earliest stages of disease and how it evolves. However, in the rare cases where antemortem tissue is available for examination (for example in cortical brain biopsy or tissue obtained during shunt insertion for hydrocephalus) there is already evidence of established amyloid and tau pathology at the time of biopsy in individuals who developed clinical AD within 4 years[84]. No subjects had evidence of Tau pathology without amyloid pathology and few had amyloid pathology without tau aggregation suggesting that both plaques and tangles predate clinical AD by many years.

1.1.8.1.2 Familial Studies

Earlier in this chapter the autosomal dominant genetic mutations that inevitably lead to clinical AD were introduced. In this group, age of symptomatic onset can be accurately predicted by age of onset in one's affected parent[67]. By tracking presymptomatic biomarker changes in mutation carriers, this group have provided an invaluable source of information about the earliest changes in AD. In the 1990s Fox and colleagues demonstrated that structural MRI changes, a surrogate marker of neurodegeneration, can predict age of clinical onset by at least 2 years[85, 86]. More

recently cross-sectional group data from the Dominant Inherited Alzheimer's Disease Network (DIAN) has been published. This is a large multicentre, multimodal observational study of individuals at risk of AD by virtue of carrying one of these autosomal dominant mutations[87]. In mutation carriers CSF amyloid changes were demonstrated 20 years before clinical onset. Brain amyloid deposition, CSF tau elevation and MRI hippocampal atrophy were seen 15 years before symptoms onset. Regional glucose hypometabolism on FDG-PET changes occurred 10 years before symptoms. These results provide support for the idea of a cascade of events leading to neurodegeneration many years before the individual is clinically affected. Importantly, no mutation carriers developed clinical symptoms without having evidence of brain amyloid deposition on amyloid PET scanning, supporting the idea that amyloid deposition is both critical for AD and occurs very early on in AD pathobiology.

It is also important to acknowledge that there may be important differences between familial and sporadic AD. The pattern of brain amyloid deposition seen on amyloid PET differs between familial and sporadic AD[88] and the pathophysiology of amyloid accumulation is likely to be different, with relative overproduction of pathological Aβ moieties being seen in familial AD[89] and failure of clearance of these species being a feature of sporadic disease[90]. Interestingly, the DIAN study, composed mainly of presenilin (PSEN) mutation carriers, suggests FAD mutation carriers have early changes in CSF amyloid levels not seen later in the disease, which may occur more than 20 years before clinical onset of dementia. This finding has also been observed in a young Colombian cohort of individuals with PSEN1 mutations[91]. The significance of this finding and its relevance are yet to be established.

1.1.8.1.3 Longitudinal studies in ageing and sporadic AD

Large-scale, multicentre prospective longitudinal studies such as the Alzheimer Disease neuroimaging initiative (ADNI; full description provided in general methods) and the Australian Imaging Biomarkers and Lifestyle (AIBL) study have used large numbers of subjects at various stages of health or disease to understand the longitudinal course of AD. When considering the earliest biological changes in AD, the study of apparently cognitively healthy control subjects is of particular interest. Many of these studies have confirmed that around one-third of elderly individuals will have either a CSF[73] or amyloid load on amyloid PET imaging[92] within the AD range. Whilst it is not yet known whether these individuals developed clinical AD, it implies that a subgroup have early asymptomatic amyloid pathology, occurring many years before symptoms. Furthermore, healthy controls with evidence of amyloid pathology have accelerated rates of atrophy (i.e. neurodegeneration)[93], in keeping with there being an inverse relationship between [11C]-PIB uptake and hippocampal volume in ADNI controls[94]. Using serial PET imaging, Villemagne *et al*[95] have calculated rates of amyloid deposition for an individual over their disease course, estimating that it takes 12 years for a healthy individual with low amyloid deposition to reach the threshold for PIB PET positivity and a further 19.2 years to develop clinical AD. Taken together, these results suggest that the opportunity to detect the earliest AD biomarkers may extend back as far as middle age.

1.1.9 Current hypothetical models of biomarker changes

Based on the amyloid cascade hypothesis and the syntheses of information gleaned from neuropathological, familial and observational studies of AD, in 2010 Jack and

colleagues published a hypothetical model to describe the temporal relationship of the pathological events in AD[83]. This model relates the disease stages of AD to biomarker changes that occur in a temporally ordered manner that can be depicted as a series of non-linear sigmoid shaped curves predating the onset of symptoms by many years (Figure 1.8). The earliest proposed biomarker changes—in keeping with the amyloid cascade hypotheses—are decline in Aβ1-42 in CSF or deposition of fibrillar amyloid demonstrated using PET imaging. Amyloid accumulation is then followed by synaptic dysfunction, identified by FDG-PET and functional magnetic resonance imaging (fMRI); neuronal injury evidenced by CSF T-tau or P-tau; and then structural brain changes demonstrated by atrophy on structural MRI. Importantly, all these changes predate cognitive decline. Since its first publication, and in light of new data, this model has undergone various modifications, including acknowledgement that tau pathology may precede brain amyloid deposition[78]. A recent study of cognitively normal individuals[96] has shown that neurodegeneration may occur without evidence of amyloid deposition on PIB PET. Possible alternative explanations are that PIB PET scans could be falsely negative, as they do not measure the more toxic soluble form of Aβ[97], that the threshold for determining that a test is abnormal may vary between tests, or that a degree of neurodegeneration may occur in the context of healthy ageing that is indistinguishable, perhaps at least in its earliest stages, to AD.

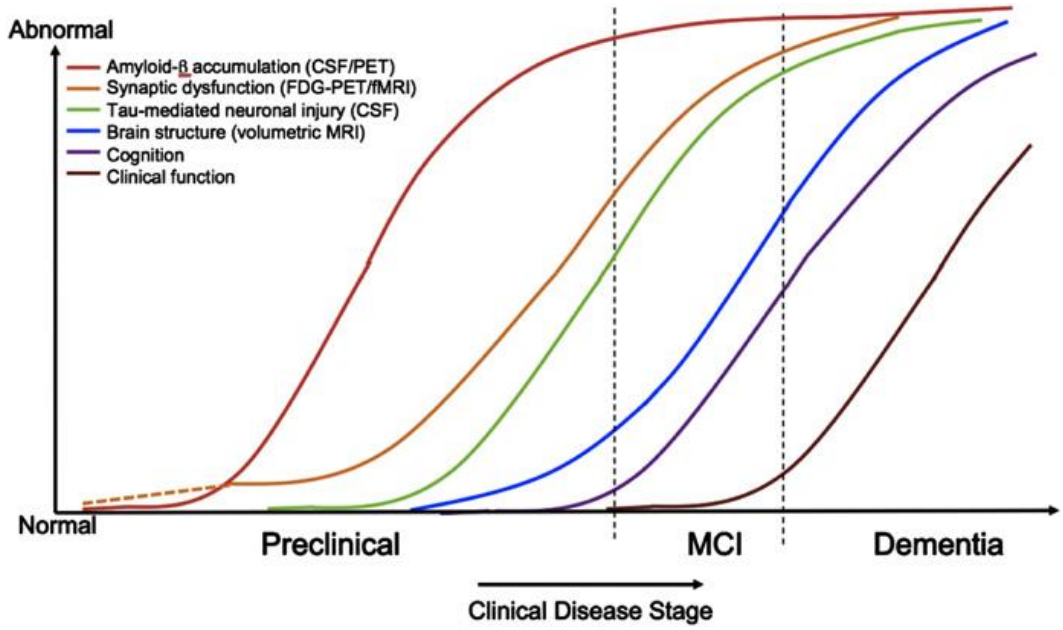

Figure 1.8 The Jack model of AD, a hypothetical model describing the temporal relationship of biomarker changes in AD adapted by Sperling *et al*[90]. MCI: mild cognitive impairment; CSF: cerebrospinal fluid; FDG: fludeoxyglucose; PET: positron emission tomography.

1.1.10 Diagnostic Criteria

The original diagnostic criteria for AD were the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) criteria, known as the NINCDS-ADRDA criteria[98]. They took account of an individual's clinical history and examination, neuropsychological profile and laboratory findings and provided good sensitivity and specificity of around 81% and 70% respectively[99] when validated using pathology proven cohorts. However these criteria had several limitations. To meet criteria for AD they required an individual to be demented, at which stage significant neurodegeneration has already occurred. They did not take account of atypical AD presentations, excluding people with visual, language led or behavioural dysexecutive forms of AD; they had a lower age limit of 40 and upper age of 90 at

symptom onset; they did not take account of genetics and importantly they did not incorporate biomarkers. Over subsequent decades the role of imaging and fluid biomarkers has become clearer. In 2007 the diagnostic framework changed, moving from a clinicopathological to a clinicobiological model of AD where revised criteria incorporated biomarkers. These biomarkers are discussed fully later in this chapter but in brief structural MRI, molecular and functional imaging and CSF biomarkers are now used to support a diagnosis of probable AD in the prodromal phase of disease[100]. The role of these biomarkers is discussed later in the context of the most recent revised AD criteria. Later criteria also recognize atypical non-amnestic presentations of AD.

1.2 Biomarkers for Alzheimer's disease

In the first part of this introduction an overview of the epidemiological, pathological, pathophysiological and clinical features of AD is given. The author also hopes to have alluded to the limitations of current knowledge and some of the practical problems facing clinicians in the diagnosis and prognosis of AD. In this section a case is made for using biomarkers as objective measures of AD pathobiology to help deal with these challenges. The following questions are considered. What is a biomarker? What would an ideal biomarker be capable of? What biomarkers are currently available? How are biomarkers currently used in clinical practice? In the final section CSF is discussed specifically, as one of the most promising sources of biomarkers and the focus of this thesis.

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention[101]. An ideal biomarker is reproducible, stable over time, widely available and reflects directly the relevant disease process[102]. For AD, biomarkers may be used to (1) distinguish different aspects of the underlying pathology; (2) detect presymptomatic pathological changes; (3) predict decline or conversion between clinical disease states(4); and/or monitor disease progression and response to treatment.

It is hoped that eventually biomarkers will facilitate presymptomatic detection of AD. This is an important challenge for the field since available biomarker evidence suggests that AD is a continuum whereby the earliest stages of AD pathophysiology go unnoticed for many years. By the time cognitive symptoms become apparent, significant neurodegeneration has already occurred and any potential therapeutic agents are less likely to be effective[103]. Biomarkers of presymptomatic AD would allow testing of "the right drug at the right time"[104]. However, before the field is in a position to accurately diagnose and monitor presymptomatic AD, a thorough understanding of which biomarker modalities are most useful in symptomatic disease and how they are best measured, validated and implemented into practice is needed.

In this section an overview of currently available biomarkers will be given, covering imaging and fluid biomarkers. The focus will then move to fluid biomarkers for AD, and specifically CSF. The latter section will discuss: currently available CSF biomarkers; emerging CSF biomarkers; confounders in CSF biomarker measurement; practical challenges of implementing CSF biomarkers into practice;

how biomarkers are incorporated into consensus criteria and finally the case for further CSF biomarker research.

1.2.1 Currently Available Biomarkers

1.2.1.1 Imaging Biomarkers

Modern imaging techniques include structural scans (magnetic resonance imaging scans (MRI) and computerized tomography (CT)) and functional techniques (detailed later in this chapter) are attractive biomarkers as they produce high quality images, can be used to compare subjects and measure changes within individuals.

1.2.1.1.1 Structural MRI

Structural brain imaging is recommended for all patients being investigated for dementia according to UK, European and US guidelines[105]. Brain imaging is imperative for excluding 'surgical lesions' such as space occupying lesions, but is increasingly used to aid in a positive diagnosis of a specific cause of dementia. Whilst CT can provide useful information, MRI is generally well tolerated and safe[106] and provides good grey/white matter differentiation without the need for ionizing radiation. MRI in particular can usefully assess vascular damage, other causes of white matter signal changes with a wide variety of causes and spongiform and gliotic changes as seen in prion disease. The pattern of regional brain atrophy has positive predictive value for different dementias and is incorporated into diagnostic criteria for several dementia syndromes[105]. Atrophy can be assessed using simple visual rating scales such as the Scheltens score for measuring medial

temporal lobar atrophy[107] or a range of more complex quantitative manual or automated techniques. Serial imaging – particularly with MRI which provides superior grey white matter differentiation without radiation exposure is widely used as safety and outcome measures in clinical trials[108], with rates of atrophy considered as surrogate markers for neurodegeneration[109]. Whole brain atrophy and hippocampal atrophy measures have been most widely used to track AD neurodegeneration[110] whilst a number of other techniques have been employed[111].

The Boundary Shift Integral (BSI) is a semi-automated method devised to measure volumetric change of a segmented area on matched pairs of structural T1 MRI scans. Following registration (digital matching of serially acquired scans) measurement of the BSI allows for measurement of the change in the boundary of the structure of interest, which is a more accurate estimation of volumetric change than simple subtraction[112]. The method has been adapted to measure changes in ventricular and hippocampal volume and fully automated methods have also been developed[113].

1.2.1.1.2 Functional Imaging

Positron emission tomography (PET) using 18-F-flourodeoxyglucose (FDG) and single photon emission tomography (SPECT) using tracers such as 99mTc-hexamethylpropyleneamine (HMPAO), allows for visualisation and quantification of patterns of brain hypometabolism and hypoperfusion which show characteristic patterns that differ in different dementia syndromes[114]. FDG PET tracer uptake is widely used as a surrogate marker of synaptic function[115]. Dopamine transporter scanning can be used to determine central dopaminergic depletion, as seen in DLB,

Parkinson's disease dementia and is incorporated in diagnostic criteria for these conditions.

1.2.1.1.3 Functional MRI

Functional MRI (fMRI) measures alterations in regional cerebral blood flow using a linked blood–oxygen-level-dependent (BOLD) signal change in the magnetic properties of cerebral venous blood[116]. fMRI techniques can measure intrinsic fluctuations in BOLD signal in the waking brain at rest ('resting state' or rsfMRI) or BOLD changes in response to articular stimulus or task in the scanner ('activation' fMRI). fMRI is an attractive biomarker since it is non-invasive, can be carried out at the same time as structural MRI[117] and has the potential ability to probe the functional integrity of brain networks early in the disease. However, at present fMRI techniques require considerable expertise and a dedicated infrastructure to implement and analyse, which limits their widespread application as biomarkers.

1.2.1.1.4 Molecular PET Imaging

Amyloid PET imaging provides a means of visualising fibrillar amyloid during life, and thus diagnose amyloid pathology in the presymptomatic phase of the disease. The first tracer to bind amyloid (^{11}C-PIB) was developed at the University of Pittsburgh but clinical utility is limited by the tracer's relatively short half-life (~20 minutes)[118]. Since then a number of ^{18}F amyloid tracers with longer half lives have been developed, and to date three have been licensed including florbetapir (*Amyvid*, Eli Lilly and Company), florbetaben (*Neuraceq*, Piramal Imaging) and flutemetamol (*Vizamyl*, GE Healthcare). There is now evidence to show that amyloid PET readings are correlated with plaque burden at autopsy[119-121]. Its routine use is

however restricted mainly due to cost and availability. Amyloid PET is now licensed for use in clinical practice in the United States and in Europe to aid diagnosis in specific clinical scenarios. Good practice guidelines issued by the Alzheimer Association recommend that it may be used in young onset disease (onset before age 65), persistent or unexplained mild cognitive impairment or in cases with an atypical clinical course or with mixed aetiology[122]. Amyloid PET can, on a research basis provide an independent means of validating CSF measures of amyloid pathology. However there are limitations; the substrate for tracer binding is the tertiary beta-pleated sheet conformation of fibrillar amyloid, and so non-plaque pathology, or plaques containing less fibrillar amyloid will not necessarily stain positive. It is not entirely clear what constitutes a positive amyloid scan; scans can be interpreted visually or using a fully automated protocol which compares intensity in the cerebellum or pons, generating the standardized uptake value ratio (SUVR). Amyloid accumulation and amyloid positivity on PET scan is thought to occur ~15-20 years before symptom onset, after which point the PET imaging findings remain static, suggesting that as a biomarker it will have limited capacity to detect disease modification, except perhaps direct amyloid clearance. A recent study has suggested that amyloid PET may be less sensitive than CSF amyloid measures in early disease[123]. Finally, a significant proportion of apparently healthy elderly individuals (perhaps 1 in 3) have a positive amyloid scan, the significance of which is not yet clear[97, 124].

A number of Tau PET tracers have been developed with one, 18 F T807 (AV1451) reaching phase 2 trials as a diagnostic tracer in vivo[125]. This tracer binds tau isoforms found in AD, and can successfully differentiate between AD and healthy controls in vivo. It is however not yet clear whether it can distinguish AD from

other primary tauopathies[125]. It is also not clear how in vivo tau PET measures correlate with CSF tau or post mortem tau pathology. However it remains an attractive potential biomarker because it has potential to diagnose AD neurodegeneration prior to symptom onset, track disease progression[126] with case reports suggest it may have value in explaining some of the phenotypic diversity within AD[34].

1.2.1.2 Fluid Biomarkers

Biofluids such as urine, blood, saliva, and cerebrospinal fluid are attractive biomarkers for a number of reasons: large numbers of tests can be carried out on a single sample; different techniques can be applied to the same sample; large numbers of individuals' samples can be batched and analysed concurrently and they can be frozen and reanalysed many years later as new assays are developed. Ongoing techniques used in biomarker discovery range from hypothesis driven techniques using commercially developed enzyme-linked immunosorbent assays (ELISAs) to more experimental hypothesis generating 'omics' techniques using mass spectrometry.

An ideal fluid biomarker for AD would detect early neuronal dysfunction in the brain many years before neurodegeneration or symptom onset and would spill over the blood brain barrier so that it could be measured in more accessible fluids such as blood, urine or saliva. It would be present at stable levels, without diurnal variation, and not be influenced by handling practices during collection. As yet, no such biomarker exists. In this section an overview of available clinical and research

biomarkers is provided, with a discussion of their potential advantages and their limitations.

1.2.1.3 Blood and urine biomarkers

Fluids that are most easily acquired are the most desirable from the perspective of patient acceptability and tolerability and if they are to be rolled out to primary and secondary care. For this reason there is a great deal of interest in blood and urine biomarkers for AD. However to date reliable blood and urine biomarkers have been elusive and none are currently used in clinical practice. A number of blood based biomarkers have been proposed but results have been difficult to validate in independent studies[127] and none is measured routinely in either clinical practice or on a research basis. There are several possible reasons for this. Assuming many of the processes occur only on the brain, the blood brain barrier is extremely effective at preventing traffic of proteins between CSF and blood so any brain derived proteins or metabolites are likely to be present in extremely small quantities[128], and whilst the blood brain barrier may be compromised in AD, the extent to which this occurs in AD is still not clear[128]; if there is a leak into peripheral fluids any biomarkers may become bound to larger proteins in peripheral blood and are therefore not detectable; proteins may be rapidly degraded by the innate immune system in peripheral blood or metabolized by the liver[128] and finally proteins of interest such as amyloid may also be generated in peripheral tissue and so blood levels may not necessarily reflect brain metabolism[129]. It is also possible that other potential confounders such as seasonal variation, activity and medical co-morbidities may influence measurement[128].

Despite these potential problems and limitations, some studies have reported differences in concentration between AD and controls in more than 2 independent cohorts using ELISA based assays. These include serum APOE concentrations[130] [131, 132] which were found to be lower in AD than healthy controls; β-2 Microglogulin[133, 134] and brain natriuretic peptide concentrations[132] which were reported to be higher. The neurofilament light protein can be measured in plasma, and plasma and CSF levels seem to correlate closely[135]. As CSF NFL is an emerging marker of disease progression in AD and other neurodegenerative diseases[136] this may yet prove to be a promising blood biomarker for disease progression. New technical developments including an ultrasensitive immunoassay for Total Tau hold promise that differences between plasma tau in AD and controls can be reliably detected [137].

In urine, concentrations of brain derived proteins and metabolites are likely to be even lower than in blood and to date no biomarkers have been validated. However the development of more sensitive immunoassays and other mass spectrometry based assays mean that this may change[138].

Salivary biomarkers are also of great potential utility because of ease of acquisition and participant tolerability. Like urine, biomarker concentrations are likely to be low, but already differences in salivary tau measured using targeted mass spectrometry have been demonstrated[139].

1.3 Cerebrospinal Fluid

Although CSF is somewhat more difficult to acquire than blood or urine, it has an important role as a source of AD biomarkers because of its close proximity to, and intimate relationship with, the central nervous system. In this section the normal physiology of CSF production, an outline of why it is important, how it is sampled and analysed and what role each of the commonly measured biomarkers play in diagnosis and prognostication will be discussed.

1.3.1 Normal physiology and function of CSF

CSF is a clear colourless and translucent fluid, which lies within the subarachnoid space of the brain and spinal cord between the arachnoid mater and the pia mater. Approximately 500ml is produced in 24 hours (~20mL produced per hour) and there is around 150ml around the brain and spinal cord at any given time with approximately 25ml in the ventricles. CSF is produced within the choroid plexus of the lateral, 3rd and 4th ventricles of the brain. The choroid plexus consists of finger-like projections of pia mater covered by ependymal cells where CSF secretion occurs by a combination of active transport and filtration. CSF passes through the ventricular system and along the cranial axis propelled by pulsatile waves generated by pulsations from cerebral and spinal blood vessels, constantly moving following the gradient of CSF pressure[140]. It is then reabsorbed through arachnoid villi adjacent to the transverse sinus, other major veins and around the cranial nerves where it is reabsorbed into blood[141]. More recently an alternative reabsorption pathway has been described[142], suggesting that the brain is supplied by a glymphatic

system that allows waste products to diffuse along a series of perivascular channels from CSF into the vascular system.

In normal human physiology CSF and blood are separated by the blood brain barrier, tightly fused brain capillary endothelial cells and astrocytes[143] which permit transport of small lipid soluble molecules with a mass of less than 400-600 Daltons, but prevent larger or insoluble molecules from moving between these spaces[144].

CSF has several important roles: it acts a mechanical buffer to protect the cerebral cortex; it provides immunological support to the brain and transports nutrition (glucose)[24] and allows for the removal of waste products[145].

1.3.2 Why is CSF of interest in Alzheimer's disease?

Cerebrospinal fluid is in close proximity to the brain and so pathological processes occurring in the brain and central nervous system may be reflected in the CSF. For example in central nervous system (CNS) infections such as meningitis or encephalitis, there may be evidence of white blood cells, increased protein levels, or it may be possible to culture bacteria or viruses. In CNS malignancy it may be possible to visualize malignant cells directly, or if there is obstruction of CSF flow due to a tumour then CSF protein levels may be elevated[146].

In neurodegenerative disease, pathological and metabolic changes are also detectable in CSF[127]. Brain derived proteins and metabolites are present in greatest concentration in CSF because of its proximity to the brain and because it is not subject to the same extracerebral confounding factors as blood and urine: the blood brain barrier; the peripheral immune system; large plasma proteins which bind

smaller molecules. CSF is therefore the most promising biofluid for AD and where most progress has been made[127] and will be the major focus of this current work.

1.3.3 Sampling CSF

CSF is usually collected by lumbar puncture, although it can also be extracted directly from the cerebral ventricles during open neurosurgery or through an external ventricular drain. LP is the safest and least invasive means of collecting CSF which involves inserting a narrow bore needle between the lumbar vertebrae, through the ligamentum flavum and dura mater and into the subarachnoid space allowing fluid to drain freely for collection. LP is usually carried out below the spinal level at which the spinal cord terminates and fluid is drawn from the lumbar cistern, typically at the level of lumbar vertebrae L3/L4, L5/L5 or L5/S1. LP can be performed sitting or supine and in most cases is associated with low frequency of serious complications[147] (Figure 1.9). The most common complications are post lumbar puncture headache (5-10%) and back pain[147] but rarely spinal haematoma, meningitis, sixth nerve palsy, hearing impairment or brain herniation can occur[147-149]. Local anaesthetic is used to anaesthetise the skin and subcutaneous soft tissues.

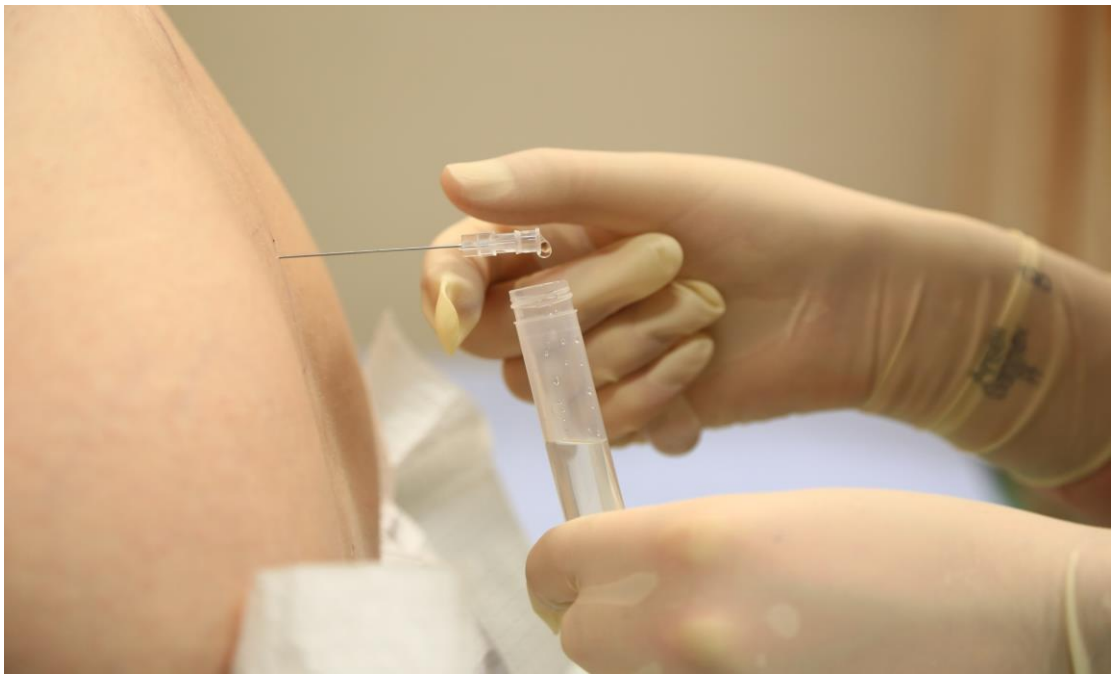

Figure 1.9 The author carrying out a diagnostic lumbar puncture according to the standard operating procedure detailed in Chapter 2 with the patient in a seated position. Photograph courtesy of Dr Alexander Foulkes. Written informed consent was given by the patient.

LP is well tolerated by individuals with cognitive impairment with low reported levels of pain and anxiety[150] and incidence of post-LP headache is lower in patients with dementia[151] than other adults. CSF can be tested serially allowing for evolving pathology to be assessed in vivo throughout the disease course and relatively large quantities can be removed for research purposes without increased risk of adverse events[152].

However, it is not possible in everyone; those on anticoagulation therapy are at greater risk of spinal haematoma[153] and lumbar puncture is avoided in individuals with basal skull abnormalities or any structural cause of raised intracranial pressure due to the increased risk of tonsillar herniation[154].

Patients should also have blood samples collected concurrently so that paired measurements of blood and CSF glucose and albumin can be made. As glucose is actively transported across the blood brain barrier levels should be directly proportional to CSF glucose levels, and should be 50-60% of serum levels in normal physiological conditions[155]. Low CSF glucose can be a marker of CSF infection or tumour[155]. CSF/Serum albumin ratio can be interpreted as a surrogate marker of blood brain barrier integrity[156].

CSF is typically collected in polypropylene containers as certain proteins may adhere to glass or plastic, and transported to the laboratory. When samples arrive in the laboratory, they are centrifuged, and samples typically undergo cytological examination within 2 hours to avoid blood cell lysis[155]. At this stage glucose and protein levels are also measured. Samples are then aliquoted and frozen at -80C prior to undergoing more specific analyses. Samples stored at this temperature can be preserved for many years[157].

1.3.4 CSF Analysis

In recent years there have been significant developments in CSF biomarker measurement techniques. To date, most CSF biomarkers have been measured using enzyme-linked immunosorbent assays (ELISA). The last decade has seen advances in the technical development of ELISA assays with the development of a Luminex technique allowing rapid, reproducible analysis of multiple analytes concurrently[158]. Other techniques such as mass spectrometry have also been used for biomarker discovery and quantification[159] but are not yet used in clinical practice.

1.3.4.1 ELISA

ELISA is a laboratory test which makes use of colour change to measure a protein of interest. Antigens are captured on a plate, usually containing 96 wells. The sample is added and binds to the antigen. A second, detection antibody is added and binds to the protein of interest. This antibody is attached to an enzyme; the substrate for this enzyme is added in the final step producing a colour change, the intensity of which is measured allowing for the protein of interest to be quantified. A range of ELISA platforms, produced by different manufacturers, which may be automated or manual, which measure a single protein or panel of proteins, are commercially available. This is a well-established technique for measuring CSF biomarkers and depending on the platform can produce highly reproducible results. One limitation of this technique is that to measure a given substance, an antibody must be commercially available.

1.3.4.2 Mass spectrometry

Mass spectrometry is a technique which measures the mass to charge ratio of protein fragments and can be used for quantifying proteins with high sensitivity. For biofluids the mass spectrometer is usually coupled with liquid chromatography which allows for the fluid to be introduced to the mass spectrometer through a column, before it is vaporized and ionised and protein fragments are separated according to their mass to charge ratio by electromagnetic fields. The ion signals are collected and measured as ion spectra which are quantified by comparing to internal standards of known quantity. This technique allows for large numbers of proteins to be measured concurrently with high sensitivity and specificity. It can be used for biomarker discovery as well as accurate quantification of proteins of interest.

Peptides can be designed 'in house' allowing for rapid development of assays for proteins of potential interest.

1.3.4.3 Other Analytical techniques

In individuals with rapidly progressive dementia and suspected prion disease the real-time quaking-induced conversion (RT-QUIC) assay is another laboratory test that can be used to differentiate individuals with sporadic Creutzfeldt Jacob disease from controls with high sensitivity and specificity (other neurological or neurodegenerative cases)[160]. This assay mixes RT-QUIC buffer, CSF and brain homogenate, which is then shaken to induce prion seeded aggregation. A positive result induces thioflavin-T fluorescence which can be measured.

1.3.5 Confounders in CSF biomarker measurement

There are several potential confounders in the measurement of analytes due to preanalytical CSF handling as well as significant analytic variability. Interlaboratory co-efficient of variation can be as great as 20-35%, which is likely to be caused by a number of factors and the lack of standardised protocols between centres[60]. A number of preanalytical variables are recognized to influence the measured biomarker concentrations of CSF[161], and are discussed later specifically in relation to amyloid. These include 'patient factors' and 'handling factors'.

1.3.5.1 Patient Factors

Diurnal variation is known to influence measured CSF Aβ concentration[162] and sleep quality may influence amyloid clearance[163], therefore when the sample is taken is

likely to be important. The site of the lumbar puncture may also be important as CSF proteins may have a rostrocaudal gradient[164] although in a study of serial 10ml acquired aliquots, measured Aβ concentration was not affected[162]. Blood contamination of CSF is important since some proteins are found in significantly higher concentrations within blood. Blood cells may lead to the degradation of CSF proteins or these proteins may become bound to plasma proteins which is known to influence Aβ concentration[161]. Whether the patient fasts prior to LP does not influence the measured concentration of Aβ[60] but it may influence other metabolic markers.

1.3.5.2 Handling Factors

The type of tube that is used to collect CSF if a significant confounder; tube material is thought to influence adsorption of some analytes such as Aβ and alpha synuclein and so polypropylene is recommended over glass and other plastics[161, 165]. The tube manufacturer is also important since different brands use different co-polymers of polypropylene with different properties and propensity for proteins to adsorb.[166] Whether a manometer is used to measure opening pressure may be a confounder since most catheters are not made of polypropylene however this has only been studied in small numbers[161]. The time delay between sample collection, the way in which the sample is transported to the lab, including transportation temperature have been studied but results are equivocal[164]. The number of freeze thaws a sample undergoes is likely to be significant for both CSF and blood[164, 167] with measured protein concentrations being lower with repeated freeze thaw cycles. However this is likely to depend on the protein, the number of cycles and the method used for

protein measurement with more sensitive methods such as mass spectrometry perhaps being less susceptible to influence[168].

1.3.5.3 Analytical Factors

Interassay variability of the laboratory ELISA methods themselves is also a problem. Round robin investigations suggest that the co-efficient of variation might be as great as ~36% between centres for any of the 'core' CSF biomarker (Aβ1-42, T-tau, P-tau) ELISA kits[169]. There may also be significant further variation between kit manufacturers or even between kit batches from the same manufacturer[170].

1.3.6 Currently available CSF biomarkers

In this section an overview of how CSF is currently used in the investigation of cognitive impairment. Later, specific CSF biomarkers are discussed individually, focussing first on those available clinically and then those in development.

1.3.6.1 Clinical utility of CSF biomarkers in individuals with cognitive impairment

In the context of individuals with cognitive impairment, lumbar puncture with cerebrospinal fluid examination extraction has, until recently been to exclude infection, malignancy and neuroinflammation using basic microscopy, protein quantification and measuring serum CSF albumin ratio. This is reflected in European guidelines [171] which recommend CSF examination in individuals with cognitive impairment presenting before the age of 55, individuals with rapid disease course, 'unusual' dementia syndromes or those who are immunosuppressed.

Typically in the degenerative dementias the cell count is not raised, there is no evidence of neuroinflammation i.e. the cell count is not raised and the blood brain barrier is intact. In the context or rapidly progressive cognitive impairment elevated total tau to P-tau ratio, S100B, 14-3-3 and real-time quaking-induced conversion (RT-QUIC) technology have positive predictive value for prion disease with a sensitivity and specificity of 87% and 100% for differentiating CJD from other neurological diseases[160]. CSF analysis using a variety of immunochemical techniques allows a range of neuronal-specific or neuronal-enriched proteins to be measured. The neuronal enriched CSF markers β-amyloid, T-tau and P-tau are widely used in the routine evaluation of patients with dementia reflecting amyloid deposition, neuronal loss and cortical tangle formation respectively. When taken together, they can be used to differentiate individuals with Alzheimer's disease pathology from those without with a high degree of sensitivity and specificity[172]. Other established biomarkers which differentiate AD from non-AD subjects which have not yet been adopted in clinical practice include the microglial and astrocytic markers YKL-40 and MCP-1; markers of neurodegeneration: neurofilament light (NFL); neuron-specific enolase (NSE); visinin-like protein 1(VLP-1) and heart fatty acid binding protein (HFABP). Finally, there is a small but significant difference in Aβ1-40, a marker of amyloid metabolism, between AD and control CSF[173].

1.3.6.2 Biomarkers of AD pathology: Aβ1-42

The established CSF biomarkers, currently used in clinical practice, are all indirect measures of AD pathology. Aβ1-42 is produced by proteolytic cleavage of its precursor, the amyloid precursor protein (APP) and it is the major constituent of amyloid plaques. CSF Aβ1-42 is low in individuals with Alzheimer's pathology and there is an inverse correlation with plaque burden[174]. One explanation is that Aβ1-

42 is sequestered within amyloid plaques reducing the measurable concentration in CSF [175, 176]. It is also reduced in individuals with mild cognitive impairment who subsequently develop and fulfill clinical criteria for Alzheimer's disease [177] and even in cognitively normal individuals who later develop AD [178, 179].

In a recent meta-analysis of 142 Alzheimer's disease cohorts and 134 control cohorts from 131 studies, which included 9949 patients with Alzheimer's disease and 6841 controls, all but one demonstrated a difference between AD and controls with a ratio of 0·56 (95% CI 0·55–0·58, p<0·0001) between mean concentrations of Aβ1-42[173].

There are, however, other causes of low Aβ1-42. Neuroinfectious and neuroinflammatory diseases such as bacterial meningitis, Lyme disease, HIV dementia, multiple sclerosis or systemic lupus erythematous alter APP metabolism and are associated with lower levels of Aβ1-42[180]. Other neurodegenerative conditions such as multi-system atrophy[181], motor neuron disease[182] and Creutzfeldt-Jakob disease (CJD)[183] may also be associated with low levels of Aβ1-42 without there being evidence of amyloid pathology at autopsy. Aβ1-40 is typically unaltered in Alzheimer's disease[184] and so the Aβ1-42/Aβ1-40 ratio may be a more specific diagnostic marker of amyloid pathology[185, 186].

1.3.6.3 Longitudinal Stability of CSF Aβ1-42

Relatively few studies have explored longitudinal stability of CSF Aβ1-42. In the ADNI dataset, data from 142 subjects (18 AD; 74 mild cognitive impairment (MCI); 50 cognitively normal subjects) who had lumbar punctures annually (mean follow-up

duration of 48 months; median follow-up duration 36 months) suggest that levels of Aβ1-42 are stable over this period across all diagnostic groups[187] and therefore not necessarily useful for tracking disease progression. For the AD and MCI groups this is reflected in the plateau in brain amyloid deposition described in the Jack model[78].

1.3.6.4 Technical aspects of CSF Aβ1-42 measurement

Of all the measured CSF analytes Aβ1-42 is particularly vulnerable to being affected by handling methods, due at least in part to its hydrophobic nature and propensity to be adsorbed to the walls of collection containers and tubing, as well as to aggregate with itself and other proteins[161]. As previously discussed several confounding factors influence biomarker concentration, particularly Aβ1-42, and include: delay in sample analysis[188]; diurnal variation and tube material[161]. Achieving analytical standardisation between laboratories is an ongoing challenge[189] and is continuously monitored in the Alzheimer's Association CSF biomarker quality control programme[169].

1.3.6.5 Total Tau (T-tau)

T-tau is a microtubule associated protein and it is located principally in neuronal axons. The role of tau in normal physiology is not completely understood but it does have a role in promoting stability of the microtubules involved in axonal transport[190]. Six tau isoforms (352-441 amino acids) are recognised[190]. Neurodegeneration associated with Tau (tauopathies) are typically considered as those with 3-repeats (e.g. Pick's disease), those with 4-repeats (e.g. progressive supranuclear palsy and corticobasal degeneration) and those with 3 and 4-repeats

(e.g. Alzheimer's disease). Tau has a number of phosphorylation sites and tau found within neuritic plaques of AD brains is in its hyperphosphorylated form[191].

Tau is elevated in AD[192] and for the last 20 years has been thought to reflect axonal degeneration, with the magnitude of the tau elevation proportionate to the intensity of axonal degeneration[193]. Inverse correlations between brain volume (hippocampus and grey matter) and T-tau[194] further support this hypothesis, suggesting that tau may be a useful surrogate measure of neurodegeneration. However, it is also now recognised that tau is also secreted by healthy neurons in the absence of cell death [195] and that tau accumulation occurs during aging, often in the absence of cognitive symptoms, a phenomenon known as primary age related tauopathy (PART)[196].

1.3.6.6 Phospho-Tau (P-tau)

P-tau is the hyperphosphorylated form of the microtubule associated protein tau. It is elevated in AD[197] and also in mild cognitive impairment [198] compared with cognitively healthy controls. A number of commercially available assays can be used to measure P-tau phosphorylated at different phosphorylation sites. The diagnostic performance of P-tau$_{181}$, P-tau$_{199}$, and P-tau$_{231}$ is similar[199]. The specificity of P-tau for AD is thought to be higher than either T-tau or Aβ 1-42 alone; indeed there are relatively few conditions that result in raised P-tau. Aside from AD, these include superficial siderosis[200] herpes simplex virus (HSV) encephalitis and is elevated in pre-term infants[201]. Finally, P-tau is correlated with cortical tangle burden[202] indicating that it may be an in vivo marker of disease severity, but to date it has not been shown to be useful for tracking disease or monitoring response to treatment.

1.3.6.7 Other CSF biomarkers in AD

There are currently a large number of potential biomarkers under investigation for AD. The following section is not exhaustive but discusses some of the most studied, most interesting and promising biomarkers in the field and how they might be used in practice.

1.3.6.8 CSF markers of blood brain barrier function

Blood brain barrier function is measured routinely in clinical practice. Its integrity may be compromised in several pathological states: trauma; infection; hypertension; in the presence of small vessel disease. The best established marker is CSF/serum albumin ratio. Blood brain barrier function is typically normal in 'pure' AD[203]. However subsequent studies have given conflicting results[204] and the significance of blood brain barrier dysfunction is not clearly understood.

1.3.6.9 S100

S100 belongs to a family of Ca2+ binding proteins that regulate intracellular levels of calcium. There are 2 sub-units; S100α and S100β; S100β is expressed by astrocytes and oligodendrocytes and has been measured clinically in order to help distinguish prion disease from other conditions[205]. The biological significance of S100β is not completely clear but it may be a marker of neuroinflammation[206]. Some evidence has shown that CSF S100β is elevated in mild to moderate AD compared with clinically severe AD[207] and is correlated with brain atrophy[208]. As S100β is elevated in a number of neurodegenerative conditions including prion disease, FTLD, traumatic cerebral brain disease and infection it is unlikely to be valuable as a diagnostic biomarker. Potentially it could be used to measure response to

treatment but evidence is lacking. The evidence for diagnostic utility of S100 in AD is summarized in the table below.

Author; Year; Journal	Subjects/Site	Main Findings
Infante *et al* 2003 [209]	43 subjects Single site	Significantly elevated in 'Dementia' Vs HC
Nooijen *et al*, 1997 [205]	159 subjects Single site	S100β significantly elevated in sCJD No difference between AD, other dementias or healthy controls
Beaudry *et al*, 1999 [210]	91 subjects Single site	S100β significantly elevated in sCJD
Petzold *et al*, 2003 [208]	31 AD 36 FTLD 49 controls with other non-inflammatory neurological diseases Single site	S100β significantly elevated in AD and FTLD versus controls S100β negatively correlated with whole brain volume.
Peskind *et al*, 2001 [207]	68 AD 25 HC Single site	No difference in CSF S100β between AD and HC. Within AD group S100β higher in those with mild/moderate disease.

Table 1.1 Key research studies determining the diagnostic utility of CSF S100 in AD and other neurodegenerative diseases. AD: Alzheimer's disease; CSF: cerebrospinal fluid; CJD: Jakob-Creutzfeldt disease; HC: healthy control; FTLD: frontotemporal lobar degeneration.

1.3.6.10 Neurofilament Light (NFL)

Neurofilament proteins are major constituents of the cytoskeletal structure of neurons. There are neurofilament heavy (NFH), intermediate (NFI) and light proteins (NFL), with neurofilament light being unphosphorylated and can be measured in CSF. NFL is considered to be an established marker of subcortical axonal degeneration.

Data from the ADNI study suggests that NFL levels are correlated with disease progression in AD; levels are correlated with cognitive function, burden of white matter disease and rates of brain atrophy[136]. However it is likely to lack diagnostic specificity since it is also modestly elevated in healthy ageing[211], in small vessel disease[212], HIV dementia and significantly elevated in FTLD[213, 214] and in amyotrophic lateral sclerosis(ALS)[215]. In multiple sclerosis levels of CSF NFL are elevated in the acute phase of a relapse[216], suggesting that is correlated with acute neuronal injury. Given this lack of specificity, it may have prognostic rather than diagnostic value in clinical practice, but could be a useful outcome measure in a therapeutic clinical trial. As previously discussed, it is one of few biomarkers that can currently also be reliably measured in blood[217].

1.3.6.11 CSF markers of amyloid processing

Amyloid Precursor Protein (APP)

APP is a type I membrane glycoprotein, which is sequentially cleaved to form the various Aβ isoforms. It is associated with neuronal network formation in the developing brain[218],[219] and with neuronal regeneration and calcium homeostasis in the developed brain[220]; Three APP isoforms are recognised (APP695, APP751 and APP770), generated by alternative splicing of exons 7 and 8 which are present in approximate ratios of 20:10:1, respectively, in the human cortex[218]. Although one study showed that a shift in the ratio of APP isoforms in CSF is recognised in individuals with AD and mild cognitive impairment compared with cognitively healthy individuals and may predict conversion from MCI to AD[221].

Beta-site amyloid precursor protein cleaving enzyme 1 (BACE-1)

(BACE-1) is an aspartic-acid protease, and its role is illustrated fully in Figure 1.7. In brief, it cleaves APP to form soluble amyloid precursor protein β (sAPPβ) and a C-terminal fragment (CTFβ), which is then cleaved to produce various anβ isoforms. To date a small number of studies have been carried out which show small increases in CSF BACE-1 in AD and MCI compared with healthy controls, which suggests that the amyloidogenic pathway could be up-regulated in AD[222,223].

sAPPα and sAPPβ

As shown in Figure 1.7, APPβ, is produced via the amyloidogenic processing of APP while sAPPα is produced by the alternative non-amyloidogenic APP processing pathway. As diagnostic biomarkers for AD both have produced disappointing results that have been conflicting or non-significant[173,224-226]. To date no longitudinal data are available for either of these biomarkers. While sAPPα and sAPPβ may have limited use diagnostically in AD, they could still prove to be useful for studying effects on APP metabolism in clinical trials. It is also interesting to observe that some sAPPβ concentrations are reduced in FTLD compared to AD, healthy controls and other neurodegenerative diseases[227], implying that amyloid processing may be altered in the presence of other non-amyloid neurodegenerative pathobiology.

1.3.6.12 CSF Markers of Neuroinflammation

YKL-40

YKL-40, also known as Chitinase-3-like protein 1, is a secreted glycoprotein associated with microglial activation. However its exact function remains unclear[228]. A number of studies have shown that CSF levels are higher in AD compared to

controls[229-232]. Moreover levels also seem to be elevated in preclinical disease[229]. There is no clear correlation between CSF YKL-40 and cognitive function[228] and studies measuring YKL-40 in serum and plasma found non-significant differences between three AD and two control populations[173]. YKL-40 is not specific to AD, and for example is elevated in individuals with multiple sclerosis[233]. It remains to be seen whether it will prove to be a reliable marker of disease activity in AD; however it does seem to be related to disease activity in multiple sclerosis (MS) and CSF levels fall with immunosuppressive treatment[233].

Complement

The role of microglia and the complement cascade are increasingly studied in AD[82]. There are approximately 30 proteins involved in the complement cascade and to date, none has found utility as a biomarker, although a number of proteins have been elevated in AD. As previously discussed the complement receptor 1 gene (CR1), an important regulator of the complement cascade has been identified on a number of GWAS studies in AD.

Other markers of neuroinflammation

A number of other markers of neuroinflammation have been shown to be elevated in AD CSF. These include the matrix metalloproteinases (MMPs), Glial fibrillary acidic protein (GFAP), Neuroserpin, α-2-macroglobulin, α-1 antitripsin, monocyte chemoattractant protein-1 (MCP-1) and others. Most have either been identified in a single clinical cohort or in more than one cohort but with conflicting results[206]. In a recent meta-analysis the only inflammatory biomarker other than YKL-40 found to have a small but significant difference in AD was MCP-1[173].

1.3.6.13 Markers of Synaptic Loss

Biomarkers that reflect synaptic loss are of considerable interest as they may reflect early biological changes in AD and may correlate well with a functional response to a disease modifying therapy. Neurogranin, a post-synaptic marker is detectable in CSF and has recently been shown to be elevated in AD[234], which may be specific to AD[172]. Other notable markers of synaptic function such as neuroendocrine peptides carboxypeptidase E and the chromogranins have also given conflicting results[206,232].

1.3.7 Practical Application of CSF biomarkers

Thus far the diagnostic and prognostic value of individual biomarkers has been discussed. In this section the clinical (and research) application of these CSF biomarkers is discussed considering some specific questions. Which biomarkers are in clinical use and why? Which clinical questions can they help to answer and how reliable are the results? Are biomarkers more clinically useful individually, in combination or as part of a formula? How are clinical cutpoints determined and validated?

As previously discussed, only a small number of these biomarkers are currently routinely used in clinical practice i.e. Aβ 1-42, T-tau and P-tau, with meta-analysis data based on large numbers of studies[173], confirming their utility.

Most clinical studies to date have compared the CSF of AD subjects with healthy controls. It is less clear what the diagnostic sensitivity and specificity of these

biomarkers are in a population of patients with memory concerns, some of whom may have other neurodegenerative diseases and some who may have other neurological or neuroinflammatory diseases which may impact on the CSF biomarker profile. Some studies have considered the diagnostic utility of AD compared to other groups of neurodegenerative diseases pooled together[235] but large studies comparing the profiles of AD CSF compared to other specific neurodegenerative diseases are not yet available. A number of small studies have compared CSF profiles of the different AD variants (memory led, language led or visual), but these studies have produced conflicting results[236-242]. The applicability of biomarker cutpoints developed using populations consisting largely of late onset memory led AD cases to atypical AD cases, many of whom are young onset, is not known. Finally, without pathology confirmation the true diagnostic accuracy of these biomarkers is not known.

In practice, combining biomarkers may improve diagnostic sensitivity and specificity[235,243], particularly when distinguishing AD from other neurodegenerative dementias such as dementia with Lewy Bodies[185]. Tau/Aβ1-42 ratio in particular is a reliable and reproducible measure which is now widely used and has been validated as a diagnostic AD biomarker in a number of large single and multicenter studies[235]. Measuring the ratio of Aβ40/42 (or Aβ42/40) to correct for individual physiological variations in amyloid metabolism may be superior to Aβ1-42 alone for differentiating AD from healthy control CSF[185, 186]. Other studies have used combinations of biomarkers as part of a weighted regression formula or other algorithm to improve diagnostic accuracy[235, 244, 245]. In some cases these formulae/algorithms have improved diagnostic accuracy when compared directly with the performance of single biomarkers. For example Mattson *et al* found that the

combination of Aβ1-42/P-tau ratio and T-tau increased sensitivity and specificity by ~5%, however there is no evidence to suggest any of these formulae are better than the simple ratio of Aβ1-42/T-tau for differentiating AD from controls or other grouped neurodegenerative diseases[235]. It has yet to be established whether larger panels of multiple biomarkers might improve diagnostic accuracy in very specific clinical situations, for example in differentiating language led AD from a language led syndrome underpinned by FTLD or differentiating AD from DLB. Furthermore it is uncertain which biomarkers these formulae or algorithms might contain.

1.3.7.1 Developing reference ranges and cutpoints

Developing normal reference ranges and reliable cut points for any of these AD biomarkers is challenging. Since most CSF biomarkers have continuous values, where the disease state is associated with either higher or lower values than the control group, a continuous variable needs to be turned into a dichotomized variable. In doing this, there are a number of possible statistical methods for defining optimum cutpoints but ultimately there is always a trade-off between sensitivity and specificity and the cutpoint will be different depending on the analytical method used, the statistical assumptions made[246] and the purpose of the test (i.e. optimizing sensitivity or specificity). The other major problem common to all of these statistical approaches is that most CSF biomarker cutpoint data is based on clinical diagnosis, rather than autopsy proven data, which means that even in the best centres, a proportion of the diagnoses will be incorrect. Moreover, since there is considerable variation in both the ELISA assays used and the way patients are classified between centres it is not straightforward to apply cutpoints derived from clinical (or research) populations in other centres.

1.3.7.2 Statistical properties of dichotomized biomarkers

Before discussing the statistical options for dealing with these problems, the relevant statistical terminology is defined:

Sensitivity and specificity: At a given cutpoint the sensitivity of a biomarker is defined as the proportion of cases who truly have the disease who have a positive test result. Conversely the specificity is the proportion of cases who truly do not have the disease who have a negative test result. Sensitivity and specificity can be calculated as follows:

Sensitivity: Number of true positive/(Number of true positives + number of false negatives)

Specificity: Number of true negatives/(Number of true negatives + number of false positives)

Accuracy: This is defined as the probability of the given cutpoint giving the correct answer. Accuracy is dependent on the disease prevalence in the general population. It can be calculated as follows:

$$(\Sigma \text{ True positive} + \Sigma \text{ True negative})/\Sigma \text{ Total population}$$

Receiver operating characteristic (ROC) curves: Sensitivity is plotted against (1-specificity) for a range of potential biomarker cutpoints. ROC curves are often used to measure a biomarkers ability to distinguish between case and control for a binary outcome[246]. Often the area under the ROC-curve (AUC) is quoted; this single value

could be interpreted as the probability of a randomly selected case (with the disease) having a different biomarker value to a healthy control subject.

Youden Index: This is calculated based on ROC curves and is determined using this formula at any given cutpoint: sensitivity + (specificity-1) [247]

Reference Range: This involves calculating the percentile values of a given biomarker for individuals with, or without, the disease in question.

1.3.7.3 Determining cutpoints

A common method for generating cutpoints is to control for sensitivity *or* specificity. This forces a decision as to whether type 1 or type 2 error is more important, which will clearly depend on the clinical context, and to decide on a fixed sensitivity or specificity value. Several previous studies have employed cutpoints based on a fixed sensitivity of 85%[243, 245], which is based on the Ronald and Nancy Regan criteria[102]. These studies favour sensitivity but disregard specificity or accuracy and so are arguably less applicable to clinical diagnostic studies where high numbers of false positive results would be unacceptable. Advantages of this method are that it is straightforward to employ and studies between centres can be easily compared. Importantly it is unaffected by disease prevalence in the population tested[246].

Another common statistical method used for calculating cutpoints is to make use of the Youden index. As previously stated, the Youden index makes use of ROC-curves and the optimum cutpoint can be determined by calculating the maximum value of the Youden index. This can be determined by calculating the greatest vertical

distance from a 45° line to the ROC-curve[248] and calculating the cutpoint where these lines intersect. This method is equivalent to finding the point of maximum accuracy assuming a disease prevalence of 50%[246]. The major disadvantage of this method is that the most accurate cutpoint is determined for arbitrarily chosen disease prevalence. For detecting AD and other neurodegenerative diseases prevalence would rarely be as high as 50% making this method of questionable value.

An alternative method is to calculate the normal reference range for a cohort of healthy controls and then to estimate a percentile above or below which might be considered normal. The disadvantage of this approach is that it requires a suitable disease free control population (noting that some elderly controls may have presymptomatic AD) and does not take account of the reference range of the affected population at all.

1.3.7.4 Other methods for generating cutpoints

Mixture effect models are data driven statistical models, which ignore disease group membership and instead place the cutpoint at the intersection of two subdistributions of data[246]. These methods have been used in ADNI and validated in independent cohorts[249] but have a number of difficulties. Like all the methods discussed, without pathological confirmation there is no gold standard. Furthermore, the crossover of sub-distributions can be wide leaving a wide range in which to select a cutpoint, creating a large grey zone in which biomarker interpretation is difficult. In practice this overlap might reflect genuine biological

uncertainty and employing a diagnostic greyzone may be a more realistic way to interpret these data.

1.3.7.5 Validating cutpoints

An attractive alternative approach to validating cutpoints in the absence of pathology proven cases is to use a surrogate imaging marker for AD pathology. Already individuals who received amyloid PIB scans during life have been autopsy proven[120, 250], and this provides an attractive opportunity to validate CSF Aβ1-42 levels. As other forms of molecular imaging, such as Tau labeled scans become more established, they too may become useful means of validating cutpoints.

1.3.8 Biomarkers in revised diagnostic criteria

Most recent criteria for AD (the latter used for research but not yet used in clinical practice)[3, 4] make use of both imaging and fluid biomarkers which reflect AD neuropathology. CSF Aβ1-42 and PIB PET are used to identify amyloid deposition; CSF Tau, atrophy on magnetic resonance imaging (MRI) and hypometabolism on FDG PET are considered surrogate markers of neurodegeneration for these criteria.

The first criteria to include biomarkers were the 2011 NINCDS–ADRDA criteria[3]. These did not incorporate biomarkers into the core criteria for probable AD, which were purely clinical, but biomarkers were used as supplementary evidence to increase the level of diagnostic certainty. Importantly these criteria do not advocate the use of biomarkers routinely on the grounds that: (1) clinical criteria alone give good diagnostic sensitivity and specificity; (2) it was felt that further biomarker

research was needed; (3) there is limited standardization in biomarkers between centres; (4) there is variability in availability of biomarkers between centres. Individuals currently require evidence of both amyloidosis and neurodegeneration to support a diagnosis of AD for these criteria.

In the later International Working Group (IWG-2) criteria[4] biomarkers are central to diagnostic criteria. Furthermore they do not need to have evidence of both amyloid pathology and neurodegeneration; a positive PIB PET scan is sufficient pathophysiological evidence of AD (Figure 1.10).

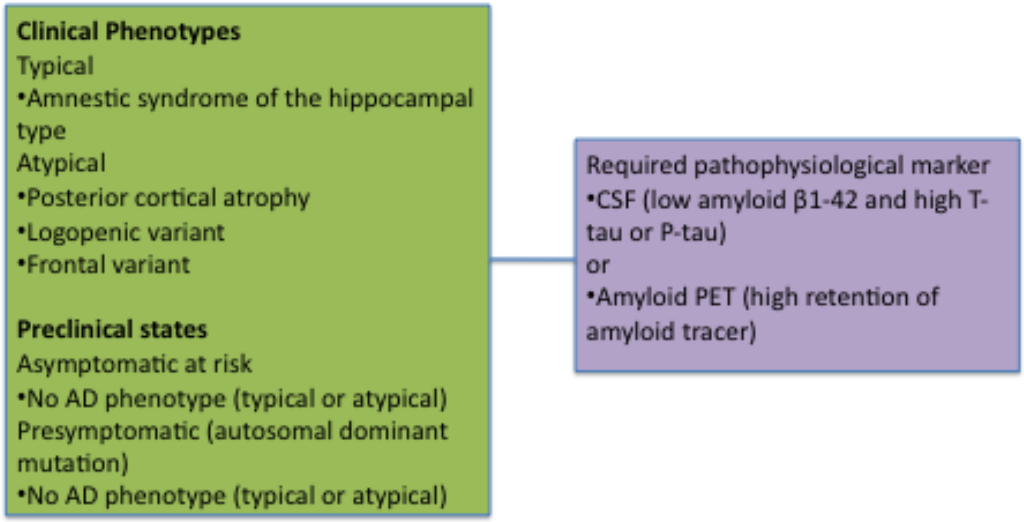

Figure 1.10 The role of biomarkers in IWG-2 criteria is summarized in Figure 1.9. At any stage of the disease and in any of the scenarios given in the green box, the diagnosis of AD also relies on the presence of a pathophysiological marker (purple box). Figure reproduced from Dubois *et al*[4].

These new criteria, which move towards using a pathobiological definition of AD and do not insist on an individual to be demented allow for a diagnosis to be given at an earlier stage in the disease (or at an earlier point in the Jack hypothetical model). This is likely to improve early recruitment to clinical trials when therapy is more

likely to be effective and if they are adopted into routine clinical practice may allow for earlier access to support for affected individuals.

1.3.9 Limitations of currently available biomarkers

As discussed earlier there are neither markers that reliably predict rate of disease progression nor prognosis in AD.

Ultimately as efforts to find a disease-modifying drug for AD are redoubled, biomarkers will be required to detect earliest evidence of neuronal dysfunction, reflect functional and cognitive function and be sufficiently closely correlated with active neurodegeneration that they are capable of changing in response to an effective disease modifying drug. (www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338287.pdf) Based on the evidence presented in this introduction, no single/combination of biomarkers currently seems capable of meeting these requirements. Even if such a CSF biomarker did exist, not all individuals would be able or willing to have a lumbar puncture. If is therefore of critical importance that new biomarkers are rapidly identified and validated in CSF using techniques that can be translated for use using other biofluids.

1.3.10 Developing new biomarkers

As increasing numbers of candidate biomarkers are being investigated, reporting standards have now been proposed so that studies can be easily appraised and compared.

It has been suggested that for Alzheimer's disease, biomarkers should have a sensitivity and specificity >85%[102] and they should be validated on pathologically confirmed cases. Agreed standards on how biomarker studies should be carried out and how results should be reported are now available (Table 1.2)[251] and is the standard to which work presented in this thesis aims to achieve.

Section	Point	Recommended reporting format
Introduction	1	General background information
	2	Specific information about biomarker of interest
Materials and Methods	3	Information on study or sample population (eligibility criteria; comparison with existing gold standard)
	4	Confirmation of approval by ethics committee
	5	Detailed account of outcome measures used
	6	Assay methods: Preanalytical handling methods and confounders Analytical methods and confounders Adequate description of kits and platforms used Performance characteristics
	7	Statistical Methods: (i) Describe statistical methods used (parametric vs non-parameteric; single or two tailed tests; report statistical package and version used) (ii) P values, mean or median, standard deviation or interquartile range should be used as standard. (iii) Correct for multiple comparisons when appropriate
Results	8	Present subject demographics Biomarker characteristics: (i) Show data on biomarkers and their relationship to existing outcome measures (ii) Report sensitivity/specificity/positive and or negative predictive value/ROC curve/odds ratio as appropriate. (iii) Prognostic biomarkers – assess predictive value or time to progression, correlate with disease state or mortality (iv) Therapeutic efficacy biomarkers – report treatment responsive/resistant individuals; pharmacodynamics analysis.
	9	A discussion of the results in relation to the original aims and objectives. Discuss in relation to other relevant studies
	10	Address limitations, sources of potential bias and suggest aims for future work

Table 1.2 Summary of guidance for biomarker research reporting, adapted from Gnanapavan *et al*, Neurology[251]

1.4 Aims and objectives

1.4.1 Aims

The aims of this thesis are:

1. to establish methods to optimise collection, handling and transportation of CSF for established biomarkers for AD in current clinical use outside of specialist centres;
2. to determine the extent to which established and emerging biomarkers can differentiate between the different neurodegenerative dementias and explain phenotypic diversity within AD;
3. to assess the role of CSF biomarkers for tracking disease progression;
4. to provide an exploratory analysis of new biomarkers for AD.

1.4.2 Objectives

The objectives of this thesis are:

1. to measure the effect of aliquot storage volume on measured biomarker concentrations;
2. to compare the methods used to transport CSF to the laboratory and the effects they have on measured biomarker concentrations;
3. to define the role of amyloid PET in determining clinical cutpoints for existing biomarkers;
4. to measure a range of cross-sectional CSF biomarkers and determine their diagnostic ability to distinguish between different neurodegenerative diseases, controls and different clinical phenotypes of AD;

5. to measure AD progression using robust longitudinal measures of brain atrophy and correlate them with cross-sectional CSF biomarkers;
6. to use quantitative mass spectrometry to develop a novel multiplex assay for rapidly identifying and validating new biomarkers of AD.

1.5 Publications arising from this chapter

RW Paterson, J Toombs, CF Slattery, JM Schott, H Zetterberg. Biomarker Modeling of Early Changes in Alzheimer's Disease, Molecular Diagnosis and Therapy. 2014 Apr;18(2):213-27

RM Ahmed, **RW Paterson*** (*joint first authors), JD Warren, H Zetterberg, JT O'Brien, NC Fox, GM Halliday, JM Schott. Biomarkers in Dementia: clinical utility and new directions. Journal of Neurology, Neurosurgery and Psychiatry. 2014 Dec;85(12):1426-34.

Chapter 2. General Methods

2.1 Cohorts

For this thesis, six distinct clinical cohorts were studied. A brief overview of each is provided and summarised in Table 2.1, followed by a detailed explanation in subsequent sections:

1. A cohort of individuals with a range of dementia diagnoses who underwent lumbar puncture and had CSF collected as part of routine clinical investigation at the National Hospital for Neurology and Neurosurgery (NHNN) between 2008 and 2012. Excess CSF samples were pseudo-anonymised, archived and subsequently used for research purposes with appropriate ethical permissions and informed consent. This cohort is hereafter referred to as 'the retrospective cohort'.

2. A cohort of individuals with a range of suspected neurodegenerative disorders who were recruited prospectively by the author. Biofluids including CSF, urine and blood were collected at a clinical-research clinic from individuals at the National Hospital for Neurology and Neurosurgery who required a lumbar puncture for clinical diagnosis, according to a standard operating procedure written by the author, and with suitable ethical permissions and informed consent. This cohort is hereafter referred to as 'the prospective cohort'.

3. A cohort of healthy elderly individuals, without cognitive concerns or objective cognitive impairment, who were recruited to a parallel study by another investigator (Dr Nadia Magdalinou) and who consented to lumbar puncture for research purposes only. CSF was collected prospectively accordingly to a standard

operating procedure. This cohort is hereafter referred to as the 'healthy control cohort'.

4. A cohort of individuals from The Alzheimer's Disease Neuroimaging Initiative (ADNI) study; an international multi-site public/privately funded multimodality longitudinal study, where a proportion of participants had CSF collected prospectively for research purposes. Participants had a diagnosis of either AD, mild cognitive impairment (MCI) or they were cognitively healthy controls. Data were shared publically on the website http://adni.loni.usc.edu/ and accessed directly by the author and used with permission. This cohort is referred to as 'the ADNI cohort.'

5. A cohort of patients with young onset AD (YOAD) and their spouses who consented to a longitudinal multimodality study over 12 months. This cohort was established by the author and Dr Catherine Slattery (CS) and subject recruitment, evaluation and lumbar punctures were carried out by the author, CS and Dr Alexander Foulkes. CSF was collected prospectively according to the same research protocol as the prospective cohort. This cohort is hereby referred to as 'the YOAD cohort.'

6. A cohort of individuals recruited from two regional Swedish memory clinics ('Swedish cohort') by Swedish neurologists Dr Johann Svenson and Dr Per Johansson. Individuals who required lumbar puncture for investigation of cognitive concerns were asked to donate extra research CSF for research. Healthy elderly controls were asked to donate CSF for research purposes only. This cohort is hereafter referred to as 'the Swedish cohort'.

Table 2.1 Summary of cohorts included in this thesis

	Cohort Name	Clinical Institution	Ethical permission	Investigators	Individuals included	Prehandling	Assays	Date of analysis/CV	Chapter
1	Retrospective	University College London NHS foundation trust	NHNN 13_LO_1155 (Retrospectively obtained)	Dr Jonathan Schott; Dr Ross Paterson	Individuals from specialist cognitive disorders clinic	Collected in polypropylene	Aβ1–42, T-tau and P-tau Elisas* Other Elisas^ LC-MS*	Contemporaneously**/ 10% January 2014/<10% May 2014/<10%	3,5,6,7,9
2	Prospective	University College London NHS foundation trust	NHNN 12_LO_1504	Dr Jonathan Schott; Dr Ross Paterson	Individuals from specialist cognitive disorders clinic	Collected in polypropylene according to SOP	Aβ1–42, T-tau and P-tau Elisas* Other Elisas^ LC-MS*	Contemporaneously**/ 10% January 2014/<10% May 2014/<10%	4,5,6,9
3	Healthy Control	University College London NHS foundation trust	NHNN 12/LO/06400	Prof Andrew Lees/ Nadia Magdalinou	Spouses of individuals recruited to an observational research study of atypical Parkinsonism	Collected in polypropylene according to SOP	All Elisas ^ LC-MS*	January 2014/<10% May 2014/<10%	5, 6, 9
4	Alzheimer's Disease Neuroimaging Initiative (ADNI)	Multiple participating centres across Unites States of America and Europe	Ethics committee consent obtained for each participating centre	Prof Michael Weiner	Individuals recruited to an observational research study of AD	Collected in polypropylene according to SOP	Luminex xMAP multiplex immunoassay panel	2011/<12.5%	8
5	Young onset Alzheimer's Disease (YOAD)	University College London NHS foundation trust	NHNN 03N049	Dr Jonathan Schott; Prof Nick Fox; Dr Catherine Slattery; Dr Ross Paterson	Individuals recruited to an observational research study of young onset sporadic AD (age of onset <65)	Collected in polypropylene according to SOP	Aβ1–42, T-tau and P-tau Elisas* Elisa ^ LC-MS*	Contemporaneously**/ 10% January 2014/<10% May 2014/<10%	5
6	Swedish cohort	Skaraborg Hospital, Lindköping,	Swedish Research Council	Prof. Johann Svenson and Dr Per M Johannson	Individuals with memory concerns and healthy controls	Collected in polypropylene according to SOP	Elisa* LC-MS*	March 2013/<10% May 2014/<10%	3, 9

*Denotes analysis carried out at UCL; **Samples were analysed within 4 weeks of sample collection in the clinical laboratory as part of the individual's clinical care; ^Denotes analyses carried out at University of Gothenburg; SOP: standard operating procedure; LC-MS: liquid chromatography mass spectrometry

2.1.1 Retrospective cohort

2.1.1.1 Individuals

Patients referred to the Specialist Cognitive Disorders Service at the National Hospital for Neurology and Neurosurgery are frequently offered a cerebrospinal fluid examination as part of the diagnostic workup of their cognitive complaints. Individuals are assessed either by a consultant neurologist in the outpatient clinic setting, or as an inpatient referral from another consultant neurologist. Since the mid 2000s, when lumbar puncture started to be routinely used in the diagnostic workup for dementia, many hundreds of patients have been assessed by the specialist cognitive disorders team and in most cases any extra CSF was frozen for method evaluation. Most individuals had detailed clinical assessments, at least one form of imaging (usually MRI), often formal neuropsychometry and in some cases clinical genetic testing. In some cases the individuals had died and donated their brains for pathological examination.

2.1.1.2 Consent and ethical considerations

Since sample collection had taken place over a number of years, the majority taken for clinical purposes, many had no research consent. Most samples were held for the purposes of clinical audit and quality control in accordance with the Royal College of Pathologist Guidelines (http://www.rcpath.org/Resources/RCPath/Migrated%20Resources/Documents/G/G035_GuidanceUseofClinicalSamples_Nov12.pdf). The clinical status of these individuals was largely unknown and it was likely that many of them would have died. As it was felt that it would be potentially distressing and inappropriate to

pursue families of these individuals in order to obtain consent retrospectively, as well as impractical given the number of samples collected over an extended time period, consent was sought from the local research ethics committee to analyse samples and to access patients clinical records held at UCLH NHS trust only to obtain necessary clinical information for correlative analyses without obtaining further consent from the subjects involved. It was agreed not to feed back any results of new assays to individuals. It was also agreed that patient identifiable data would only be accessed by members of the clinical care team named on the ethics application. Permission was granted to hold onto clinical data for 15 years and to undertake a range of established and novel biomarker techniques in those individuals where >200uL CSF remained.

The study was approved by the local research ethics committee at the National Hospital for Neurology and Neurosurgery, Queen Square (Reference number 13_LO_1155) with conditions. The conditions imposed by the committee were:

- that only the samples collected up to date that ethical approval was granted would be used in the study
- the investigators provide a list of all the biomarkers to be tested
- and to confirm that if additional biomarkers were to be tested in the future, then a Substantial Amendment would have to be submitted
- that the local Research and Development office would deal with any material transfer issues

2.1.1.3 Inclusion & exclusion criteria

Since 2006-2007 CSF samples have been routinely collected in polypropylene vessels. Prior to this other plastic containers, of varying materials and manufacturers

were used, and this is likely to be major confounder in the measurement of some CSF biomarkers, in particular β-amyloid[161]. With this in mind, samples acquired before 1/1/2008 were excluded as the provenance of the collection tubes could not be guaranteed. Individuals had to be ≥18 years old at the time of CSF collection.

Laboratory records from the neuroimmunology laboratory at the Institute of Neurology were interrogated. Any individuals assessed by a member of the specialist cognitive disorders team (who had a clinic letter documented on the hospital electronic database or a written entry by a cognitive consultant neurologist in the clinical notes) were included. Individuals with ventricular shunts at the time of lumbar puncture were excluded since amyloid moieties may adhere to the plastic shunt confounding results.

2.1.1.4 Archiving historic CSF samples

The author interrogated departmental databases and relevant clinical notes to identify all individuals who may have had a lumbar puncture following their assessment at the Specialist Cognitive Disorders Clinic. All corresponding historic samples stored within the Institute of Neurology Neuroimmunology laboratory in freezers maintained at -80C were then manually reviewed. The identification details were checked and the sample quality and volume (on visual inspection) were documented. Where possible the number of freeze thaw cycles of an individual sample were documented. Individual samples were then given a unique barcoded identifier and these data along with basic clinical information (age at lumbar puncture, sex, pre-lumbar puncture clinical diagnosis) was stored within the neuroimmunology laboratory in a Microsoft Excel database with all other clinically

identifiable details removed. A database containing patient identifiable data and the unique barcoded identifier were documented in a spreadsheet accessible only to clinicians within the department and protected by Firewall.

2.1.1.5 Clinical Diagnosis

For this cohort the author sought to define the clinical diagnosis based on the final diagnosis, which included all available clinical, paraclinical investigations (neuropsychometry, in some cases serial neuroimaging) clinical follow-up (often over many years) in addition to fluid biomarker evidence, i.e. CSF results, which would inevitably influence the treating clinician's diagnosis. Patients were classified according to a pre-agreed list of possible clinical diagnoses (Table 2.2). For individuals to be diagnosed with either probable Alzheimer's disease, Lewy Body dementia, behavioural variant frontotemporal dementia (BvFTD) or a specific language phenotype they had to fulfill contemporary clinical consensus criteria[3, 32, 252, 253] (Table 2.2). To avoid circularity, the author also sought to determine the pre-lumbar puncture clinical diagnosis. This was defined as the treating clinician's working diagnosis based on the available clinical information at that point (usually at least one set of imaging and neuropsychometry and usually 1-2 clinical assessments). Electronic clinical notes were interrogated and the last clinical correspondence prior to LP was used to determine the pre-LP clinical diagnosis. Again, patients were classified according to a pre-agreed list of possible clinical diagnoses (Table 2.2).

Clinical Diagnosis	Abbreviation
1. Amnestic AD	AD
2. Posterior cortical atrophy	PCA
3. Logopenic aphasia	LPA
4. Lewy Body Dementia	DLB
5. Behavioural variant frontotemporal dementia	BvFTD
6. Progressive non-fluent aphasia	PNFA
7. Semantic dementia	SD
8. Vascular dementia	VAD
9. Corticobasal syndrome	CBS
10. Mood disorder	
11. Neurodegenerative disorder - unclear	
12. Parkinson's Disease Dementia	PDD
13. Other	
14. Progressive Supranuclear Palsy	PSP
15. PPA language phenotype that did not fulfil standard Gorno-Tempini criteria [32]	PPA
16. Frontal variant AD	fvAD

Table 2.2 List of possible clinical diagnoses and their abbreviations

2.1.1.6 Independent review of Clinical Diagnosis

In order to independently corroborate the pre-LP diagnosis another clinician was asked to determine the pre-LP diagnosis based on consensus diagnostic criteria. They were provided with copies of the most recent relevant anonymised clinical correspondence and were asked to select from the same list of possible clinical diagnoses (Table 2.1). Independent reviews were carried out on approximately half of the cohort, randomly selected.

All notes reviews occurred over a one month period in December 2012. During the notes review several other aspects of clinical information were also recorded,

including the time from time symptom onset to lumbar puncture as reported by the patient (or collateral source; caregiver; spouse; relative); the earliest symptom was taken; the duration of neurological follow-up defined as the time from lumbar puncture to the last clinical assessment by a member of the specialist cognitive disorders team (either an outpatient clinic appointment or assessment during an inpatient admission); and the most recent mini mental state examination (MMSE) score carried prior to lumbar puncture was also documented (Appendix A). Only MMSEs carried out by a doctors or specialist nurse form the specialist cognitive disorders service were used. In most cases this assessment was carried out at the most recent outpatient clinic review.

In November 2015 records of post mortem diagnosis (where applicable) were updated. A secure password protected Microsoft Excel spreadsheet, containing details of all subjects who have died and undergone post mortem examination at the Queen Square Brain Bank was interrogated by the author. All individuals undergoing post mortem examination gave informed written consent during life, and their relatives gave consent post-mortem. The database was interrogated by name and hospital number of all individuals included in the retrospective cohort and the final pathological diagnosis was recorded. Pathological diagnosis was made according to standard pathological criteria outlined in the introduction.

2.1.2 Prospective Cohort

2.1.2.1 Individuals

Patients with a suspected neurodegenerative disease (including, but not limited to Alzheimer's disease, Parkinson's disease, Dementia with Lewy Bodies,

Frontotemporal lobar degeneration, Parkinson's disease, Huntington's disease) in whom CSF collection was planned as part of their usual clinical diagnostic work-up were asked to donate an extra 15mls of CSF, up to 50ml urine and up to 50ml of blood for research purposes at the time of their planned diagnostic lumbar puncture. Individuals referred to the Specialist cognitive disorders clinic at the National Hospital for Neurology are sometimes offered a lumbar puncture as part of the diagnostic workup of cognitive impairment.

2.1.2.2 Inclusion Criteria

Individuals had to be assessed by clinicians from the specialist cognitive disorders team, be ≥18 years of age and have a suspected neurodegenerative disorder and were willing to consent to this research study. Individuals were either required to have capacity to give consent themselves or in cases where they lacked capacity they were required to have a friend or relative in attendance. Individuals could only be included if their samples could be collected according to a standard operating procedure.

2.1.2.3 Exclusion Criteria

If it was not deemed safe for the individual to have a clinical LP they were excluded. Standard exclusion criteria included: coagulopathy or treatment with blood thinners e.g. warfarin; lumbar spinal surgery within the last 6 months prior or any lumbar spinal procedure that significantly altered the anatomy of the intervertebral spaces; history of chronic or repeat CSF leaks following previous lumbar punctures; active infectious process.

2.1.2.4 Consent and ethical considerations

The author wrote the study protocol and successfully obtained ethical permission to carry out this study. It was approved by the local research ethics committee at the National Hospital for Neurology and Neurosurgery, Queen Square (Reference number: 12_LO_1504). Informed written consent was obtained from all subjects or their appropriate legal representative. No reimbursements or inducements were given. There is no evidence that removal of additional CSF is associated with an increased rate of headache or other side-effects[254] at the time of writing, and subsequently evidence has shown that taking larger volumes (up to 30ml) is associated with lower incidence of post lumbar puncture headache[152].

2.1.2.5 CSF Collection and handling

2.1.2.5.1 Research Biobanking Standard Operating Procedure (SOP)

A SOP for CSF collection and handling was drafted by the author according to research guidelines[73, 255] (http://www.adni-info.org/Scientists/Pdfs/14-Biomarker_Sample_Collection_Processing_and_Shipment.pdf). A full version of this SOP is provided in Appendix B.

2.1.2.5.2 Identification of Individuals

Suitable research subjects were identified via the specialist cognitive disorders service at the National Hospital for Neurology and Neurosurgery or when they attended the National Hospital Day Care Unit having been referred for lumbar puncture for suspected neurodegenerative disease by a consultant neurologist from the specialist cognitive disorders team. Subjects were identified at one of two time points: (1) At the specialist cognitive disorders clinic: In the specialist cognitive disorders clinic subjects were provided with written information, asked to sign a

Data Protection Act form and consent form if they felt ready to do so; (2) At the National Hospital day care unit: the Dementia Research Fellow responsible for managing the CSF database (or deputy) would review the day care unit admission list each Friday afternoon in order to identify potential participants who may or may not have previously been identified in clinic. The consultant in charge was informed by email at least 48 hours' notice prior to the admission and given the opportunity to opt-out. The CSF laboratory technician was informed of prospective participants by email 48 hours prior to admission.

2.1.2.6 Clinical data collection

The subject-specific code stickers were attached to the front of the clinical notes at the point at which consent was obtained. Clinical details (outlined in Table 2.3) were collected using a brief questionnaire by a member of the cognitive disorders team and logged and stored along with this code in a secure clinical database by the research officer at the Dementia Research Centre.

Issue	Clinical information	Comments
1	Name	
2	Hospital Number	
3	Date of birth	
4	Date of first symptom	
5	First symptom	
6	Pre-LP diagnosis	
7	Consultant	
8	Date and time of lumbar puncture	
9	Age at lumbar puncture	
10	Manometer used	Yes/No

Table 2.3 Data collection form to be completed contemporaneously by the doctor carrying out the lumbar puncture

2.1.2.7 Lumbar puncture

The procedure was undertaken in the usual clinical setting, the Day Care Unit of the National Hospital for Neurology and Neurosurgery. The author arranged these visits (provided invitation letters; telephoned individuals to discuss the procedure in advance; coordinated a rota of clinical fellows to assist), personally supervised all procedures and performed more than 50%). The CSF samples were collected between 8am and 12 noon on a Thursday morning. In addition to samples taken for routine clinical analysis. CSF was collected in two screw-top polypropylene tubes (Sarstedt 62.610.018), which were the last tubes filled. Guidelines for CSF collection are outlined in Table 2.4. Pressure readings using a manometer were avoided unless there was a compelling clinical reason to measure it, and this was documented.

2.1.2.8 Transferring samples to the laboratory

All research samples including blood, CSF and urine were stored, transferred and processed together. Clinical samples were handled and transported separately. Samples were accompanied by the lab transfer sheet without other clinical information. The CSF laboratory technician was contacted by the doctor performing the lumbar puncture or a member of the research team immediately after the sample had been successfully collected. Samples were left in a designated metal box at the Day Care Unit reception for collection. The CSF technician personally collected all samples and delivered them to the CSF laboratory within 30 minutes.

Issue		Ideal Situation
1	Preferred Volume	In addition to CSF collected for routine clinical examination (cell count, protein, glucose, neurodegenerative markers) a further 15ml was collected between two polypropylene screw-top containers (Sarstedt 62.610.018).
2	Time of collection	Always between 8-12am to avoid potential for diurnal variation in CSF biomarkers.
3	Other samples collected simultaneously	Blood for storage; urine for storage
4	Local anaesthesia	As per usual clinical guidelines
5	If bloody	To be sent to CSF laboratory regardless
6	Storage conditions	Room temperature before, during and after spinning.
7	Transfer to laboratory	Within 30 minutes.
8	Post lumbar puncture advice	As per usual clinical guidelines
9	Manometer	Avoided unless a good clinical reason

Table 2.4. Overview of the lumbar puncture procedure. CSF: cerebrospinal fluid.

2.1.2.9 Laboratory Procedures

The samples were transferred to the lab in a clear plastic bag provided by the research group. Upon arrival the samples were logged in a standard import spreadsheet for the database. This was done either manually or by using a barcode scanner.

CSF Samples were centrifuged (3000rpm for 10 minutes) and the supernatant fluid aliquoted in volumes of 0.5ml within 1.5ml polypropylene tubes. The tubes were allocated storage positions using Item tracker software, and the individual aliquots given specific positions on the rack with individually unique identifiers. The location information was stored in the database. Unique identifiers for each aliquot generated and printed on nylon labels using a Brady BBP11 Thermal Transfer printer with Ribbon appropriate for storage conditions of up to -100C. Date and time of sampling, collector, condition, date and time of arrival, date and time of freezing and storage condition, cell count, sample quality comments (for example, indicating possible contamination) were also logged in the database. Samples were stored in a -80 freezer within one hour of arrival. The freezers had a monitoring and alarm system for ensuring that sample conditions were maintained in the event of a freezer failure.

2.1.2.10 Clinical Follow-up

Participants were phoned the following day by the author. Subjects were routinely asked whether or not they experienced a headache after the lumbar puncture, or other complications and these complications were documented on the participant questionnaire.

2.1.3 Healthy Control Cohort

2.1.3.1 Individuals

Individuals recruited to a separate research project entitled 'CSF biomarkers in Parkinsonian disorders' were included in this study to provide a valuable cohort of age matched healthy controls subjects. This study was carried out by another

neurology specialist registrar / PhD student at the Institute of Neurology, Dr Nadia Magdalinou (NM). Typically individuals were recruited from the movement disorders or specialist cognitive disorders clinic at the National Hospital for Neurology and Neurosurgery as spouses of affected individuals.

2.1.3.2 Inclusion and exclusion criteria

Individuals were aged 40-85 and had no subjective memory complaints. They were required to have no objective evidence of cognitive impairment on the Mattis dementia rating scale[256]. In addition to the standard contraindications for lumbar puncture in routine clinical practice additional contraindications included: an increased bleeding risk (Platelets <150 per cubic millimeter and an international normalized ratio (INR) <1.5 for blood clotting); history of migraines; known vertebral deformity; obesity (defined as body mass index>30); any signs or symptoms of Parkinsonism as assessed by a movement disorder specialist doctor.

2.1.3.3 Consent and ethical considerations

This study was approved by the local research ethics committee at the National Hospital for Neurology and Neurosurgery (Ethics reference number: 12/LO/06400). Permission to use the samples in this project was granted by major amendment by the same ethics committee. All individuals gave informed written consent.

2.1.3.4 CSF collection and analysis

A standardised clinical protocol developed at the University of Gothenburg for the collection and storage of CSF was followed, similar to that used for the methods described for the 'prospective cohort'. Full details are available at: (www.neurochem.gu.se/TheAlzAssQCProgram)

2.1.4 Alzheimer's Disease Neuroimaging Initiative (ADNI) Cohort

2.1.4.1 Overview

The ADNI is a multicentre public-private initiative, which was established in 2004 with 3 principal aims:

1. To be able to detect AD as early as possible and to be able to track disease progression using biomarkers.
2. To facilitate therapeutic interventions in AD.
3. To expand the ADNI data sharing policy to facilitate access to as many researchers and research groups as possible (ADNI website http://adni.loni.usc.edu/ accessed September 2014)

2.1.4.2 Individuals

Individuals were screened, recruited, assessed, scanned and followed up according to the ADNI procedure manual (http://www.adni-info.org/Scientists/ADNIStudyProcedures.aspx). In brief, they aimed to recruit 800 individuals, including 200 healthy controls, 400 individuals with mild cognitive impairment and 200 individuals with mild Alzheimer's disease. They were recruited from 50 sites across the United States of America and Canada. The general inclusion criteria were:

- Age 59-90 years of age (inclusive) at recruitment
- Have a partner able to provide a collateral history
- Be able to speak English or Spanish fluently
- Be willing to have regular follow-up, MRI, PET scans and lumbar puncture

Inclusion criteria for healthy controls:

- MMSE of 24-30 (inclusive)

- Clinical Dementia Rating (CDR)[257](Appendix C) score of 0.
- Not depressed

Inclusion criteria for individuals with mild cognitive impairment

- MMSE of 24-30 (inclusive)
- CDR of 0.5
- A subjective memory complaint and objective memory deficit measured by education adjusted scores on Wechsler Memory Scale Logical Memory II

Inclusion criteria for individuals with mild AD

- MMSE of 20 – 26 (inclusive)
- CDR of 0.5 or 1.0
- Meets National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA)[3] criteria for probable AD

All subjects were assessed at regular intervals with clinical and cognitive assessments and 1.5Tesla(T) MRI during these assessments. AD subjects were assessed at 0, 6, 12, and 24 months from baseline. Individuals with MCI were assessed at 0, 6, 12, 18, 24 and 36 months from baseline. Age matched healthy control subject were assessed at 0, 6, 12, 24 and 36 months. All individuals were offer a CSF examination at 0 and 12 months from baseline and then annually thereafter.

2.1.4.3 Consent and ethical considerations

The study was conducted in accordance with Good Clinical Practice (GCP) guidelines, the Declaration of Helsinki, US 21CFR Part 50 – Protection of Human

Subjects, and Part 56 – Institutional Review Boards, and to state and federal regulations. All patients gave informed written consent.

2.1.4.4 CSF Collection

CSF samples were collected according to the CSF collection protocol on the ADNI website, appendix to the document (www.adni-info.org/Scientists/ADNIStudyProcedures.aspx). In brief, all samples were collected following an overnight fast at breakfast time using a Sprotte 24G atraumatic needle in all individuals who were willing to have a lumbar puncture. They were carried out with the patient in either the sitting or supine position. Samples were collected in 5ml polypropylene vessels and frozen at the bedside using dry ice.

The author was not directly involved in the collection, handling or analysis of these samples. The full ADNI CSF analysis plan is available online but not reproduced as an appendix due to its considerable size. (http://adni.loni.ucla.edu/wp-content/uploads/2012/01/2011Dec28-Biomarkers-Consortium-Data-Primer-FINAL1.pdf)

2.1.5 Young Onset AD (YOAD) cohort

2.1.5.1 Overview

Subjects for the study in this part of the thesis were recruited as part of the YOAD study. This was a prospective observational cohort study designed to determine the clinical and imaging phenotype of young onset Alzheimer's disease. The author was involved in writing the study protocol, obtaining ethical consent, recruiting individuals, arranging, coordinating and running all clinical visits.

2.1.5.2 Individuals

Prior to recruitment to the study all patients had undergone a full clinical assessment as part of their clinical care including a full history (with collateral informant), comprehensive neurological examination and blood tests to exclude other treatable causes of dementia such as hypothyroidism or vitamin B12 deficiency. The study aimed to recruit 50 subjects with AD, onset before the age of 65 and 25 healthy age matched control subjects.

The general inclusion criteria were:

- Diagnosis of probable AD according to NINCDS-ARDRDA criteria[3].
- Have a partner, friend or family member able to provide a collateral history
- Be able to speak English fluently
- No contraindications to MRI
- Be willing to have regular follow-up, MRI and to complete questionnaires.
- Have consented to have data stored for research purposes under the Data Protection Act (1998)
- Have given or will have given consent to donate DNA for genetic research (Ethical approval through the Queen Square Ethics committee, reference:03N049).

Inclusion criteria for healthy control subjects

- No major psychiatric illness
- No known neurological disease
- Speaks English fluently

Inclusion criteria for AD subjects

- MMSE (or equivalent) score at recruitment of >12/30 (see Appendix A).

- The MRI scan at screening is consistent with a diagnosis of probable Alzheimer's disease.
- Fulfils criteria for probable AD dementia of intermediate or high certainty based on NIA AD criteria incorporating biomarkers[3].
- On the basis of a medical history and physical examination the participant is considered to be otherwise healthy.
- Speaks English fluently

2.1.5.3 Consent and ethical considerations

The study was conducted in accordance with Good Clinical Practice guidelines, the Declaration of Helsinki, US 21CFR Part 50 – Protection of Human Subjects, and Part 56. Written informed consent was obtained. Separate written informed consent was obtained for lumbar puncture. Separate caregiver consent was signed and dated indicating agreement to their own participation as an informant. Participants lacking capacity were not recruited to this research study. However if a participant lost capacity during the course of the study, in accordance with Section 30-33 of the Mental Capacity Act participants were still be able to continue to participate when a suitable consultee was identified. All participants agreed for their general practitioners to be informed of their involvement by letter.

The study was approved by the local research ethics committee at the National Hospital for Neurology and neurosurgery (reference12/LO/0005).

2.1.5.4 Study Design

An overview of the study design is provided as an appendix (Appendix D). In brief, individuals attended 3 visits over the period of 1 year and were offered a lumbar puncture at the final visit.

2.1.5.5 CSF Collection and handling

CSF collection and transfer to the laboratory were carried out using the same standard operating procedure as samples acquired for the prospective cohort. On the sample collection sheet the study name was indicated. Safety data (cell count and protein levels) were made available on the hospital clinical data repository (CDR) and reviewed by the author or deputy with 24 hours of lumbar puncture. Results of neurodegenerative markers were not made available on CDR and were not communicated to participants, as agreed with the local ethics committee.

2.1.6 Swedish Cohort

2.1.6.1 Overview

This cohort was collected by Prof. Johann Svenson and Dr Per M Johannson from a regional memory clinic at Skaraborg Hospital in Sweden. A subset were fully assessed and are described in detail[258]. Individuals were referred by their general practitioners with cognitive impairment and were assessed by a single clinician (JSv) and diagnosed with a range of neurodegenerative diagnoses. Healthy age matched controls were either spouses of affected individuals or recruited from an advert in a local newspaper. This cohort also included CSF samples from individuals who

sought medical advice at another regional Swedish memory clinic because of cognitive impairment. As only the age and gender of these individuals was known, subjects were categorized neurochemically as having AD or non-AD CSF.

2.1.6.2 Consent and ethical considerations

Permission was granted by the Swedish Research council to use these samples for research purposes and all individuals gave written informed consent.

2.1.6.3 CSF Collection and Handling

Samples were collected in polypropylene vessels, and aliquoted within 1 hour. They underwent 1 freeze thaw cycle prior to use.

2.2 Cerebrospinal Fluid Analysis

2.2.1 Enzyme-linked immunosorbent assays (ELISA)

In this section details are given of the assays used to quantify the CSF biomarkers discussed in this thesis. In brief *enzyme-linked immunosorbent assays* (ELISA) were used to test Aβ, T-tau and P-tau and some of the other more novel candidate biomarkers. Where possible, established and validated commercially available assays were used. For panels of novel biomarkers, multiplex assays were used, which allows panels of multiple analytes to be measured simultaneously. In ADNI, the extended panel of CSF analytes was measured using a multiplex assay employing xmaPR technology©(Luminex, Austin, Texas, USA). This technique makes use of bead

technology, which seeks to optimize antibody/antigen capture allowing for smaller volumes of CSF to be measured with a higher sensitivity. The author was not involved in these analyses. For the prehandling experiments (Chapter 3) Aβ1-42 and T-tau were assayed on a Meso Scale Discovery 6000 platform, using MSD Human Aβ1-42 and MSD Human Total Tau kits, respectively (Meso Scale Discovery, Gaithersburg, Maryland, USA). Details of the analytical procedures used are provided in each chapter. For ELISAs used in more than one chapter the methods are described in full below. CSF ELISAs were carried out or supervised by board certified laboratory technicians. The author was involved in planning all experiments and carrying out some of the ELISAs with technician supervision.

2.2.1.1 Innotest (Fujeribio) β amyloid(1-42) ELISA

2.2.1.1.1 Principle

The principle of this test is to capture the β amyloid peptide with the monoclonal antibody, 21F12 (IgG2a) and then incubate it with a second antibody, 3D6 (IgG2b). The antibody antigen complex is then detected by a horseradish peroxidase labelled streptavidin. The colour intensity can then be measured using a microplate reader (Figure 2.1).

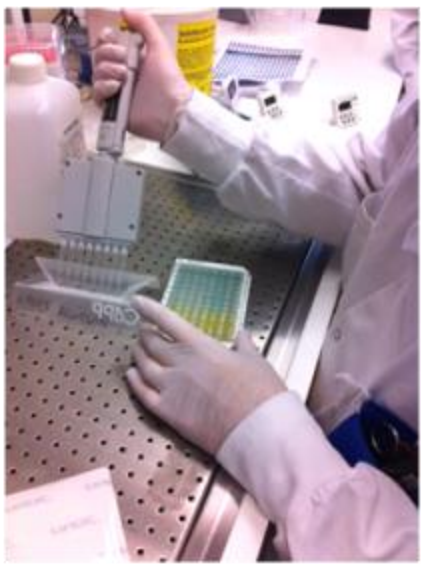

Figure 2.1(a) A microplate reader used at the neurochemistry laboratory at the Sahlgrenska hospital in Goteborg; **(b)** The author pipetting under the supervision of Ulrika Tornqvist, board certified laboratory technician in the same department.

2.2.1.1.2 Analytical procedure

CSF aliquots were defrosted at the benchside at room temperature approximately 60 minutes prior to starting the test. A preparation plate was prepared to reduce dispensing time. 80uL of CSF was pipetted into each well. All samples were then transferred to the pre-coated plate containing the first antibody using a multichannel pipette, using a reverse pipette technique to reduce bubbles. The second antibody in buffer solution (75uL) was then added to each well using the same technique. The plates were then shaken at 700 RPM and incubated for 60 minutes at 25°C. The plates were then washed five times with phosphate buffer and deionized water. At this stage the horseradish peroxidase labelled streptavidin containing solution was added and the plates sealed and incubated for 30 minutes at 25C. The plates were then washed a further five times before adding tetramethyl benzidine containing solution to provide colour. It was then incubated for 30 minutes in the dark. To stop the reaction, a stop solution was added (0.9N sulfuric acid) and the plates were read using a microplate reader with 450nm filter.

2.2.1.1.3 Control procedure

The manufacturer provides 12 calibrators of increasing Aβ concentration in order to produce a standard curve. Four manufacturers controls and an internal control of known Aβ were also included on each plate. For the controls the percentage difference was calculated as follows: (absorbancy (con1-con2)/((con1+con2/2)0*100). Values <20% are considered acceptable by the manufacturer.

2.2.1.1.4 Normal range

Values for the 10th and 90th percentile for this assay are 627 – 1322 pg/ml, based on a cohort of healthy control subject at the National Hospital for Neurology.

2.2.1.2 Innotest (Fujeribio) T-tau ELISA

2.2.1.2.1 Principle

Like the β amyloid 1-42 assay the principle of this test is to capture Total Tau (T-tau) (which includes all 6 isoforms, 352 to 441 amino acids, found in the brain) with a monoclonal antibody AT120. Two secondary antibodies BT2 or HT7 were incubated to create an antibody/antigen complex that could be detected by a horseradish peroxidase labelled streptavidin.

2.2.1.2.2 Analytical Procedure

The procedure was similar to the β amyloid 1-42 assay but used 25uL CSF and was analysed in duplicate. Overnight incubation was required and the plate was also read using a microplate reader with 450nm filter.

2.2.1.2.3 Normal Range

Values for the 10th and 90th percentile for this assay are 146 – 595 pg/ml based on a cohort of healthy control subject at the National Hospital for Neurology. The manufacturer does not provide a normal reference range.

2.2.1.3 Innotest (Fujeribio) P-tau ELISA

2.2.1.3.1 Principle

The principle of this test is to capture phosphorylated Tau (181) using HT7 antibody. It is then incubated with a secondary antibody AT270. The antibody-antigen complex can be detected by a horseradish peroxidase labelled streptavidin.

2.2.1.3.2 Analytical Procedure

The procedure used 75uL of sample (or 75uL control sample) in duplicate. As with the T-tau assay, the plate required to be incubated overnight once the secondary antibody was added.

2.2.1.3.3 Normal Values

Values for the 10^{th} and 90^{th} percentile for this assay are 24 – 68 pg/ml based on a cohort of healthy control subjects at the National Hospital for Neurology. The manufacturer provides a clinical cutpoint of <62 pg/ml.

2.2.1.4 Neurofilament Light Uman Diagnostics (Umea, Sweden) ELISA

2.2.1.4.1 Principle

The principle of this test was to capture and detect CSF neurofilament light using 2 specific antibodies[259].

2.2.1.4.2 Analytical procedure

As previously described, CSF samples were thawed by the bench for 60 minutes. CSF samples were then transferred to a pre-plate, with 105uL in each well which was then diluted with the same volume of buffer diluent as provided by the manufacturer. The pre-coated plates were first washed with a wash buffer. Then

100uL of each sample was added in duplicate to the plate using a multichannel pipette. The plates were then incubated at room temperature and agitated at 800RPM for 60 minutes. The plates were then washed 3 times with wash buffer. The solution containing the second antibody is then added and allowed to incubate for a further 45 minutes with agitation. The plates were rewashed 3 times and at this stage the horseradish peroxidase labelled streptavidin containing solution was added and the plates sealed and incubated for 30 minutes at 25C. The plates were washed 3 times and tetramethyl benzidine containing solution was added for 15 minutes with agitation, before sulfuric acid was added to stop the reaction. The plate was then read using a microplate reader with 450nm filter. As previously described the manufacturer provides controls in addition to internal standards. Inter-assay co-efficients of variation were then calculated as follows: (mean/standard deviation*100).

2.2.1.4.3 Reference range

The manufacturer provides a cutpoint for healthy controls between the age of 40 and 60 of <830pg/mL based on a cohort of 18 individuals with no history of neurological disease.

2.2.1.5 YKL-40 ELISA

2.2.1.5.1 Principle

The principle of this test is to capture CSF YKL-40 using recombinant human YKL-40 antibodies and then to add a detection antibody, which is a recombinant antibody raised against YKL-40. The kit was provided by R & D systems (R&D systems, Minneapolis, MN). The antibody-antigen complex was then labeled and read using a

microplate reader with a 450nm filter.

2.2.1.5.2 Analytical procedure

Samples were defrosted at the benchside for 60 minutes at 25°C. The analytical procedure is identical to the Innotest assays (Ghent, Belgium). 50uL sample and control were required for each well. Incubation time allowed was 2 hours. All samples were run in duplicate.

To the author's knowledge there are no published reference ranges for this biomarker.

2.2.1.6 Amyloid β Triplex assay (X-38, X-40, X-42)

2.2.1.6.1 Principle

The principle of this multiplex test is to capture CSF amyloid β X-38, X-40 and X-42 in CSF using the pre-prepared MULTI-SPOT® Abeta Peptide 3-Plex Plate (Meso Scale Discovery, Gaithersburg, Maryland, USA) then use a specific preparation of detection antibodies, SULFO-TAG™ 6E10 (Meso Scale Discovery, Gaithersburg, Maryland, USA) which allows the three antigens to be detected and quantified using the Mesoscale discovery instrument. Since the antibodies selectively detect amino acids ending in 38, 40 and 42 respectively, the nomenclature X-38/40/42 is used.

2.2.1.6.2 Analytical procedure

Samples were thawed at the benchside for 60 minutes at room temperature and a pre-plate was prepared where 30uL of sample were added to each well. The 96 well plate was prepared by adding a blocker solution and incubating for 60 minutes at

25°C and then washed 4 times using Tris buffer solution. The detection antibody and 25uL sample (and controls + standard curve samples) were then added using a multi-channel pipette. The plate was then sealed and incubated for 2 hours (with agitation) before it was washed 4 times with Tris buffer wash. A 'read' buffer was then added and the sample was ready to be read by the Meso scale platform.

2.2.1.6.3 Analysis and interpretation

Samples were run in duplicate; CVs calculated and standard curve produced using the manufacturers' samples.

Mesoscale Discovery (MSD) also produced ELISA kits for AβX-42 and T-tau and these kits were used with the MSD platform in place of the Innotest ELISAs in Chapter 3. The Mesoscale (MSD) platform was also used to measure sAPPα and sAPPβ in chapters 6 and 7 using the same protocol as the AβX-38/40/42 assay.

The manufacturer does not provide a normal reference range for this assay.

2.2.2 Mass Spectrometry

In this thesis multiple reaction monitoring (MRM) assays were used to identify and quantify peptides, corresponding to proteins in CSF. CSF was digested into fragmented peptides using the protease trypsin. These peptides were subsequently analysed using an ultra-performance liquid chromatography system (Waters, Manchester, UK) coupled to a triple quadrupole mass spectrometer using 2 different approaches: an unbiased *discovery-based* proteomics approach for hypothesis generation and a *targeted* proteomics approach to detect and quantify proteins

thought to be of interest in Alzheimer's disease and other neurodegenerative diseases.

2.2.2.1 Selecting Proteins of interest

A panel of proteins known to be of interest in neurodegeneration was available in the laboratory having been designed and published by Dr Wendy Heywood *et al* [260] to detect markers of neurodegenerative diseases: Lewy Body Dementia; Parkinson's disease and frontotemporal dementia. This panel was developed using the Label-Free Proteomic Analysis (2D-LC-MS^e) method (Waters, Manchester, UK). For this, 400uL of pooled CSF from patients with a range of suspected neurodegenerative disorders including AD, Parkinson's disease, Frontotemporal dementia, collected from 3 different centres in Europe (University of Milan, University of Gothenburg and University College London) was used. These samples were prospectively collected according to a standard operating procedure in polypropylene vessels. Low abundant proteins were enriched by depleting abundant plasma proteins using a 'Top 20' plasma protein depletion column (Sigma-Aldrich, Dorset, UK). Depleted CSF was double digested with LycC and trypsin (Sigma-Aldrich, Dorset, UK) and peptides were fractionated using the high-Ph fractionation technique as previously described [261]. Yeast enolase standard was added and the fractionated peptides were analysed using label free quantitation on a Waters Quadrupole Time of Flight (QToF) Premier mass spectrometer coupled to a NanoAquity liquid chromatography system (Waters, Manchester, UK). Proteins were then identified using Waters ProteinLynx Global server v 2.5 and a UniProt human reference proteome database. Results were controlled for false discovery rate set at 4% and only peptides with >95% confidence were analysed further. Proteins with a mass of >800kDA were

excluded. Other proteins identified on literature search by Heywood et al[260](Chapter 9), when this panel was developed in 2014 were also included in the panel. These included proteins of interest in AD and other neurodegenerative diseases[80, 262-269]. The final list of proteins included in the panel is provided in appendix F.

2.2.2.2 Designing peptides for proteins

This work was carried out by Dr Wendy Heywood at the Institute of Child of Health. Once the list of proteins of interest was determined, suitable corresponding peptides that could be detected in lysed CSF were required. For each protein the amino acid sequence was determined using the online protein directory UniProt for homosapiens (www.uniprot.org). The sequence was fragmented using Skyline software[270] to generate at least 3 shorter peptide transitions (fragment ions). Transitions containing cysteine were avoided where possible since cysteine required modification during sample preparation. Cysteine containing transitions were later capped as later described. These transitions were then entered into the UniProt Basic Local Alignment Search Tool (BLAST) (blast.ncbi.nlm.nih.gov) to ensure that they were unique to the protein of interest (proteotypic). The amino acid codes for these transitions were sent for manufacture (Genscript, New jersey, USA).

2.2.2.3 Capping Cysteine Residues

Cysteine residues are reactive and readily form disulphide bonds with other cysteine residues making it difficult to adequately digest proteins. Reduction and S-carboxymethylation was used to cap cysteine residues to facilitate complete protein

digestion. This was carried out by adding Dithiothreitol(DTE) and Iodoacetic Acid (IAA).

2.2.2.4 Standard Preparation

The internal standard was prepared by combining the following peptides in one standard mix pot:

1. 20ng yeast enolase whole protein (Sigma, UK) was added from a 10ng/uL stock in digest buffer; 2uL added (1mL)
2. T-ApoE aquapeptide was added from 10nmol/ml stock diluted 1/10 (1pmol/uL); 3pmol added (1.5mL)
3. T-tau aquapeptide made to 50pmol/ul; 1uL added (0.5mL)
4. Beta-amyloid 42 aquapeptide was of unknown concentration: Stock was diluted by ¼ and 1ul for assay (0.5mL)

The same internal standard mix pot was used for both discovery and validation phase of each experiment.

2.2.2.5 CSF digestion

This work and subsequent steps were carried out by the author under the supervision of Dr Wendy Heywood and Dr Amanda Heslegrave. Aliquots of 100uL of CSF were freeze dried then re-suspended in 12uL of digest buffer (100mM Tris, pH 7.8, 6M urea, 2M thiourea, 2% ASB14). 7uL of standard mix and 1uL of β-amyloid 1-42 aquapeptide were added followed by 1.5uL Dithioerythritol (DTE) solution for protein reduction, then vortexed for 20 seconds. The samples were then

shaken at room temperature for 1 hour and 3uL Indole-3-acetic acid (IAA) solution, to block reformation of disulphide bonding, added followed by 20 seconds vortexing. The samples were then incubated for 45 minutes at room temperature and mixed with 165.5ul deionised water (DDH2O). Finally 10uL (0.1ug/uL) of Promega trypsin (Wisconsin, USA) was added and samples were left to digest overnight, incubated at 37°C. Samples were then transferred into glass vials for analysis immediately prior to analysis.

2.2.2.6 Standard curve preparation

To prepare the standard curve 700ul of pooled CSF was freeze dried. 7uL standard mix and 7 x 14ul of digest buffer were added. This was split into 7 aliquots and reconstituted in 100ul digest buffer.

Standard curve samples were prepared as follows:

0 – 0uL mix & 165.5ul water added

2 -2uL of 1/10 dilution (1pmol/ul), 163.5ul water added

5- 5ul of 1/10 dilution, 160.5ul water added

10- 1ul neat solution 164.5ul water added

20-2ul neat solution, 163.5ul water added

The same standard curve samples were used for both discovery and validation cohorts.

2.2.2.7 Targeted Proteomic Multiple Reaction Monitoring (MRM) Liquid Chromatography-Mass spectrometry (LC-MS) assay

A multiplexed 10 minute, targeted proteomic triple quadrupole, peptide MRM-based assay was used to measure approximately 50 proteins in each experiment. The same Waters Xevo quadropole mass spectrometer and Cortecs column (Waters, Manchester, UK) was used for all experiments (Figure 2.2). Full details of the experiment design are given in chapter 9.

This involved injecting 35uL of CSF digest onto a Waters CORTECS UPLC C18 + Column, 90 Å, 1.6 µm, 3 mm × 100 mm column attached to a C18+ VanGuard pre-column and dynamic multiple reaction monitoring took place over 10 minutes. Samples were run in duplicate. Quality control (QC) samples, consisting of pooled CSF, were run after every 5 subjects, and CV<10% were considered acceptable. QCs were also run in triplicate at the start of each run to coat the column, and the source tip was cleaned in methanol and formic for at least 20 minutes.

Targetlynx software (Waters, Manchester, UK) was used to analyse chromatograms. Relative peptide quantity was determined by comparing area under the curve of each chromatogram peak with that of a heavy labeled spiked peptide or yeast enolase peptide. The absolute value was determined using the standard curve, where absolute mass could be determined by calculating the peptide's molecular mass. Data were exported to Microsoft Excel and then relative and absolute masses were determined in Graphpad Prism (California, USA).

Figure 2.2 Waters Xevo TQ-S tandem quadrupole mass spectrometer ('Tom') with Cortecs column situated in Dr Kevin Mills' laboratory at the UCL Biological Mass Spectrometry Centre at the Institute of Child Health, Guilford Street. All LC-MS work in this thesis was carried out on this device.

2.3 Imaging

For this thesis two imaging modalities were used: MRI (Chapter 8) and amyloid PET (Chapter 5). Full details are provided in the methods section of each of these chapters respectively.

2.4 Genetic Testing

In this thesis reference is made to genetic testing. All genetic results reported were carried out on symptomatic individuals, on a clinical basis and analysed in the Clinical Pathology accredited Neurogenetics laboratory at the National Hospital for Neurology and Surgery. All individuals gave informed written consent.

2.5 Data Management

To allow accurate management and analysis of data produced by the study of each of these cohorts bespoke databases were designed. Clinical details from the retrospective cohort were pseudo-anonymised and the link code was held in a password protected spreadsheet accessible only to the named investigators in the study. Sample details were held in pseudo-anonymised form on a password protected Microsoft Access database at the Neuroimmunology laboratory within UCL Institute of Neurology.

2.6 Statistical Analysis

Statistical analyses were carried out in collaboration with statisticians at the London School of Hygiene and Tropical Medicine, Dr Jonathan Bartlett, Dr Jennifer Nicholas, Dr Teresa Poole and Prof Chris Frost. Different methodologies were used dependent on the experiments performed and are described fully in each chapter. Most were performed either on STATA version 12.1 (Stata Corporation Texas, USA) and latterly STATA version 14.1 or SPSS version 21 (IBM Corp, Armonk, New York, USA). Certain methodological principles were applied throughout this work, as described in the following subsections.

2.6.1 Testing for normality of data

In order to reliably use parametric statistical methods or linear regression modeling data requires to have a normal, or Gaussian distribution. For this thesis the normality of data was visually inspected using histograms or the inverse normal plots of data using the "qnorm" function in STATA, rather than employing specific statistical methods to detect non-normality, which might fail in smaller data sets[271].

In some cases, non-normally distributed data were log transformed to permit the use of parametric tests.

2.6.2 Handling missing values

Where values are missing, for example if a CSF ELISA failed to capture, this is stated and the case is discarded for that specific analysis. No imputed values are used.

2.6.3 Handling truncated values

Owing to the limitations in sensitivity of some CSF assays at the extremes of the standard curve, some assays employ truncated maximum or minimum values. Where this is the case this is declared in the methods section and non-parametric statistical methods were used.

2.6.4 Adjusting for multiple comparisons

The approach to dealing with multiple comparisons varied depending on the statistical question. In chapter 8, where large numbers of biomarkers were tested and large numbers of comparisons made adjustments were made using the false discovery rate[272].

Chapter 3. CSF collection, handling and processing: Aliquot volume

To optimize the diagnostic accuracy of existing AD biomarkers it is of critical importance to understand the factors that might confound their measurement and to consider how one might control for them.

In archiving samples from the 'retrospective cohort', it became apparent that samples were stored in aliquots of significantly different volumes, simply dependent on the volume that remained after clinical analysis had taken place. Before using these samples for further research, the author sought to establish the impact of aliquot volume on measured CSF biomarker concentration.

3.1 Introduction

There is considerable variability in the prehandling and laboratory handling methods of cerebrospinal fluid before it is analysed for Aβ1-42, T-tau & P-tau. In the laboratory at the National Hospital for Neurology, samples collected for clinical analysis over the preceding years had not been collected according to a standard operating procedure leading to potential inconsistency in prehandling methods and potential for inconsistency in the measured concentrations of these analytes between analyses, between individuals and between different laboratories[273]. Known confounding factors in the measurement of Aβ1-42 and T-tau concentrations include delay in sample analysis[274], diurnal variation[162], CSF contamination with blood or

breakdown of the blood brain barrier[161] and choice of storage tube material[166]. Of the commonly measured analytes, Aβ1-42 seems to be most influenced by these external factors, due at least in part to the hydrophobic nature of Aβ and its propensity to be adsorbed to the walls of collection containers, as well as to aggregate with itself and other proteins[161]. The use of polypropylene tubes mitigates against this problem to some degree, but does not guarantee satisfactory pre-analytic behaviour since most polypropylene tubes are made of copolymers to which Aβ adsorbs to different degrees depending on the tube manufacturer[166]. It was hypothesized that apparent Aβ1-42, T-tau and P-tau concentrations could also be altered by varying the aliquot volume in which CSF is stored, and hypothesized that increasing the ratio of CSF volume to surface area of polypropylene storage container, analyte adsorption would be decreased. At a practical level it was considered whether addition of a buffer-containing detergent might lead to reduced tube surface adsorption and more complete measurement of analyte concentrations.

The aim of this chapter was to determine whether aliquot volume influences the measured concentration of Aβ1-42, T-tau and P-tau and if so, whether it might be lessened by adding a buffer detergent (Tween).

3.2 Contributions and collaborations

CSF sample collection was carried out by clinicians at the Sahlgrenska Hospital, Gothenburg, Sweden. Study design was by the author and Jamie Toombs. Laboratory work was led by Jamie Toombs with assistance from the author. The author and Jamie Toombs wrote first draft of the manuscript. Figures are courtesy of Jamie Toombs. The author carried out the statistical analysis with the support of statistician Dr Jennifer Nicholas.

3.3 Materials and Methods

3.3.1 Sample pools

CSF aliquots of different volumes were tested for Aβ1-42, T-tau, and P-tau. Experiments were conducted over five rounds. For each round, two pools of CSF were tested: the first, from a cohort of anonymised individuals with CSF biomarker profiles consistent with Alzheimer's disease (AD) from the 'Swedish' cohort. These were classified according to biomarker cutpoints that, taken together, and at the time of the experiment were determined to have high sensitivity and specificity for AD[275]: Aβ1-42 <530 ng/L, T-tau >350 ng/L, P-tau >60 ng/L. An exception had to be made for the AD pool used in Rounds 4 and 5 due to insufficient available quantities. This pool was composed of CSF that met the criteria for Aβ1-42 and T-tau, but not P-tau. The second pool was composed of anonymised non-AD control (CTRL) CSF (all three biomarker concentrations within normal range according to local clinical guidelines). Pooled CSF was used because of the large CSF volume requirement of the study design. Different CSF pools were used in the different Rounds: Round 1 was unique, Rounds 2 and 3 were identical except for the addition of a detergent buffer (Tween 20 [0.05%]) to the CSF of Round 3, Rounds 4 and 5 used identical pools.

To confirm that pooled CSF would behave in the same way as that of individual subjects, a series of aliquots of differing volumes was created from the CSF of single subjects (individual, IND) and tested alongside the pooled samples. The individual samples were collected from the 'prospective cohort'. In Round 2, volume was insufficient to allow for P-tau measurement or Tween comparison.

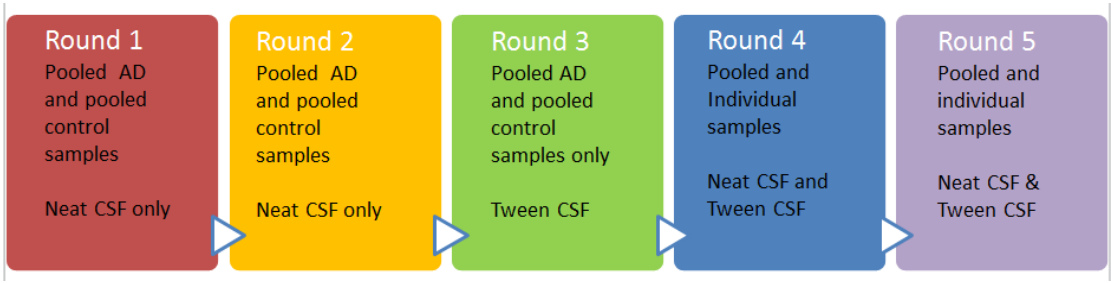

Figure 3.1 Outline of experiment design

3.3.2 Sample treatment

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3.3.3 Schedule and CSF analysis

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3.3.4 Statistical analysis

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3.3.5 Surface area calculation

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3.4 Results

3.4.1 Aβ1-42

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Figure 3.2 Restricted due to copyright.

3.4.2 T-tau

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Figure 3.3 Restricted due to copyright.

3.4.3 P-tau

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Figure 3.4 Restricted due to copyright.

3.4.4 Tube surface area

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Figure 3.5 (a) Restricted due to copyright.

3.5 Discussion

This study shows that the storage volume of CSF samples has a significant impact on the measured concentration of Aβ1-42, and to a lesser extent P-tau but not T-tau. Furthermore the introduction of a buffer detergent to CSF samples at the initial aliquoting stage may be an effective solution to this problem.

One possible explanation for these findings is that Aβ1-42 is hydrophobic which causes it to adsorb to the walls of the storage vessel. It is also possible that sample volume might influence amyloid kinetics and the conversion of Aβ1-42 to oligomers and fibrils in vitro[276]. Since standard CSF Aβ1-42 ELISA measures free Aβ1-42 only, a change in ratio of free Aβ1-42 to oligomers or fibrils may confound measurement. In this section, other factors that may have influenced results such as surface area are considered.

3.5.1 Surface area

There was an inverse relationship between the measured concentration of Aβ1-42 and the surface area per volume ratio of the storage tubes tested. This suggests that surface adsorption may be a key factor in the observed trend for measured Aβ1-42 concentration to increase with storage volume.

In the pooled AD CSF of Round 1 there was an association between volume and measured T-tau concentration, but this could not be reproduced in subsequent assays and therefore seems unlikely to be a real finding. The control and individual pools for T-tau consistently demonstrated no effect between volume and

concentration, and this was also the case for most P-tau pools. This is perhaps not surprising since the 6 tau isoforms that comprise 'T-tau' are small molecules, highly soluble and not hydrophobic like Aβ[190] and therefore more likely to remain in solution than to adhere to polypropylene. Figure 3.4 shows that although neat Aβ1-42 concentration and surface area seem related, the correlation is not perfect, suggesting that other factors may play a role.

It is challenging to explain why samples stored in 1500µL aliquots had lower measured biomarkers concentrations; inconsistent with the overall trend. It is possible that when volume reaches a tube's maximum capacity, the remaining space is insufficient to allow thorough mixing by the method of vortexing used. It is also possible that the sample undergoes greater agitation since it is more likely to be in contact with the vessel lid. Greater sample agitation can be associated with greater protein denaturation which in turn facilitates amyloid adsorption[277]. This trend requires to be explored further.

3.5.2 Tween 20

Tween 20 is a nonionic detergent widely used in biochemistry for a number of different purposes including cell lysis, as a blocking agent for immunoassays and in this case as a emulsifying agent(Sigma Aldrich, Dorset, UK: www.sigmaaldrich.com). Tween 20 treated Aβ1-42 samples had considerably higher concentrations of Aβ1-42 than neat samples. Tween 20 also seemed to lessen any effect of differing aliquot volume. This observation may support the hypothesis that Aβ1-42 adsorbs to the vessel wall and suggests that a greater proportion of Aβ1-42 molecules were free in the solution of the storage tube after treatment. These

findings are consistent with another study[161]. However it is also important to acknowledge that Tween could profoundly alter the behaviour of CSF amyloid, and could potentially alter the behaviour of amyloid oligomers or fibrils, misrepresenting the physiological Aβ1-42 concentration in vitro. Furthermore, if Tween 20 were to be added routinely to samples then Aβ1-42 cut points for clinical practice, clinical trials and research studies would require to be adjusted accordingly. To introduce this practice universally between laboratories would be a major undertaking, especially if the relatively cheap and simple practice of standardizing aliquot storage volume would produce a similar result.

Tween 20 tau sample results very closely reflected the results of neat samples, suggesting negligible quantities of tau are lost to surface adsorption, and that Tween 20 does not appreciably alter detection.

3.5.3 Temperature

It is possible that the observed relationship between volume and measured analyte concentration is due to the effect of variation in thaw times. It is known that the number of freeze/thaw cycles have an effect on the measured protein concentration of a sample[188] although the mechanism for this is not clear. Every sample in this study underwent two freeze thaws or less and this did not vary within rounds. All aliquots were thawed together at room temperature for approximately 1 hour, but large volume samples thaw more slowly (-80°C - ~21°C took approximately 60 minutes for a 1500µL aliquot compared with a few minutes for the smallest volumes) and so spent less time at room temperature, and therefore less time in solution than the low volume samples. It may be that the additional time spent at room

temperature for lower volume samples allowed more proteins (particularly Aβ1-42) to denature, become proteolysed, aggregate, or adhere. Furthermore, it has been demonstrated that the conversion of Aβ1-42 to amyloid oligomers (which are thought to be pathogenic but cannot be measured using standard Aβ1-42 ELISA) is influenced by a number of factors including incubation time in aqueous medium[278]. This is, however contrary to the findings of another similar study which has shown that CSF, not previously frozen, that rests at room temperature for 24 hours has significantly higher levels of measured Aβ1-42 than CSF that is frozen immediately[279]. In the same experiment they saw no difference in measured T-tau or P-tau concentration. There is therefore no compelling evidence to suggest that temperature is a major contributing factor to the differences observed between samples of different volume.

3.5.4 pH

Finally, given that pH is known to be a major factor to influence Aβ1-42 aggregation and oligomerization[280], we considered whether pH could have varied between aliquots of differing volumes. While pH was not explicitly measured, no reagents or vessels known to have a non-neutral pH were used. CSF pH is usually closely physiologically regulated so we do not anticipate any significant variation between rounds, even for individual samples[281].

3.6 Further Work

Adsorption of Aβ1-42 to vessel wall proportionate to the volume to surface ratio is a credible explanation for the results observed in this experiment. It is also consistent

with what is known about the properties of Aβ1-42. The significant relationship between T-tau AD pooled CSF and volume to surface area observed in Round 1, but not in other rounds, was unexpected and may simply be a chance finding. It is worth considering that the T-tau assay detects six isoforms of the protein, and it may be that some are more vulnerable than others to the variables discussed, however there is currently no commercially available ELISA kit available to measure these isoforms and this experiment would arguably be better carried out on a more sensitive platform such as single molecule array technology (SIMOA, Quanterix, MA, USA).

3.7 Conclusions

Aβ1-42 is now widely used as a diagnostic marker for Alzheimer's pathology. It is therefore concerning that a twofold difference can exist between the measured Aβ1-42 concentrations of the 50µL and 1500µL volumes in both AD and control CSF. This could easily result in misclassification of individuals in both clinical and research settings, and is a source of variance that, to our knowledge, has not previously been considered or investigated. As the field moves towards 'analytical harmonization' of CSF between centres[189], this study suggests that aliquot volume should also be standardized within and between centres. Furthermore, as the addition of a readily available buffer detergent appears to neutralise the effect of Aβ1-42 adsorption (and potentially that of other biomarkers), the addition of Tween 20 to aliquots immediately before sample storage could also be explored as a practical solution to the problem.

In this thesis the retrospective cohort samples were collected in inconsistent aliquot volumes, which may be a confounder when interpreting Aβ1-42 values. All samples collected prospectively in the 'prospective' and 'healthy control' cohort were

collected according to a standard operating procedure where aliquot volumes were 1000uL, as a result of this work.

3.8 Publications arising from this chapter

J Toombs* and **RW Paterson***, MP Lunn, JM Nicholas, NC Fox, MD Chapman, JM Schott, H Zetterberg. A potential confound in cerebrospinal fluid biomarker measurement: aliquot volume. Clin Chem Lab Med. 2013 Dec;51(12):2311-7. *joint first author

3.9 Publications related to this chapter

J Toombs and **RW Paterson,** MP Lunn, JM Nicholas, NC Fox, MD Chapman, JM Schott, H Zetterberg. The impact of Tween 20 on repeatability of amyloid β and tau measurements in cerebrospinal fluid. Clin Chem Lab Med. 2013 Dec;51(12):2311-7

J Toombs and **RW Paterson**, JM Schott, H Zetterberg. Amyloid-beta 42 adsorption following serial tube transfer. Alzheimer's Research and Therapy, 2014 Jan 28;6(1):5

Chapter 4. Transferring CSF from bedside to laboratory; does careful pre-analytical handling of CSF affect measured Aβ1-42, T-tau, P-tau in clinical practice?

4.1 Introduction

The ultimate aim of CSF biomarker research is to develop biomarkers that can be used in clinical practice to improve accuracy of diagnosis, estimate prognosis and monitor response to treatment. It is therefore important to establish whether biomarkers discovered and developed in research populations collected under optimal standardized conditions are applicable and reproducible in 'real life' clinical populations, possibly acquired outside major neurological centres. One of the major differences between clinical and research samples that was identified in our institution, is the way in which samples are handled between collection at the bedside and arrival in the laboratory. The aim of the second section of this chapter was to establish whether the 'normal' clinical conditions in which 'real life' samples are collected could confound measurement of the established CSF biomarkers.

In clinical practice in UK hospitals CSF samples are currently collected by portering staff, who deliver samples to the laboratory on a non-urgent basis. Samples can pass through the hands of doctors, nursing staff, porters, specimen reception staff and laboratory scientists before being aliquoted and frozen. Having established the importance of aliquot storage volume in addition to the other known pre-handling confounding variables, the purpose of this experiment was to determine whether

earlier events in a sample's journey could also influence measured biomarker concentrations. The aims were to determine: a) if the time interval between sample collection and transport to the lab affected measured biomarker concentration; b) if careful handling using a dedicated porter reduces disruption and protein adsorption, and c) if early cooling at the bedside alters measured biomarker concentration.

4.2 Contributions and collaborations

CSF sample collection was carried out by the author as part of sample collection for the 'prospective cohort'. Study design was by the author with statistical support from statistician, Dr Jennifer Nicholas. Sample delivery was undertaken by Jamie Toombs, Dr Amanda Heslegrave or porters and laboratory reception staff at the National Hospital for Neurology and Neurosurgery (NHNN). Laboratory work was carried out by board certified technicians at the Department of Neuroimmunology at NHNN. First draft of the manuscript was written by the author with minor revisions made by other co-authors.

4.3 Methods

4.3.1 Subjects

Individuals with cognitive symptoms and suspected neurodegenerative disease who were scheduled to have a lumbar puncture as part of their normal clinical care at the day care unit of the NHNN were recruited. They consented to donate CSF for research purposes as outlined in the methods section describing the 'prospective cohort'.

4.3.2 Methods

Individuals had a lumbar puncture between the hours of 8 and 12 noon by a doctor

from the specialist cognitive disorders team using a 22G Quincke needle. No manometer was used. Four samples, each containing ~1ml volume were collected for clinical analysis; up to 15ml was collected between two Sarstedt 10ml polypropylene (cat. 62.610.018) tubes for storage for future biomarker discovery. For this analysis the clinically acquired CSF was used. These samples were labeled sequentially: *,1,2,3. The first tube labeled * was not used in this analysis as it was most likely to have been contaminated with blood.

Three transport methods were used for each individual's samples with the order randomized using a random number generator command in STATA (College Station, Tx, USA) (1) samples were collected by a designated laboratory technician within 10 minutes of collection and transferred to the laboratory in a cool box containing wet ice at 4°C; (2) samples were collected by a designated laboratory technician who picked up the sample within 10 minutes of collection and transferred to the laboratory at room temperature; (3) samples were transferred to the laboratory via the routine portering service. (4) A further group of individuals (n=10) had their third sample deliberately "mistreated," being quarantined at room temperature for between 24 hours and 1 week. In the laboratory, samples were centrifuged at 1750 relative centrifugal force for 5 minutes at room temperature and frozen at -80°C within 15 minutes of arrival. Each sample was analyzed for Aβ1–42, T-tau, and P-tau using an INNOTEST enzyme-linked immunosorbent assay (Ghent, Belgium). Laboratory staff were blinded to the transfer method.

4.3.3 Statistics

Sample size calculations were based on prior ELISA-based measures of Aβ1–42, T-tau, and P-tau from 456 individuals with suspected neurodegenerative disease, where

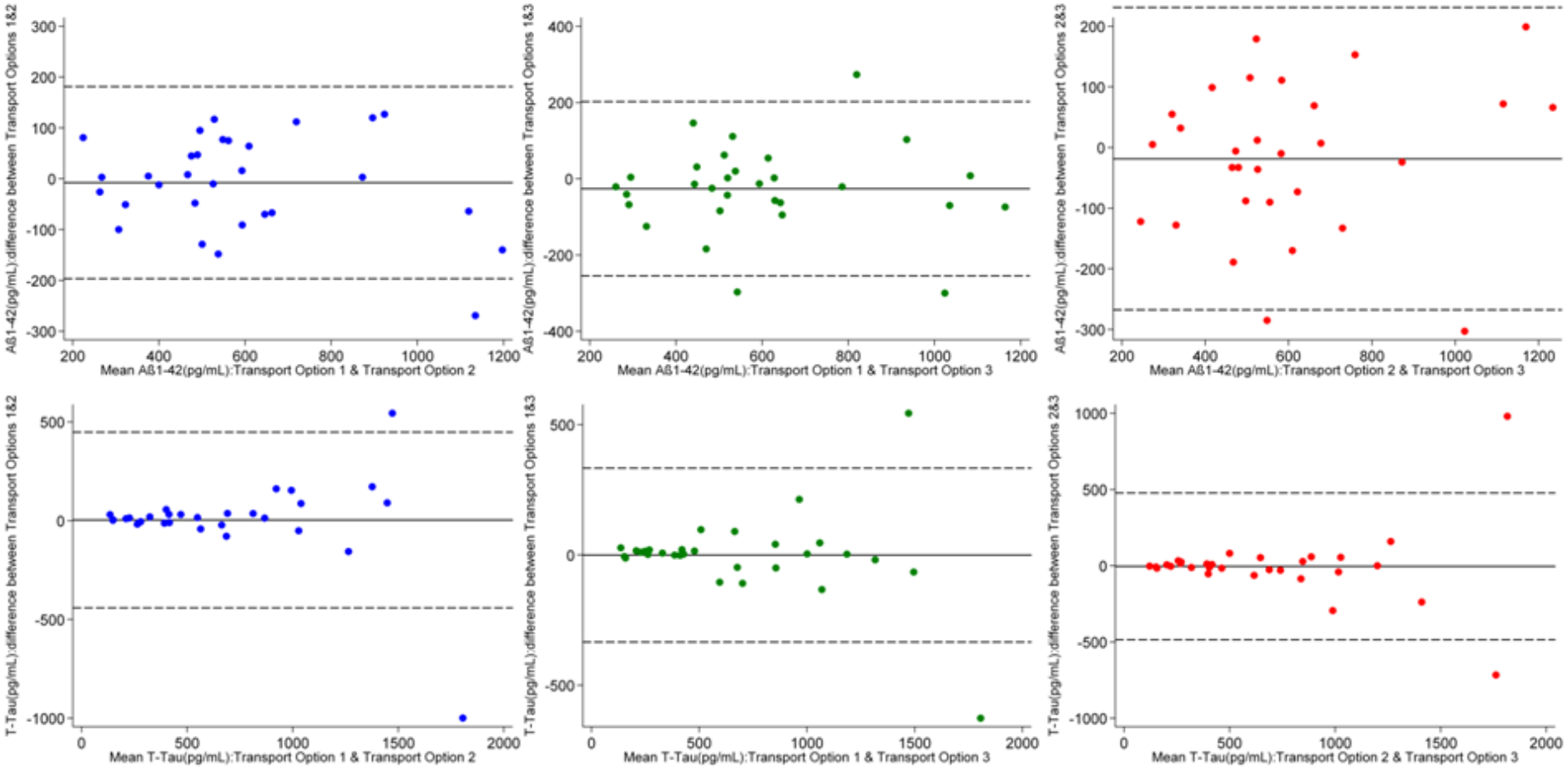
the mean (standard deviation) concentration (pg/mL) for Aβ1–42 =520 (122), T-tau =556 (442), and P-tau =70.3 (37.2). Assuming a correlation between results from the transfer methods of 0.9, 30 participants were needed to detect a difference of 20% in sample concentration with 90% power and 5% risk of a type 1 error. Wilcoxon matched-pairs signed-rank tests were used to compare the level of each biomarker concentration between transport methods, and reproducibility was compared between transport methods using Pitman's test of equality of variance for paired samples. Variance between transport methods is represented using Bland Altman plots. Spearman's pairwise correlation co-efficient was determined for differences in biomarker concentration and delivery time. All analyses were conducted in STATA version 12.1 (College Station, Tx, USA).

4.4 Results

Thirty subjects were included in the initial analysis comparing transfer methods 1–3, including patients with suspected Alzheimer and a range of non-Alzheimer pathologies. Samples randomized to transport options 1 and 2 all arrived simultaneously in the laboratory within 30 minutes of collection. Samples randomized to transport option 3 arrived a median of 24 minutes (range, 13–55) later. There was no significant difference in measured CSF Aβ1–42, T-tau, and P-tau concentrations between any of the transport methods, and no evidence that variance of CSF Aβ1–42 or T-tau differed between the trans-port methods. There was significant variance of P-tau between transport methods 1 and 2 and between methods 1 and 3, this association being driven by a single data point (Figure 4.1).

CSF Analyte	Transport Option 1*	Transport Option 2*	Transport Option 3*	*Comparison of options 1 and 2*		*Comparison of options 1 and 3*		*Comparison of options 2 and 3*	
				P diff. level	*P diff. variance*	*P diff. level*	*P diff. variance*	*P diff. level*	*P diff. variance*
Aβ1-42^	563 (436-775)	565 (448-696)	553 (450-691)	0.84	0.24	0.13	0.88	0.52	0.42
T-Tau^	486 (263-874)	455 (276-842)	546 (261-999)	0.15	0.36	0.59	0.39	0.73	0.79
P-Tau^	61 (42-96)	56 (43-97)	61 (43-105)	0.68	0.74	0.72	0.04	0.97	0.03

Table 4.1 Measured CSF biomarker concentrations for each transport method, results of paired Wilcoxon signed-rank tests used to compare transport methods and Pitman's test for equality of variance; ^pg/mL; *Median (Interquartile range); CSF: cerebrospinal fluid; Aβ1-42, amyloid beta 1-42; T-tau: total-tau; P-tau: phosphorylated tau; Option 1: Designated courier transported on wet ice; option 2: designated courier at room temperature; option 3: standard hospital porter. Samples are from the prospective cohort


Aß1-42(pg/mL):difference between Transport Options 1&2
Mean Aß1-42(pg/mL):Transport Option 1 & Transport Option 2
Aß1-42(pg/mL):difference between Transport Options 1&3
Mean Aß1-42(pg/mL):Transport Option 1 & Transport Option 3
Aß1-42(pg/mL):difference between Transport Options 2&3
Mean Aß1-42(pg/mL):Transport Option 2 & Transport Option 3
T-Tau(pg/mL):difference between Transport Options 1&2
Mean T-Tau(pg/mL):Transport Option 1 & Transport Option 2
T-Tau(pg/mL):difference between Transport Options 1&3
Mean T-Tau(pg/mL):Transport Option 1 & Transport Option 3
T-Tau(pg/mL):difference between Transport Options 2&3
Mean T-Tau(pg/mL):Transport Option 2 & Transport Option 3

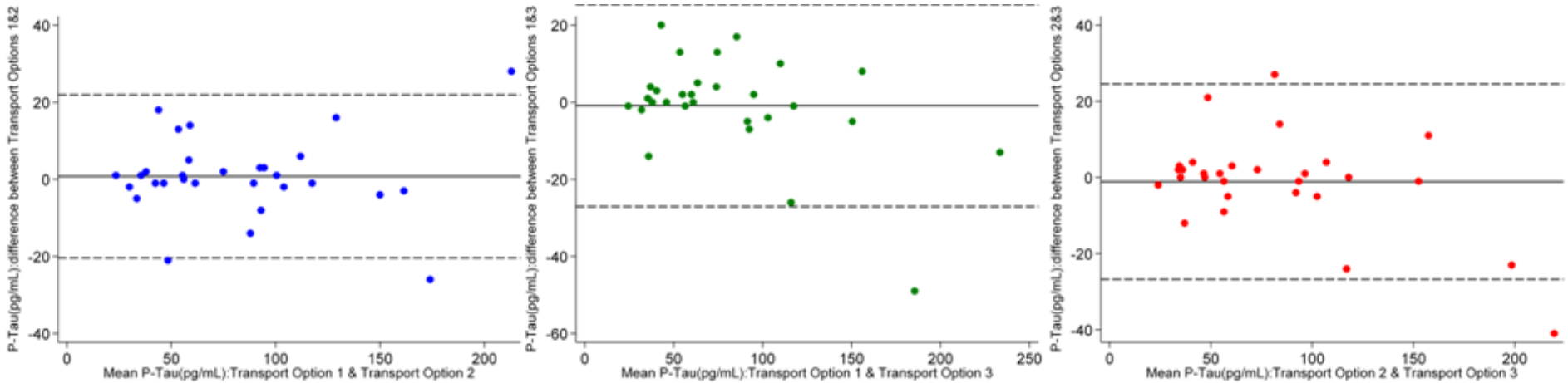


Figure 4.1 Bland-Altman plots for measured CSF Aβ1–42, T-tau, and P-tau concentrations comparing transport options 1 and 2, 1 and 3, and 2 and 3. Solid line indicates mean difference between methods; dashed lines represent 95% reference range for difference between methods. Abbreviations: CSF, cerebrospinal fluid; Aβ1–42: amyloid β 1–42; T-tau, total-tau; P-tau, phosphorylated tau

Measured T-tau concentration was weakly negatively correlated with transport time for samples transported by porter compared with those sent by courier at room temperature (Spearman's r 20.42) and on wet ice (Spearman's r 20.39). There was no correlation between Aβ1-42 or P-tau concentration and transport time. For the 10 individuals who had samples sent by transport method (1) and following quarantine (method 4), the latter samples arrived at the laboratory 1440 minutes (range,1440–4320) after collection (Table 4.2). There was also no significant difference in measured CSF Aβ1–42, T-tau, and P-tau concentrations between transport methods 1 and 4. There was a moderate negative correlation between Aβ1–42 concentration (Spearman's r 20.52) and weakly positive correlation with T-tau (0.43) and P-tau concentrations (0.37) and transport time, although these correlations were not statistically significant.

	Transport Option 1*	**Transport Option 4***	***Comparison of options 1 and 4***	
Transfer time(mins)	30 (30-33.5)	1440 (1440-4320)		
			P diff. level	***P diff. variance***
Aβ1-42^	563 (448-713)	565 (463-927)	0.20	0.39
T-tau^	471 (273-874)	345.5 (252-538)	0.36	0.50
P-tau^	60 (39-96)	50.5 (33-56)	0.26	0.73

Table 4.2 Measured CSF biomarker concentrations for transport option 1 and 4 (n=10), results of paired Wilcoxon signed-rank tests used to compare transport methods and Pitman's test for equality of variance; ^(pg/mL);*Median (Interquartile range).Aβ1-42: amyloid beta 1-42; T-tau: total-tau; P-tau: phosphorylated tau; Option 1: designated courier transported on wet ice; option 4: standard hospital porter, where samples were deliberately mistreated at room temperature for greater than 24 hours. Samples are from the prospective cohort.

4.5 Discussion

The clinical utility of CSF biomarkers for diagnosing AD pathology in individuals with cognitive impairment and suspected neurodegeneration is now well established in the research setting[127] but it is less clear to what extent Aβ1–42, T-tau, and P-tau concentrations can be reliably measured and interpreted in 'real-life' clinical cohorts where samples cannot always be collected according to gold-standard practices. This randomized study shows that CSF samples collected in polypropylene vessels can be transferred without cooling, in a time frame and manner appropriate for routine clinical practice, without significantly altering the measured concentration of the

most useful neurodegenerative markers, and supports the findings of a prior smaller study suggesting that biomarker concentrations may remain stable at room temperature for up to 24 hours[161]. We found no consistent or significant correlation between transfer time and biomarker concentration for Aβ1–42 or P-tau. P-tau was negatively correlated with delivery time for delivery options 1–3 but this association was not observed for the quarantined samples (option 4) when greater time differences were studied. Although not powered to study changes over this period, this study suggests there may be no effect of transfer time on Aβ1–42, T-tau, or P-tau even when samples were quarantined for up to a week. The conclusions from this prospective, blinded, randomized study have significant implications for future use of CSF as a clinical diagnostic tool. In many countries, use of CSF sampling in the investigation of dementia is restricted to specialist neurology centers. As biomarkers are increasingly used as part of clinical diagnostic criteria and there is a drive to identify AD in the earliest preclinical phase of the illness, regional hospitals and memory centers are likely to want to make use of CSF sampling to aid early diagnosis, identify individuals for trials, and to improve the likelihood of successful therapeutic intervention. These results show that, provided samples are collected appropriately and in suitable tubes and can reach a laboratory for aliquoting and freezing within a reasonable time frame, robust results can be obtained. It also provides reassurance that the samples collected historically in this centre, and analysed later in this thesis as part of the 'retrospective cohort' are not vulnerable to this particular confounding variable.

Potential limitations of this study are the relatively small sample size which means that it is not powered to detect very small differences which could be clinically meaningful in certain clinical or research circumstances. We have tested a small selection of biomarkers in clinical use, so these findings may not be

generalisable to all proteins.

4.6 Conclusions

In recent years, there have been moves to improve harmonization in CSF collection and handling methods between centres to help standardize clinical cutpoints and facilitate multicenter observational research studies and trials of disease modifying drugs, yet there is significant variation in the time taken to transfer samples from bedside to laboratory between centres for research biobanking[157]. These data demonstrate that harmonization of this particular variable may be less vital than other preanalytical factors such as test-tube material and brand. A relative weakness of this study is sample size. A significantly larger study could be powered to detect smaller differences between transport groups. It is therefore recommended that clinical CSF is collected according to a standardized operating procedure using polypropylene collection tubes. These data suggest that samples need not be transferred to the laboratory on ice and that transfer times of up to and beyond 24 hours may not alter the validity of Aβ1–42, T-tau, and P-tau measurement.

4.7 Publications related to this chapter

RW Paterson, J Toombs, MD Chapman, JM Nicholas, AJ Heslegrave, CF Slattery, AJ Foulkes, CN Clark, CA Lane, PS Weston, MP Lunn, NC Fox, H Zetterberg, JM Schott. Do cerebrospinal fluid transfer methods affect measured amyloid β42, total tau, and phosphorylated tau in clinical practice? Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring. 2015 Jul 2;1(3):380-4.

Chapter 5. Using Amyloid PET to determine clinical cutpoints for established CSF biomarkers

In the previous chapter the clinical utility of the core CSF biomarkers for differentiating between AD, healthy elderly and other neurodegenerative markers was investigated, based largely on clinical diagnosed cases. The aim of this chapter was to explore the use of amyloid PET imaging as a surrogate for pathological confirmation to further refine clinical cutpoints for the core CSF biomarkers.

5.1 Introduction

The 'core' molecular biomarkers of AD Aβ1-42, T-tau and P-tau are increasingly used to determine whether an individual has evidence of AD pathology *in-vivo*. As previously demonstrated, it is challenging to determine clinical cutpoints for abnormal levels of these biomarkers in clinical practice for a number of reasons. Firstly, CSF sampling for clinical diagnosis is usually carried out some months or years after conversion to AD dementia and therefore doesn't necessarily reflect earlier or later stages of the disease. The second major problem is that there are few 'gold standard' pathology confirmed studies, which have followed individuals through to death. One exception is a study of the ADNI cohort which was sufficiently large to include a small number of pathology confirmed cases[73], however even in this study it was not feasible to have autopsy confirmation close to the time of CSF sampling. Studies that rely on clinical diagnostic classification alone are potentially confounded by a number of variables including diagnostic circularity where the clinical diagnosis is informed by the CSF findings, or individuals with

clinical non-AD having unexpected subclinical or secondary amyloidosis. A third major problem is between centre variation: some of the factors that contribute to pre-analytical handling variation are discussed in chapter 3, but even controlling for many of these variables there is still significant variance in measured concentrations of CSF Aβ1-42, T-tau and P-tau between centres using the same samples, with CV typically 15-20%[282]. Defining normal values of these molecular biomarkers in healthy controls is also challenging for similar reasons; pathological confirmation close to sampling is rarely practical; a proportion of healthy elderly individuals will have asymptomatic amyloidosis[94]. Finally, while a single cutpoint is convenient for clinical diagnostic purposes, it seems biologically unlikely that a single cutpoint will have sufficient sensitivity and specificity. For example there is variation in rates of production and clearance of amyloid, even between healthy individuals[63].

Several large studies have estimated cutpoints using different approaches. One approach is use very large cohorts of clinically diagnosed cases and to compare cases with presumed AD with either controls or other neurodegenerative diseases such as bvFTD. This has been done for example by Duits *et al*[235] and they were also able to collect a small number of pathology confirmed cases and determined a cutpoint of 0.52 for Tau/Aβ1-42 ratio determining a sensitivity and specificity of around 90%. Ultimately this and other similar studies are confounded by the fact that diagnoses are based on clinical diagnostic criteria and it is difficult to ensure that raters are blinded to CSF results. An alternative approach is to use data driven models. Aβ1-42 and T-tau values produce a bimodal distribution if individuals with and without AD are included. If these values are plotted for sufficient numbers of subjects unbiased mixture modelling can be used to estimate a cutpoint[283]. Such studies require very large numbers of subjects, usually across multiple centres and laboratories and they

are not necessarily helpful in determining the clinical cutpoint for any given single centre.

An alternative way of helping to establish amyloid status is to correlate CSF biomarkers with Amyloid PET. This modality has now been pathologically proven in 2 major studies and so could be considered a reasonable alternative to the gold standard of pathology proven confirmation[120]. Although several tracers are now available they are not widely used. In clinical practice they are recommended in a minority of cases[122] and they have yet to be adopted in the UK by NHS England. Visual rating scores of Florbetapir F18 amyloid PET has been shown to correlate loosely with amyloid burden at autopsy[120]. Fibrillar amyloid can also be quantified by comparing with the PET signal with intensity in the cerebellum (semiautomated quantitative analysis of the ratio of cortical to cerebellar signal; SUVR). Mattsson[284], Palmqvist[285] and others have shown that SUVR and CSF Aβ1-42 are closely correlated, particular in the midrange of values where the cutpoints are likely to lie[286, 287]. The objectives of this chapter are to determine whether comparing CSF molecular biomarkers and amyloid PET might provide a useful means of determining clinical CSF cutpoints in a local population of individuals with suspected AD, individuals anticipated to be healthy controls, or suspected to have other neurodegenerative diseases.

5.2 Contributions and collaborations

This work was done in collaboration with Dr Philip Weston. CSF sample collection was carried out by the author. Recruitment to the amyloid PET sub-study was carried out by Dr Philip Weston and the author. Amyloid PET scans were carried out and interpreted by colleagues at the Institute of Nuclear Medicine, University

College London Hospital NHS foundation trust and the Transitional Imaging Group, Centre for Medical Image Computing, University College London, London, UK. Figure 5.1 is courtesy of Dr Philip Weston.

5.3 Subject and Methods

5.3.1 Ethics statement

This study was approved by the Queen Square Ethics Committee.

5.3.2 Subjects and CSF collection

A total of 23 individuals were recruited: 19 patients with a range of dementia syndromes recruited from the 'Prospective cohort' and 'YOAD cohort'; each individual had a diagnostic lumbar puncture as part of their clinical evaluation. Individuals were chosen to represent a spectrum of neurodegenerative disease and clinical phenotypes. Individuals with capacity to consent to the scan and who were willing to travel across London to the PET scanner were preferentially selected. A further four healthy controls were recruited from the 'Healthy control cohort' and had a lumbar puncture for research purposes only. These individuals were selected to ensure quality control as their amyloid PET scans were expected to be negative. CSF samples were collected using a 22G Quincke needle according to the standard operating procedure described in Chapter 2. Each sample was analysed for Aβ1-42, total tau and P-tau using INNOTEST ELISAs (Ghent, Belgium). Individuals with CSF Aβ1-42 levels in a potential border zone range of 400-700pg/ml were preferentially chosen.

5.3.3 Amyloid PET

All participants had an F18 florbetapir PET scan on a Siemens 3T PET/MR unit, with a 50-minute dynamic acquisition commencing immediately after intravenous injection of 370MBq of florbetapir. A volumetric T1-weighted MRI scan was acquired concurrently. Attenuation correction was performed using synthetic CTs generated from the MR images[288]. A single static PET image, reconstructed from the last 10 minutes of the PET acquisition, was used for the analysis. PET images were registered to the MRI, and segmented using a semi-automated parcellation tool [289].

The four age-matched healthy controls previously had a florbetapir PET/CT scan as part of another study, with a separate T1-weighted MRI acquisition. These images were processed in the same way as described above, excluding the generation of synthetic CTs.

PET images were analysed in two ways. First, three trained nuclear medicine physicians blinded to the clinical diagnosis visually rated the images positive/negative according to clinical criteria[120]. Secondly, a semiautomated quantitative analysis of the ratio of cortical to cerebellar signal (SUVR) was calculated by comparing uptake in six predefined cortical regions[120] to the whole cerebellum. A positive/negative SUVR cut-off of 1.10 was used as described[290].

5.3.4 Statistical Analysis

Statistical analyses were performed in STATA v.12.1 (College Station, TX, USA). Independent of clinical diagnosis, we compared CSF Aβ1-42, tau/Aβ1-42, and P-tau in subjects rated amyloid positive/negative on visual reads, and based on SUVR. Linear regression was used to assess the relationship between CSF and SUVR. The time interval between lumbar puncture and PET scan was included as a covariate.

5.4 Results

Patients and controls were well matched for age (63.7±7.6 vs. 62.9±7.0). Nine patients had amnestic, and ten non-amnestic (five posterior cortical atrophy, four progressive aphasia, and one behavioural) clinical syndromes (Table 4.1). CSF examination was carried out prior to amyloid PET scan, with a median delay of 145 days (range 32-427). Across all subjects, CSF Aβ1-42 ranged from 343-1199ng/L, tau/Aβ1-42 0.11-2.54, and P-tau 14-227g/L.

	n	Aβ1-42 (pg/mL) range	Tau/Aβ1-42 range	P-tau (pg/mL) range
Positive visual read	17	343-729	0.54-2.54	26-227
Negative visual read	6	630-1199	0.11-0.34	14-40
SUVR positive	14	343-633	0.88-2.54	49-227
SUVR negative	9	403-1199	0.11-0.58	14-49
Concordant positive	14	343-633	0.88-2.54	49-227
Discordant	3	403-729	0.54-0.58	26-49
Concordant negative	6	630-1199	0.11-0.34	14-40

Table 5.1 CSF measurements of Aβ1-42, T-tau/Aβ1-42 and P-tau. Patients are divided based on (i) a positive/negative visual PET read, (ii) a positive/negative PET SUVR (using a cut-off of 1.10), and (iii) whether there is concordancy or discordancy between the two different PET results. Subjects were from the prospective, healthy control and YOAD cohorts.

17/23 participants were rated as amyloid-positive on visual assessment. SUVRs ranged from 0.87-1.66. At an SUVR cut-off of 1.10, 14/23 were amyloid-positive. Comparing SUVR and clinical reads, 20 were concordant (14 positive, 6 negative); and 3 discordant. The discordant group all had positive amyloid reads, negative SUVRs (0.88, 1.05 and 1.03), and tau/Aβ ratios between 0.54 and 0.58.

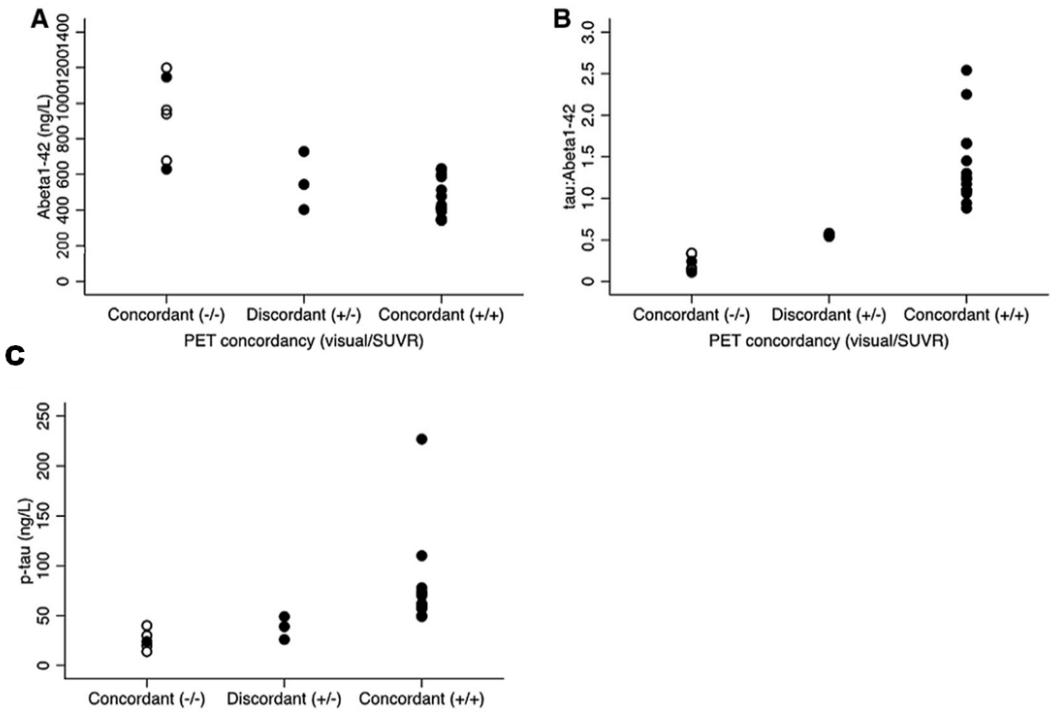


Figure 5.1 Distribution of CSF values for A) Aβ1-42, B) tau/Aβ1-42 and C) P-tau. Participants have been split, depending on the result of both visual PET read and PET SUVR in to either concordant (-/-) (both PET outcomes negative), discordant (+/-) (one PET outcome positive and one negative), or concordant (+/+) (both PET outcomes positive). Patients are represented by black points, with controls in white.

The SUVR correlated with CSF Aβ1-42 (R^2=0.26, p=0.013), CSF tau/Aβ1-42 (R^2=0.47, p<0.001) and CSF P-tau (R^2=0.34, p=0.005), with no evidence for an influence of duration between CSF sampling and scanning.

At a CSF Tau/Aβ1-42 ratio cutpoint of 0.52[235] the sensitivity and specificity for a positive amyloid scan based on visual reads were 100% (95%CI 80-100) and 100% (54.1-100) respectively; and based on SUVR, 82% (57-96) and 100% (54-100).

5.5 Discussion

As shown in previous studies there was good correlation between CSF and PET measures of Aβ1-42. However individuals with CSF Aβ1-42 values in the midrange were deliberately selected, where linear associations with amyloid PET are more likely[287]. Discordant cases all had Aβ1-42, Tau/Aβ1-42 and P-tau values in the midrange, supporting the idea that a grey zone could be required when interpreting CSF values. The visual rating scale and SUVR results were broadly concordant although in a handful of cases individuals had a positive PET read with a negative SUVR. There are a number of possible explanations: scans could have been misread; there may have been errors in calculating SUVR, or perhaps simply this discordance reflects genuine biological uncertainty since 2/3 discordant cases had SUVR close to the cutpoint. If so, it seems likely that SUVR has a lower sensitivity in detecting amyloid positivity than visual rating.

If the individuals with discordant results are disregarded, then there was almost complete separation (19/20 correctly classified) of individuals at a CSF Aβ1-42 of 630pg/ml. There was perfect separation on both tau/Aβ1-42 ratio (positive: ≥0.88, negative: ≤0.34), and P-tau (positive: ≥49, negative: ≤40pg/ml). This CSF Aβ1-42 cutpoint is similar to that generated in other studies[291,292]. Similarly the tau/Aβ1-42 ratio values generated here support the cutpoints used in large multicentre data generated studies[235] used to dichotomise individuals according to amyloid status in later chapters of this thesis. The P-tau cutoff is somewhat lower than the value quoted by the manufacturer (63pg/mL). One possible explanation for this is that several of our subjects have atypical presentations. Work carried out in chapter 7 suggests that individuals with posterior cortical atrophy for example have lower levels of CSF P-tau, which may reflect less extensive cortical Tau pathology.

There are limitations of this study. The number of subjects is relatively small. Furthermore there are limitations in using amyloid PET to validate CSF biomarker cutpoints; although amyloid PET has been pathologically validated, it remains an imperfect and indirect means of generating cut-points for CSF biomarkers and ultimately pathological validation is required.

Finally, it is noteworthy and reassuring that the cutpoints for Aβ1-42 , Tau/Aβ1-42 ratio and P-tau, determined using the clinically diagnosed cases in the previous chapter all fall broadly within the grey-zones determined in this study, using an independent cohort.

5.6 Conclusion

It is possible to confirm that CSF biomarkers of AD pathology and amyloid PET results were broadly concordant and that in the absence of local pathology proven cases, PET is therefore likely to be a valuable means of validating CSF biomarkers in clinical practice. Cases with discordant PET results had CSF values in the midrange indicating that the concept of a diagnostic grey zone is likely to be useful.

5.7 Publications arising from this chapter

PSJ Weston, **RW Paterson**, M Modat, N Burgos, MJ Cardoso, N Magdalinou, M Lehmann, JC Dickson, A Barnes, JB Bomanji, I Kayani, DM Cash, S Ourselin, J Toombs, MP Lunn, CJ Mummery, JD Warren, MN Rossor, NC Fox, H Zetterberg, JM Schott. Using florbetapir positron emission tomography to explore cerebrospinal fluid cut points and gray zones in small sample sizes. Alzheimer's and Dementia: Diagnosis, assessment and disease monitoring, 2015 Nov 2;1(4):440-446.

Chapter 6. CSF in the differential diagnosis of Alzheimer's disease: clinical utility of a panel of biomarkers in a specialist cognitive clinic

6.1 Introduction

CSF biomarkers are increasingly used to support a diagnosis of Alzheimer's disease (AD). CSF Aβ1-42, T-tau and P-tau have been most widely studied[173], have proven utility in differentiating AD from healthy controls individually or in combination in a number of clinical[235, 245, 274, 293] and much smaller number of pathology proven studies[73, 173]. As a result, these measures are included in contemporary clinical[3] and research (IWG2) diagnostic criteria.

Aside from these established biomarkers, a variety of other CSF measures relevant to neurodegeneration are now available, including markers of amyloid processing (AβX-38, AβX-40 and AβX-42, sAPPα, sAPPβ), large fibre axonal myelination (neurofilament light chain, NFL), and neuroinflammation (S100β and Chitinase-3-like protein 1 also knows as YKL-40). A recent large meta-analysis has confirmed that YKL-40 and NFL are elevated in clinically diagnosed AD CSF compared to controls; and that there is also a small but significant differences in AβX-40 [173].

Whilst most prior studies have distinguished patients from controls, or individuals with mild cognitive impairment who subsequently develop dementia, a major challenge in clinical practice is to distinguish neurodegenerative diseases from each other. The role that CSF biomarkers may have in this context is much less well established. Some previous studies have shown low specificity of Aβ1-42, and P-tau in differentiating AD from Dementia with Lewy Bodies (DLB) or frontotemporal

dementia (FTD)[243, 274]. Such studies have also usually investigated the standard panel of AD biomarkers [199, 243] and found that in some clinical situations biomarker combinations improve diagnostic specificity. Single biomarker studies suggest CSF NFL and YKL-40 may have utility in distinguishing FTD from AD and DLB [232, 294]. Others have pooled non-AD dementias for comparison with AD [235, 295]. This approach, whilst appropriate for identifying AD, does not provide information about the various other non-AD dementias, and has much less clinical utility.

The aims of this study were to determine the diagnostic utility of a panel of 11 CSF biomarkers in a large sample of clinically diagnosed neurodegenerative dementias; to determine which individual or combinations of biomarkers would provide greater diagnostic accuracy in distinguishing these diseases from one another; and to validate these findings in an independent cohort.

6.2 Contributions and Collaborations

Data was collected by the author. Independent clinical diagnosis was determined by Dr Catherine Slattery (CFS). The study was designed was by the author. Sample analysis was carried out at in the clinical chemistry laboratory at the University of Gothenburg by the board certified technicians assisted by the author. Statistical analysis was carried out by the author with the help of Teresa Poole.

6.3 Methods

6.3.1 Ethics statement

The study was conducted in accordance with local clinical research regulations and was approved by the local Queen Square Ethics Committee.

In this chapter two independent cohorts, the 'prospective' and 'retrospective' cohorts were studied.

6.3.2 Test ('retrospective') cohort

All individuals referred to the specialist cognitive disorders service at the National Hospital for Neurology and Neurosurgery who had a diagnostic CSF examination between 1st January 2008 and 31st December 2012 were included. Electronic patient records were interrogated retrospectively by a single operator (RWP) to determine the pre-lumbar puncture (LP) clinical diagnosis, most recent clinical diagnosis, time in months from reported symptom onset to lumbar puncture (earliest symptom reported by individual or their family or caregiver), time in months from lumbar puncture to most recent clinical assessment by a member of the specialist cognitive disorders team, and mini mental state examination (MMSE) score prior to LP.

Individuals fulfilling consensus criteria for the following clinical diagnoses were included in the analysis including: amnestic, logopenic aphasia (LPA) and posterior cortical atrophy (PCA) variants of AD[3]; DLB[44]; bvFTD[253]; progressive nonfluent aphasia (PNFA) and semantic dementia (SD)[32]. Diagnoses were based on available clinical correspondence and imaging, eight cases were autopsy proven and two further cases were found to have presenilin 1 mutations. Individuals with language led phenotypes were classified according to the consensus criteria for primary progressive aphasia[32]. To avoid circularity, the pre-LP clinical diagnosis (ie not using the CSF result) was used for the purpose of establishing biomarker utility. A second, independent, clinician (CFS) repeated the blinded clinical diagnostic

classification on a randomly selected group of 119 subjects in order to assess consistency in the classification process.

6.3.3 Validation ('prospective') cohort

All individuals referred to the specialist cognitive disorders service at the National Hospital for Neurology and Neurosurgery who had a diagnostic CSF examination between 16th May 2013 and 16th May 2016 and who at the time of the LP fulfilled consensus criteria (confirmed as described above) were recruited as a validation cohort. Of those classified with AD, a small number (n=12) had amyloid PET scans, all of which were positive providing supportive evidence of amyloid pathology.

6.3.4 Healthy controls

Healthy controls were recruited for research purposes only as described in chapter 2. The healthy controls did not have subjective memory complaints at the time of LP or at a follow-up phone call one year later.

6.3.5 Sample treatment and analysis

CSF was collected according to the methods described in chapter 2, which have now been published[296]. The amount of available CSF differed between individuals, and as a result not all biomarker measurements could be obtained for all members of the retrospective cohort (see table 6.1a for full details).

Total tau (T-tau), phosphorylated tau (P-tau) and β-amyloid 1-42 (Aβ1-42) were analyzed using INNOTEST enzyme-linked immunosorbent assays (ELISAs) (Fujirebio Europe N.V., Gent, Belgium). Other markers of amyloid processing were

measured using the MSD Aβ Triplex assay (Meso Scale Discovery, Rockville, MD) which is a multiplexed method in which C-terminally specific antibodies are used to selectively capture Aβ forms ending at amino acids 38, 40 and 42 respectively, which are then quantified using the 6E10 detector antibody. This assay is thus not specific to the 1st amino acid of the Aβ peptides (the epitope of 6E10 lies within amino acids 3 to 8 in the Aβ sequence) and the measured Aβ isoforms are therefore called AβX-38, AβX-40 and AβX-42. Neurofilament light chain (NFL) concentrations were determined using the NF-light method (UmanDiagnostics, Umeå Sweden), YKL-40, also known as chitinase-3-like protein 1 (CHI3L1), was measured using the Human Chitinase 3-like 1 Quantikine ELISA Kit (R&D systems, Minneapolis, MN). Amyloid precursor protein soluble metabolites α and β (sAPPα, sAPPβ) were measured using a commercial duplex immunoassay with electrochemiluminescence detection (Meso Scale Discovery, Rockville, MD). β-amyloid 1-42 (Aβ1-42), T-tau and P-tau assays were carried out in batches according to local clinical NHNN neuroimmunology laboratory standard operating procedures to achieve CV<10%. Other assays were carried out at a single time point in the Neurochemistry laboratory of University of Gothenburg and Institute of Neurology, UCL by board-certified laboratory technicians.

6.3.6 Statistical analysis

Data distribution was assessed and values greater than or less than an assay's reliable detectable range were assigned maximum or minimum values. Medians and interquartile ranges were used to describe demographic, clinical characteristics and CSF biomarker data by diagnostic group. Missing CSF biomarker values were assumed to be Missing Completely at Random. CSF biomarkers were compared

between diagnostic groups using log transformed data, due to skewed and/or truncated data, and generalised least squares linear regression analyses, to allow for different variances in each group (an extension of the t-test/ANOVA model that allows different variances in each group). These global tests for differences across groups were assessed for: i) all groups, including healthy controls; ii) dementia-only groups; and iii) dementia-only groups with adjustment for age, sex, and time from symptom onset to lumbar puncture. Post-hoc pairwise comparisons between diagnostic groups were made when the initial (unadjusted) global test across dementia-only groups was statistically significant ($p<0.05$). There was no adjustment for multiple testing.

Non-parametric receiver operating characteristic (ROC) curves and the area under the curve (AUC) were used to quantify how well each biomarker discriminated between Alzheimer's disease and each of the other diagnostic groups (or combinations of groups). As expected, the group sizes varied greatly, reflecting the prevalence of these conditions in the population. Assuming that a biomarker is associated with disease, AUC can be considered a simple measure of the probability that a randomly selected case would have a higher biomarker value than a control, assuming higher values are associated with disease (and vice versa if lower values are associated with disease)[246]. Cutpoints and their conservative exact binomial confidence intervals were estimated for a fixed sensitivity of 85% as used in other studies[243] and similar to the recommended sensitivity of 80% suggested by the Reagan consensus report[102], and the associated specificities were calculated. ROC curves using combinations of up to five of the best performing (based on AUCs) biomarkers were used to calculate AUCs where group sizes were sufficiently large (defined as >10 subjects in each of two groups being compared) to avoid over-fitting, with bias corrected bootstrapped confidence intervals for the AUC (2,000

replications). The estimated cutpoints of five of the biomarkers were validated by calculating the sensitivity and specificity using the prospective cohort dataset. All analyses were carried out using STATA Version 14.1 (Texas, USA).

6.4 Results

6.4.1 Subject demographics

A total of 418 subjects were included; 275 in the retrospective cohort and 143 in the prospective cohort. The retrospective cohort comprised 245 patients with dementia and 30 controls ('healthy control' cohort). The patients' diagnoses were: AD (n=156, including 27 PCA and 12 LPA); DLB (n=20); bvFTD (n=45); PNFA (n=17); and SD (n=7). All groups had a similar duration of disease (time from symptom onset to LP) except for the SD group who presented later (Table 6.1a). The mean age of the DLB group was at least 5 years older than each of the other disease groups and the proportion of males was higher for DLB and SD than for other groups. As expected, MMSE was lower in the dementia groups compared with healthy controls.

The diagnoses in the 143 individuals in prospective cohort were: AD (n=104); DLB (n=5); bvFTD (n=12); PNFA (n=3); SD (n=9) and controls (n=10).

6.4.2 Diagnostic concordance

Clinical correspondence for 119 randomly selected subjects in the retrospective cohort was reviewed (the number of notes reviews achieved by CFS in a fixed 1 month period). There was a 95.8% concordance between the pre-LP diagnosis of the treating clinician and the diagnosis made by the independent rater blinded to the LP results (classifying by clinical diagnostic category, i.e. AD, bvFTD or other).

6.4.3 CSF biomarker concentrations

The biomarker profile of each diagnostic group (based on Pre-LP diagnosis) is presented in Table 6.1a for the retrospective and healthy control cohorts and shown graphically in Figure 6.1. The number of individuals tested for each biomarker is given in the table in parentheses.

In the prospective cohort data were available for eight biomarkers: Aβ1-42 (n=143); T-tau (n=143); T-tau/Aβ1-42 ratio (n=143); P-tau (n=131); AβX-38 (n=141); AβX-40 (n=141); AβX-42 (n=140); AβX-40/AβX-42 ratio (n=140).

	AD (n=156)	DLB (n=20)	BvFTD (n=45)	PNFA (n=17)	SD (n=7)	Controls (n=30)
Age at lumbar puncture (years)	62.5 (57-68)	70.0 (68-75)	61.0 (57-66)	65.0 (61-69)	62.0 (57-68)	63.5 (50-67)
% Male	42.3	75.0	60.0	47.1	71.4	46.7
Time from symptoms onset to LP (months)	36 (24-60) [n=154]	36 (18.5-48)	36 (24-60) [n=44]	36 (24-48)	60 (18-72)	N/A
MMSE	22 (17-25) [n=142]	22 (18-28) [n=15]	24 (18-27) [n=42]	25 (9.5-28) [n=8]	27 (16-27) [n=7]	30 (30-30)
Duration of neurological follow-up (months)	12 (6-24)	11 (4-29.5)	11 (6-23)	12 (4-24)	23 (11-43)	N/A
Aβ1-42 (pg/mL)	310.5 (218.0-451.5)	357.5 (327.0-490.0)	638.0 (396.0-871.0)	440.0 (308.0-696.0)	767.0 (633.0-859.0)	953.0 (771.0-1199.0)
T-Tau (pg/ml)	674.5 (430.0-973.5)	338.5 (185.0-489.0)	289.0 (187.0-389.0)	501.0 (367.0-744.0)	319.0 (229.0-458.0)	303.5 (189.0-402.0)
T-Tau/Aβ1-42 ratio	2.3 (1.2-3.7) [n=154]	0.8 (0.4-1.5)	0.4 (0.3-0.7) [n=44]	1.1 (0.7-2.1)	0.5 (0.3- 0.6)	0.3 (0.2-0.4)
P-Tau-181 (pg/L)	86.4 (59.4-111.8) [n=119]	47.1 (38.1-64.3) [n=16]	49.2 (37.0-64.0) [n=39]	62.5 (49.8-100.1) [n=13]	50.9 (25.5-58.6)	47.8 (39.3-65.4) [n=26]
S100β	0.19 (0.16-0.26) [n=70]	0.24 (0.15-0.34) [n=15]	0.24 (0.18-0.32) [n=23]	0.20 (0.15-0.30) [n=6]	0.30 (0.21-0.38) [n=4]	N/A
NFL(ng/L)	1191.5 (857.6-1584.0) [n=119]	929.6 (839.9-1650.1) [n=17]	1788.4 (839.9-3334.6) [n=38]	1974.9 (1627.7-3490.5) [n=12]	2400.0 (1687.5-3584.7) [n=6]	649.0 (515.9-849.5)
YKL-40 (ng/mL)	163 (127-194) [n=114]	158 (134-186) [n=16]	163 (135-244) [n=35]	192 (140-207) [n=10]	179 (132-256) [n=5]	111 (93-164) [n=29]
AβX-38 (ng/L)	1462.0 (1101.4-2025.5) [n=117]	1214.2 (840.1-1529.2) [n=15]	1306.0 (1106.2-1658.8) [n=34]	1653.8 (1251.7-2046.7) [n=12]	1751.4 (1442.0-1777.0) [n=5]	2183 (1980.8-3058.6) [n=29]
AβX-40 (ng/L)	3635.1 (2911.0-4584.4) [n=117]	2916.1 (2235.6-3718.2) [n=15]	3439.5 (2714.7-4274.9) [n=34]	3900.6 (3175.7-4355.6) [n=12]	3965.4 (3702.2-4537.6) [n=5]	5478.3 (4888.3-7615.2) [n=29]
AβX-42 (ng/L)	164.6 (109.1-231.6) [n=117]	182.1 (170.9-281.7) [n=15]	284.5 (195.2-369.4) [n=34]	183.0 (117.4-343.6) [n=12]	346.0 (309.9-372.1) [n=5]	592.2 (469.7-749.8) [n=29]
AβX-40/X-42 ratio	23.3 (18.9-28.0) [n=117]	18.1 (11.2-21.2) [n=15]	12.1 (10.6-13.9) [n=34]	19.4 (11.6-24.9) [n=12]	11.5 (10.7-11.8) [n=5]	9.4 (8.7-10.9) [n=29]
APPα (ng/mL)	348.8 (254.9-532.7) [n=119]	218.6 (175.8-368.1) [n=16]	270.7 (164.9-328.7) [n=37]	374.3 (316.1-467.3) [n=12]	379.5 (281.7-479.8) [n=5]	426.4 (322.0-654.5)
APPβ (ng/mL)	202.2 (151.2-325.8) [n=119]	138.0 (115.0-175.2) [n=16]	128.0 (107.4-187.1) [n=37]	220.3 (178.8-298.1) [n=12]	181.9 (171.4-236.4) [n=5]	258.6 (182.0-372.0)

Table 6.1a Demographic and biomarker data for all diagnostic groups, based on pre-lumbar puncture (LP) diagnosis**.** Samples are from the Retrospective and Healthy control cohorts. Median and interquartile range are reported. Where data were missing, the number of subjects for which data were available is indicated within parentheses. AD: Alzheimer's disease; DLB: dementia with Lewy Bodies; BvFTD: behavioural variant frontotemporal dementia; PNFA: progressive non-fluent aphasia; SD: Semantic dementia; HC: healthy control; MMSE: mini-mental state examination.

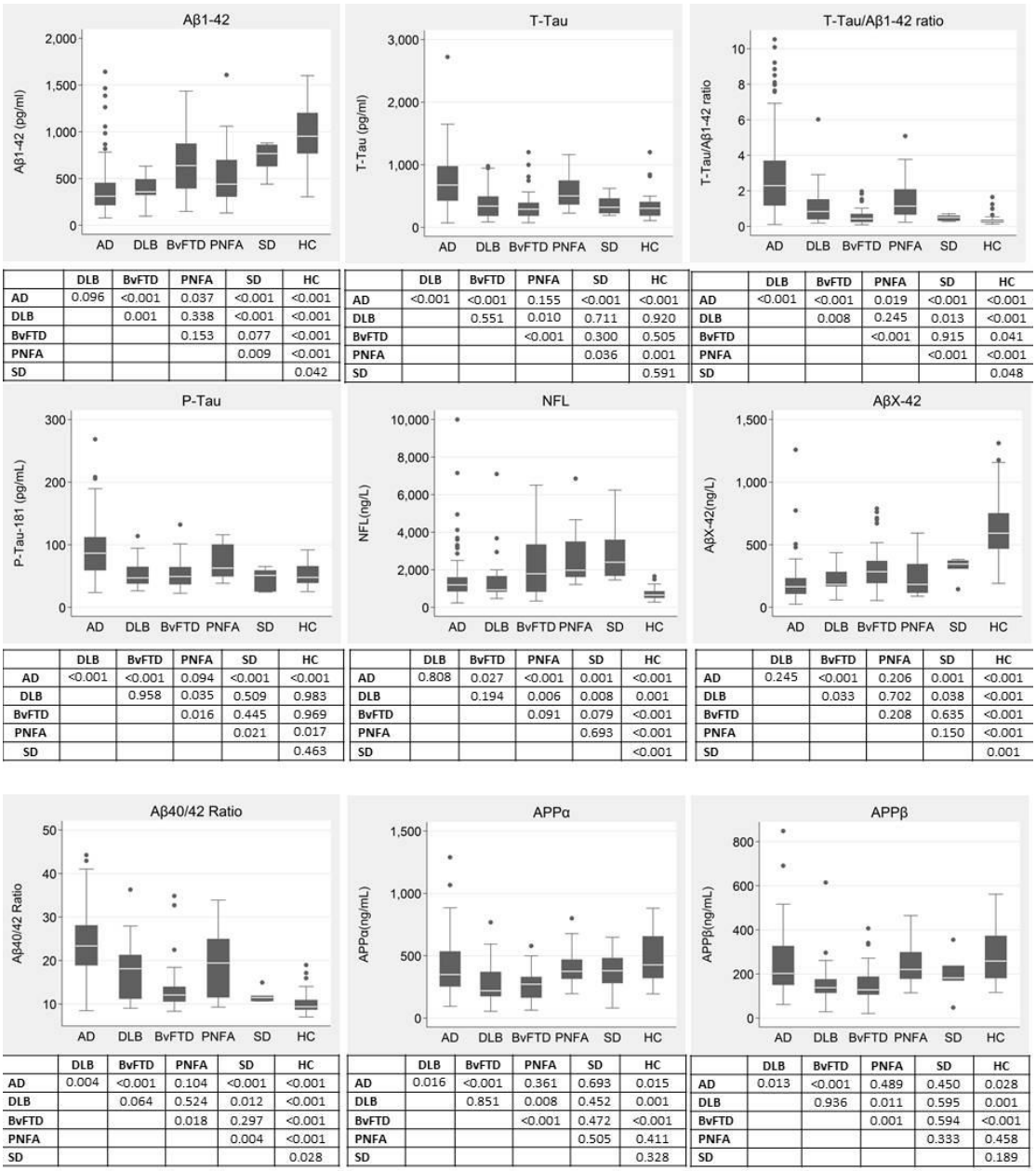
Comparisons between the groups based on regression analyses are shown in Table 6.1b. There was evidence of a significant difference ($p<0.05$) in mean values between disease groups for all tested biomarkers when healthy controls were included in the analyses. When the analyses were repeated without the healthy control group this was still the case for nine out of 13 biomarkers. After also adjusting for age, sex and time from symptom onset to LP, (without the healthy control group), the results were consistent except for one biomarker (YKL-40) where there was weak evidence of a difference between groups ($p=0.04$) in the adjusted results (Table 6.1b).

	Global test* with HC (p-value)	**Global test* no HC (p-value)**	**Adjusted** global test* no HC (p-value)**
Aβ1-42	<0.0001	<0.0001	<0.0001
T-tau	<0.0001	<0.0001	<0.0001
T-tau/Aβ1-42 ratio	<0.0001	<0.0001	<0.0001
P-tau-181	<0.0001	<0.0001	<0.0001
S100β	N/A	0.36	0.54
NFL	<0.0001	<0.0001	<0.0001
YKL-40	0.0038	0.51	0.04
AβX-38	<0.0001	0.43	0.17
AβX-40	<0.0001	0.57	0.30
AβX-42	<0.0001	0.0001	0.0002
AβX-40/X-42 ratio	<0.0001	<0.0001	<0.0001
APPα	<0.0001	<0.0001	0.0001
APPβ	<0.0001	0.0001	0.0001

* Test of the null hypothesis that all disease groups have the same mean biomarker value; ** Adjusted for age, sex and time from symptom onset to lumbar puncture;.

Table 6.1b Regression analyses comparing biomarkers between all disease groups classified according to pre-lumbar puncture diagnosis, with and without healthy controls. Samples are all from Retrospective and Healthy control cohorts. Biomarker data is log transformed to achieve normal distribution. HC: healthy controls.

Pairwise comparisons between diagnostic groups were carried out where the (unadjusted) global test across dementia-only groups was statistically significant ($p<0.05$) (Figure 6.1). A summary of where there was evidence of a difference in mean biomarker levels is presented in Table 6.2 for each pairwise comparison.



	DLB	BvFTD	PNFA	SD	HC
AD	0.096	<0.001	0.037	<0.001	<0.001
DLB		0.001	0.338	<0.001	<0.001
BvFTD			0.153	0.077	<0.001
PNFA				0.009	<0.001
SD					0.042

	DLB	BvFTD	PNFA	SD	HC
AD	<0.001	<0.001	0.155	<0.001	<0.001
DLB		0.551	0.010	0.711	0.920
BvFTD			<0.001	0.300	0.505
PNFA				0.036	0.001
SD					0.591

	DLB	BvFTD	PNFA	SD	HC
AD	<0.001	<0.001	0.019	<0.001	<0.001
DLB		0.008	0.245	0.013	<0.001
BvFTD			<0.001	0.915	0.041
PNFA				<0.001	<0.001
SD					0.048

	DLB	BvFTD	PNFA	SD	HC
AD	<0.001	<0.001	0.094	<0.001	<0.001
DLB		0.958	0.035	0.509	0.983
BvFTD			0.016	0.445	0.969
PNFA				0.021	0.017
SD					0.463

	DLB	BvFTD	PNFA	SD	HC
AD	0.808	0.027	<0.001	0.001	<0.001
DLB		0.194	0.006	0.008	0.001
BvFTD			0.091	0.079	<0.001
PNFA				0.693	<0.001
SD					<0.001

	DLB	BvFTD	PNFA	SD	HC
AD	0.245	<0.001	0.206	0.001	<0.001
DLB		0.033	0.702	0.038	<0.001
BvFTD			0.208	0.635	<0.001
PNFA				0.150	<0.001
SD					0.001

	DLB	BvFTD	PNFA	SD	HC
AD	0.004	<0.001	0.104	<0.001	<0.001
DLB		0.064	0.524	0.012	<0.001
BvFTD			0.018	0.297	<0.001
PNFA				0.004	<0.001
SD					0.028

	DLB	BvFTD	PNFA	SD	HC
AD	0.016	<0.001	0.361	0.693	0.015
DLB		0.851	0.008	0.452	0.001
BvFTD			<0.001	0.472	<0.001
PNFA				0.505	0.411
SD					0.328

	DLB	BvFTD	PNFA	SD	HC
AD	0.013	<0.001	0.489	0.450	0.028
DLB		0.936	0.011	0.595	0.001
BvFTD			0.001	0.594	<0.001
PNFA				0.333	0.458
SD					0.189

Figure 6.1 Boxplots and whiskers (25th-75th percentiles) and outliers of measured biomarker concentrations presented by disease group (pre-lumbar puncture diagnosis) and unadjusted pairwise comparisons (p-values). X-axis: pre-lumbar puncture diagnosis. AD: Alzheimer's disease; DLB: Lewy Body Dementia; BvFTD: Behavioural variant frontotemporal dementia; PNFA: progressive non-fluent aphasia; SD: semantic dementia; HC: healthy controls.

	Aβ1-42	T-tau	T-tau/Aβ1-42	P-tau	NFL	AβX-40/X-42	APPα	APPβ
AD vs DLB		+	+	+		+	+	+
AD vs bvFTD	+		+	+	+	+	+	+
AD vs PNFA	+	+	+		+			
AD vs SD	+	+	+	+	+	+		
DLB vs bvFTD	+		+					
DLB vs PNFA		+		+	+		+	+
DLB vs SD	+		+		+	+		
bvFTD vs PNFA		+	+	+		+	+	+
bvFTD vs SD								
PNFA vs SD	+	+	+	+		+		

Table 6.2 Summary of the biomarkers that are significantly different between neurodegenerative diseases. Biomarkers listed distinguish between groups with $p<0.05$; individuals are classified by pre-LP clinical diagnosis and values are unadjusted. Subjects included are from the retrospective and healthy control cohorts. AD: Alzheimer's disease; DLB: dementia with Lewy Bodies; BvFTD: behavioural variant frontotemporal dementia; PNFA: progressive non-fluent aphasia; SD: Semantic dementia.

As shown in Table 6.2, T-tau/Aβ1-42 ratio was significantly elevated in AD CSF compared to each of the other neurodegenerative diseases tested. T-tau, P-tau and AβX-40/X-42 ratio were also significantly higher in the AD cohort CSF compared to all of the other disease groups, except for PNFA. Aβ1-42 concentrations were lowest in AD and DLB groups but this biomarker by itself could not distinguish between these two disease groups. Measured concentrations of NFL were

significantly elevated in all neurodegenerative diseases compared to healthy controls; furthermore, concentrations were higher in the SD, PNFA and the bvFTD groups, which each had significantly higher concentrations than the AD and DLB groups (Figure 6.1). APPα and APPβ were significantly lower in DLB and bvFTD compared to AD, PNFA or healthy controls (Figure 6.1). AβX-38 and AβX-40 concentrations were lower in all neurodegenerative diseases compared to healthy controls ($p<0.01$) but there were no pairwise significant differences between each of the neurodegenerative diseases. Similarly, YKL-40 concentrations were elevated across all neurodegenerative disease groups tested relative to healthy controls but not between diseases.

6.4.4 Diagnostic accuracy of CSF biomarkers

Area under the ROC curve (AUC), sensitivity and specificity were used to compare how well single biomarkers distinguished between AD, the commonest form of dementia, and each other neurodegenerative disease and healthy controls. A summary of up to the 'top 5' biomarkers, as determined by AUC, are given in Table 6.3, with the highest AUCs varying between 0.79 and 0.95. Using a pre-determined fixed sensitivity of 85%, we generated cutpoints for each biomarker and the associated specificities, which varied between 24% and 100% (Table 6.3).

Diagnostic Groups compared	***Biomarker***	***AUC (95% CI)***	***Optimal cutpoint (95% CI) and specificity (%) at a fixed sensitivity of 85%***
AD vs DLB	P-tau (pg/L)	0.79 (0.68-0.90)	>48.9 (95% CI: 42.4-58.7) (50%)
	T-tau (pg/mL)	0.78 (0.67-0.88)	>312.0 (95% CI: 261.0-391.0) (50%)
	T-tau/Aβ1-42 ratio	0.77 (0.66-0.88)	>0.64 (95% CI: 0.52-1.01) (40%)
	AβX-40/X-42	0.73 (0.59-0.88)	>16.8 (95% CI: 11.4-18.1) (47%)
	APPβ (ng/mL)	0.73 (0.58-0.87)	>136.4 (95% CI: 115.3-144.6) (44%)
AD vs BvFTD	T-tau/Aβ1-42 ratio	0.89 (0.85-0.94)	>0.64 (95% CI: 0.52-1.01) (70%)
	AβX-40/X-42 ratio	0.86 (0.77-0.94)	>16.8 (95% CI: 11.4-18.1) (85%)
	T-tau (pg/mL)	0.83 (0.76-0.90)	>312.0 (95% CI: 261.0-391.0) (64%)
	Aβ1-42 (pg/mL)	0.78 (0.70-0.87)	<529.0 (95% CI: 479.0-647.0) (60%)
	P-tau (pg/L)	0.78 (0.70-0.86)	>48.9 (95% CI: 42.4-58.7) (46%)
AD vs PNFA	NFL (ng/L)	0.84 (0.76-0.93)	<1877.0 (95% CI: 1609.8-3149.6) (50%)
	T-tau/Aβ1-42 ratio	0.67 (0.54-0.80)	>0.64 (95% CI: 0.52-1.01) (24%)
	Aβ1-42 (pg/mL)	0.65 (0.50-0.80)	<529.0 (95% CI: 479.0-647.0) (35%)
AD vs SD	AβX-40/X-42 ratio	0.92 (0.86-0.97)	>16.8 (95% CI: 11.4-18.1) (100%)
	T-tau/Aβ1-42 ratio	0.91 (0.86-0.96)	>0.64 (95% CI: 0.52-1.01) (86%)
	Aβ1-42 (pg/mL)	0.91 (0.84-0.98)	<529.0 (95% CI: 479.0-647.0) (86%)
	NFL (ng/L)	0.87 (0.78-0.96)	<1877.0 (95% CI: 1609.8-3149.6) (67%)
	P-tau (pg/L)	0.85 (0.75-0.94)	>48.9 (95% CI: 42.4-58.7) (29%)

Table 6.3 Diagnostic performance of the 'top 5' biomarkers, comparing AD with other neurodegenerative diseases and controls determined using ROC curves. Subjects included are from the retrospective and healthy control cohorts.

Diagnostic Groups compared	***Biomarker***	***AUC (95% CI)***	***Optimal cutpoint (95% CI) and specificity (%) at a fixed sensitivity of 85%***
AD vs HC	AβX-40/X-42 ratio	0.95 (0.92-0.99)	>16.8 (95% CI: 11.4-18.1) (93%)
	Aβ1-42 (pg/mL)	0.93 (0.88-0.98)	<529.0 (95% CI: 479.0-647.0) (90%)
	T-tau/Aβ1-42 ratio	0.93 (0.89-0.97)	>0.64 (95% CI: 0.52-1.01) (83%)
	T-tau (pg/mL)	0.81 (0.73-0.90)	>312.0 (95% CI: 261.0-391.0) (53%)
	P-tau (pg/L)	0.80 (0.71-0.88)	>48.9 (95% CI: 42.4-58.7) (54%)
AD vs Non-AD neurodegenera-tive diseases	T-tau/Aβ1-42 ratio	0.82 (0.77-0.88)	>0.64 (95% CI: 0.52-1.01) (56%)
	AβX-40/X-42 ratio	0.79 (0.72-0.87)	>16.8 (95% CI: 11.4-18.1) (68%)
	T-tau (pg/mL)	0.77 (0.71-0.83)	>312.0 (95% CI: 261.0-391.0) (51%)
	P-tau (pg/L)	0.76 (0.70-0.83)	>48.9 (95% CI: 42.4-58.7) (41%)
	Aβ1-42 (pg/mL)	0.73 (0.67-0.80)	<529.0 (95% CI: 479.0-647.0) (48%)
AD vs all others (including HC)	T-tau/Aβ1-42 ratio	0.85 (0.80-0.90)	>0.64 (95% CI: 0.52-1.01) (63%)
	AβX-40/X-42 ratio	0.84 (0.79-0.90)	>16.8 (95% CI: 11.4-18.1) (76%)
	T-tau (pg/mL)	0.78 (0.73-0.84)	>312.0 (95% CI: 261.0-391.0) (51%)
	Aβ1-42 (pg/mL)	0.78 (0.73-0.84)	<529.0 (95% CI: 479.0-647.0) (59%)
	P-tau (pg/L)	0.77 (0.71-0.83)	>48.9 (95% CI: 42.4-58.7) (45%)

Table 6.3 (continued). Diagnostic performance of the 'top 5' biomarkers, comparing AD with other neurodegenerative diseases and controls determined using ROC curves. Individuals are classified by pre-LP clinical diagnosis. Subjects included are from the retrospective and healthy control cohorts. Biomarker with greatest diagnostic accuracy is in bold. AD: Alzheimer's disease; DLB: dementia with Lewy Bodies; BvFTD: behavioural variant frontotemporal dementia; PNFA: progressive non-fluent aphasia; SD: Semantic dementia; AUC: area under curve.

6.4.5 Diagnostic utility of combinations of biomarkers to predict diagnosis

Combining the biomarkers identified in Table 6.3 into a single model for each of the comparisons, the diagnostic utility of these combinations was again determined using the AUC (Table 6.4). In each case the new AUC was informally compared with the AUC of the relevant best performing single biomarker. There was no evidence that including more than one biomarker in the model improved the AUC. For one disease group comparison (AD vs PNFA) the combined biomarkers reduced the AUC.

Diagnostic Groups compared	Biomarkers included in the model	AUC (95% CI)	Comparison AUC (95% CI) for a single biomarker
AD vs DLB	P-tau; T-tau;T-tau/Aβ1-42 ratio; AβX-40/X-42 ratio; APPβ	0.83 (0.70-0.92)	0.79 (0.68-0.90) (P-tau)
AD vs BvFTD	T-tau/Aβ1-42 ratio; AβX-40/X-42 ratio; T-tau; Aβ1-42; P-tau	0.90 (0.81-0.94)	0.89 (0.85-0.94) (T-tau/Aβ1-42 ratio)
AD vs HC	AβX-40/X-42 ratio; Aβ1-42; T-tau/Aβ1-42 ratio; T-tau; P-tau	0.95 (0.88-0.97)	0.95 (0.92-0.99) (AβX-40/X-42 ratio)
AD vs PNFA*	NFL; T-tau/Aβ1-42 ratio; Aβ1-42	0.74 (0.52-0.82)	0.84 (0.76-0.93) (NFL)
AD vs Non-AD neurodegenerative cases (excluding HC)	T-tau/Aβ1-42 ratio; AβX-40/X-42 ratio; T-tau; Aβ1-42; P-tau	0.84 (0.75-0.89)	0.82 (0.77-0.88) (T-tau/Aβ1-42 ratio)
AD vs All others (including HC)	T-tau/Aβ1-42 ratio; AβX-40/X-42 ratio; T-tau; Aβ1-42: P-tau	0.86 (0.79-0.91)	0.85 (0.80-0.90) (T-tau/Aβ1-42 ratio)
* Only three biomarkers were found to be significantly different (see Table 6.2)			

Table 6.4 Diagnostic utility of combinations of biomarkers to predict disease group determined using ROC curve analysis. AD: Alzheimer's disease; DLB: dementia with Lewy Bodies; BvFTD: behavioural variant frontotemporal dementia; PNFA:

progressive non-fluent aphasia; HC: healthy control. Subjects included are from the retrospective and healthy control cohorts

6.4.6 Validation

In the independent prospective cohort we calculated sensitivity and specificity of the 'top-five' biomarkers including Aβ1-42, T-tau, T-tau/Aβ1-42, P-tau and AβX-40/X-42 using the optimal cutpoints determined in the retrospective and healthy control cohorts that provided a sensitivity of 85% (Table 6.5). Sensitivities were very consistent with the 85%, ranging from 83-88% for all biomarkers compared between all groups except for Aβ1-42 where the sensitivity was lower (71%). Given small sample sizes for groups other than AD, specificities were not informative.

		Retrospective cohort (used to estimate the cut-point)(data from table 6.3)		*Prospective cohort (using retrospective cohort cutpoint)*	
Diagnostic Groups compared	***Biomarker***	***Optimal cut-point for 85% sensitivity***	***Specificity***	***Sensitivity***	***Specificity***
AD vs DLB	P-tau (pg/L)	>48.9	(50%)	83%	25%
	T-tau (pg/mL)	>312.0	(50%)	87%	20%
	T-tau/Aβ1-42 ratio	>0.64	(40%)	88%	20%
	AβX-40/X-42	>16.8	(47%)	83%	20%
AD vs BvFTD	T-tau/Aβ1-42 ratio	>0.64	(70%)	88%	75%
	AβX-40/X-42	>16.8	85%	83%	75%
	T-tau (pg/mL)	>312.0	(64%)	87%	33%
	Aβ1-42 (pg/mL)	<529.0	(60%)	71%	67%
	P-tau (pg/L)	>48.9	(46%)	83%	67%
AD vs PNFA	T-tau/Aβ1-42 ratio	>0.64	(24%)	88%	100%
	Aβ1-42 (pg/mL)	<529.0	(35%)	71%	100%
AD vs SD	AβX-40/X-42	>16.8	(100%)	83%	88%
	T-tau/Aβ1-42 ratio	>0.64	(86%)	88%	89%
	Aβ1-42 (pg/mL)	<529.0	(86%)	71%	100%
	P-tau (pg/L)	>48.9	(29%)	83%	78%
AD vs HC	AβX-40/X-42	>16.8	(93%)	83%	80%
	Aβ1-42 (pg/mL)	<529.0	(90%)	71%	80%
	T-tau/Aβ1-42 ratio	>0.64	(83%)	88%	89%
	T-tau (pg/mL)	>312.0	(53%)	87%	78%
	P-tau (pg/L)	>48.9	(54%)	83%	78%
AD vs all others (including HC)	T-tau/Aβ1-42 ratio	>0.64	(63%)	88%	76%
	AβX-40/X-42	>16.8	(76%)	83%	74%
	T-tau (pg/mL)	>312.0	(51%)	87%	50%
	Aβ1-42 (pg/mL)	<529.0	(59%)	71%	77%
	P-tau (pg/L)	>48.9	(45%)	83%	70%

Table 6.5 Diagnostic accuracy of Aβ1-42, T-tau, T-tau/Aβ1-42 ratio, P-tau and AβX-42/X-40 ratio in retrospective and prospective cohorts based on pre-LP diagnostic classifications. AD: Alzheimer's disease; DLB: dementia with Lewy Bodies; BvFTD: behavioural variant frontotemporal dementia; PNFA: progressive non-fluent aphasia; SD: Semantic dementia; HC: healthy control. Subjects included are from the retrospective, prospective and healthy control cohorts.

6.5 Discussion

In this large single centre clinical study of 418 subjects it was possible to establish that the 'core' CSF biomarkers of AD pathology, T-tau, Aβ1-42, P-tau and T-tau/Aβ1-42 ratio, can differentiate AD subjects from healthy controls with a similar degree of discrimination of about 80-85% sensitivity and specificity (as indicated by AUC) and with similar clinical cutpoints to those findings reported in previous studies[235]. These results were based on classifying individuals clinically, using consensus criteria uninfluenced by biomarker data. It was also determined that these core biomarkers and other more novel markers, notably AβX-40/42 ratio and NFL, had some diagnostic utility in distinguishing AD from other neurodegenerative diseases, although their clinical diagnostic performance depended on the specific disease groups compared. We found that by using combinations of multiple biomarkers, there was no meaningful improvement in diagnostic utility over the best performing single biomarkers.

T-tau/Aβ1-42 ratio was the single most reliable measure to distinguish AD from the other neurodegenerative diseases as well as healthy controls, at a cutpoint of 0.64 and fixed sensitivity of 85% providing 83% specificity to distinguish AD from controls, and 66% specificity to distinguish AD from all other neurodegenerative cases.

Whilst CSF Aβ1-42 alone distinguished AD from controls (85% sensitivity, 90% specificity with a cutpoint of <529pg/ml), it was relatively poor in distinguishing AD from neurodegenerative diseases (85% sensitivity, 57% specificity), in keeping with other studies [297]. In the prospective cohort our estimated cutpoint, which achieved a sensitivity of 85% in the retrospective cohort, only achieved a sensitivity

of 71%. One explanation is that the retrospective cohort CSF samples, which were collected and stored in a non-standardised way, were particularly affected by pre-analytical handling variability, to which Aβ1-42 is particularly susceptible [161], resulting in falsely low and inconsistent measured Aβ1-42 values so giving different sensitivity at the same cutpoint. Several studies have found that AβX-42/X-40 ratio is superior to Aβ1-42 alone in differentiating AD from other clinically or pathologically determined neurodegenerative diseases[185, 186, 298]and my findings broadly support this observation in a larger independent cohort showing improved specificity to distinguish AD from DLB, BvFTD, SD, healthy controls and all other neurodegenerative diseases combined. Aβ1-40 is the most abundant form of amyloid and less likely than Aβ1-42 to aggregate, and thus may be the best measure of function of the amyloidogenic APP pathway [299]. Using a ratio of AβX-40/X-42 or AβX-42/X-40 theoretically corrects for the inter-individual physiological differences in amyloid processing. In all of the clinical scenarios explored in this study, AβX-42/X-40 was better at discriminating than Aβ1-42 alone; and also showed slightly improved specificity compared to T-tau/Aβ1-42 ratio to distinguish AD from DLB, BvFTD, SD, and healthy controls

In this study, P-tau alone was the best performing single biomarker for differentiating AD from DLB, and a model using multiple biomarkers did not improve on diagnostic utility. Our findings were similar to a previous meta-analysis which compared P-tau levels in AD and DLB and reported similar diagnostic sensitivity and specificity[297]. P-tau is thought to be more specific to AD pathology than other forms of Tau; Tau, an intraneuronal protein released following neuronal death, and in its phosphorylated form is more likely to restructure to form paired helical filaments[300], which become neurofibrillary tangles, one of the pathological

hallmarks of AD. Conversely, 'Pure' DLB is characterised not by tau or amyloid pathology but by Lewy Bodies and intraneuronal inclusions of alpha synuclein[301]. However in pathology proven DLB, DLB and AD pathology often co-exist[302] which is likely to explain the overlap in P-tau and other biomarker values between AD and DLB cases. This may also explain why I and others found that all biomarkers had low specificity in differentiating AD from DLB [243].

None of the established biomarkers tested achieved potentially useful diagnostic sensitivity or specificity for differentiating AD from PNFA, except for NFL (sensitivity 85%, specificity 50%). LPA, the language variant of AD most likely to mimic PNFA, is usually underpinned by AD pathology and this has previously been demonstrated in this cohort[296]. PNFA is classically associated with non-AD pathology but 10-30% of cases will have evidence of AD pathology at autopsy[303 304]. In this cohort one PNFA case had an autopsy proven diagnosis of mixed pathology: AD; cerebral amyloid angiopathy and Lewy Body disease. A degree of biomarker overlap was therefore expected, to reflect this pathological heterogeneity and is likely to explain why T-tau, P-tau and Aβ1-42 when used together actually reduced AUC.

There were also significant differences between the PNFA cohort and other FTD syndromes, bvFTD and SD. SD is the most pathologically homogeneous of the syndromes, being typically underpinned by TDP43 type C pathology[46], while bvFTD is caused by a range of molecular pathologies including, rarely, AD[46]. In both groups, mean measured concentrations of T-tau and P-tau were lower, and mean Aβ1-42 levels higher than the PNFA cohort, consistent with the fact that these syndromes are relatively less likely to have AD pathology at post mortem[305]. The finding that NFL was highest in SD is consistent with a number of previous

studies[214 213, 294]. NFL is a marker of large axonal neurodegeneration[136] which is elevated in a number of non-AD subcortical disease processes[306], particularly in FTD and motor neurone disease[307]. These findings taken together suggest a possible association between CSF NFL concentrations and TDP43 pathology. Since NFL levels were significantly higher in SD and PNFA compared to AD, it may have a clinical role in helping to distinguish AD from non-AD language cases.

It was found that YKL-40 showed some utility in differentiating AD from healthy controls, in keeping with findings from other studies[173,229]. YKL-40 is a non-specific marker of microglial activation and likely to be correlated with rate of disease progression[228], rather than with a specific neurodegenerative process[230] which may explain why I did not find major differences between AD and DLB, BvFTD, PNFA or SD.

Neither APPα nor APPβ, were found to be useful in differentiating AD from healthy controls, again in keeping with several previous studies[173]. Mean APPα and APPβ were both significantly lower in DLB and BvFTD cohorts compared to AD and healthy controls, similar to findings of a previous study by Gabelle *et al* [227] which demonstrated lower concentrations of APPβ in FTLD compared to control CSF. The significant of this is unclear, but raises the possibility amyloid metabolism may be altered in some non-AD pathology.

It was found that combining several different biomarkers did not materially increase diagnostic accuracy when comparing different dementias from one another; and that individually Aβ1-42/T-tau, AβX-42/X-40 P-tau and NFL were the measures that provided maximal accuracy to distinguish the various dementias from one another.

Strengths of the study include it being from a single centre study, with large numbers of well phenotyped individuals with a broad range of neurodegenerative diseases classified using consensus criteria independently by two raters; and use of both a control and independent validation cohort. Samples were analysed using an extended panel of biomarkers quantified on a single run to reduce sources of error; and robust statistical analyses were employed.

The study has some weaknesses. The retrospective cohort was retrospectively studied and samples were clinically acquired and therefore not necessarily handled according to a prospectively determined standard operating procedure, although broadly similar findings were made in a prospective cohort with CSF collected under research conditions. The numbers of samples in some clinical groups were comparatively small but is likely to represent broadly the proportion of patients with various forms of dementias who might undergo diagnostic CSF examination. Without post-mortem confirmation of the pathological diagnosis in all cases we cannot be certain of the underlying pathology, or, for example in the case of DLB there were more than one underlying pathologies. Very few CSF studies in dementia have pathological confirmation of diagnosis, and this is thus an inevitable limitation of most work in the literature. The author mitigated this limitation as far as possible both by using a relatively large clinical sample, employing clinical diagnostic criteria independently assessed by two clinicians both pre- and post-LP, and through use of an independent validation cohort. However, even taking these factors into account there are still conceptual limitations in using the pre-lumbar puncture clinical diagnosis to classify subjects; while this approach avoids circularity, it may also lead to biological misclassification. For example asymptomatic individuals with asymptomatic prodromal AD could be misclassified as healthy; individuals with atypical clinical phenotypes may fulfill more than one set of clinical criteria.

Furthermore, clinical syndromes that might be underpinned by one of a number of neurodegenerative pathologies (such as PNFA or PCA) may give rise to inconsistent heterogenous biomarker profiles leading to wide confidence intervals and difficult clinical interpretation. Future work might use a combination of clinical syndromic classification in combination with the more established CSF biomarkers to refine diagnosis as far as possible. Ideally one would also like to correct for important confounders such as white matter disease, which could be quantified using appropriate imaging sequences and processing pipelines.

Finally, whilst only a small proportion of the cases in this cohort had pathology confirmation or amyloid imaging, the diagnosis was consistent in all cases except for the case of PNFA with mixed AD/CAA/DLB pathology.

6.6 Conclusions

This work establishes the biomarker profiles of an extended panel of ELISA biomarkers in a range of neurodegenerative dementias and healthy controls. It is shown that CSF T-tau/Aβ1-42 ratio reliably discriminates AD from controls and from all of the other most common neurodegenerative diseases and AβX-40/42 ratio performs similarly. Aβ1-42 on its own does not have high diagnostic accuracy, but in the correct clinical context, NFL and P-tau have diagnostic utility in the differential diagnosis of dementia.

6.7 Publications arising from this chapter

R.W. Paterson *et al.* CSF in the differential diagnosis of Alzheimer's Disease: Clinical Utility of an Extended Panel of Biomarkers in a Specialist Cognitive Clinic. Alzheimer's & Dementia (Conference Abstract, Alzheimer's Association International Conference, 2016)

Chapter 7. CSF in the differentiation of AD clinical phenotypes

7.1 Introduction

Alzheimer's disease (AD) is usually associated with an initial decline in episodic memory followed by progressive cognitive deficits that reflect a symmetrical, generalised loss of function of association cortices[308]. However there is also considerable symptomatic heterogeneity particularly in young onset cases[36]. In some cases, this heterogeneity is sufficient to define discrete syndromic variants, including posterior cortical atrophy[26, 27, 309] and logopenic aphasia[32]. Whilst previously only amnestic presentations were recognised in diagnostic criteria for Alzheimer's disease, newer criteria including those from the International Working Group (IWG-2) which combine biomarkers and clinical phenotypes, distinguish "typical", i.e. memory-led Alzheimer's disease from "atypical" Alzheimer's disease, the latter comprising posterior (visual or biparietal), logopenic (language) and frontal (behavioural) variants[4]. Whilst the various Alzheimer's variants are, by definition, underpinned by the same core pathology, i.e. the accumulation of Aβ plaques and tangles composed of hyperphosphorylated tau protein and have a broadly similar widespread cortical distribution of Aβ,[310-312] there appears to be differences in the distribution of tau pathology[34], neuronal cell loss[313, 314], and network disruption[315] between variants. Aside from the fact that atypical AD variants are over-represented in young onset cases[36], and evidence for genetic differences in some of the atypical forms of the disease[79, 316, 317], The aim of this study was to use an extended CSF panel to assess differences between IWG-2 typical and atypical AD, and further to investigate whether amnestic, posterior cortical atrophy, logopenic

and frontal variants of AD were associated with different CSF profiles. The author hypothesized that there would be differences in markers of neurodegeneration and amyloid processing between AD subtypes to reflect different distribution of tau deposition and neuronal disruption. It was also considered that there might be differences in the role of neuroinflammation and large-calibre myelinated axon involvement to explain clinical heterogeneity.

7.2 Contributions and collaborations

The CSF ELISAs were carried out at the University of Gothenburg Laboratory in Sweden by board certified technicians under the supervision of Prof Henrik Zetterberg. Statistical analysis was supervised by Dr Jennifer M Nicholas. Neuropsychology testing was carried out by Prof Sebastian Crutch or one of the research neuropsychologists at the Dementia Research Centre.

7.3 Subjects and methods

7.3.1 Ethics statement

This study was approved by the Queen Square Ethics Committee.

7.3.2 Subjects

Subjects in this study are from the 'Retrospective' cohort described fully in chapter 2. All subjects had had a clinical CSF examination as part of their diagnostic work-up; had a CSF profile consistent with Alzheimer's disease (Aβ1-42<550pg/mL and tau/Aβ1-42 ratio≥0.5)[235]; and fulfilled IWG-2 criteria for Alzheimer's disease[4], summarized in Figure 7.1.

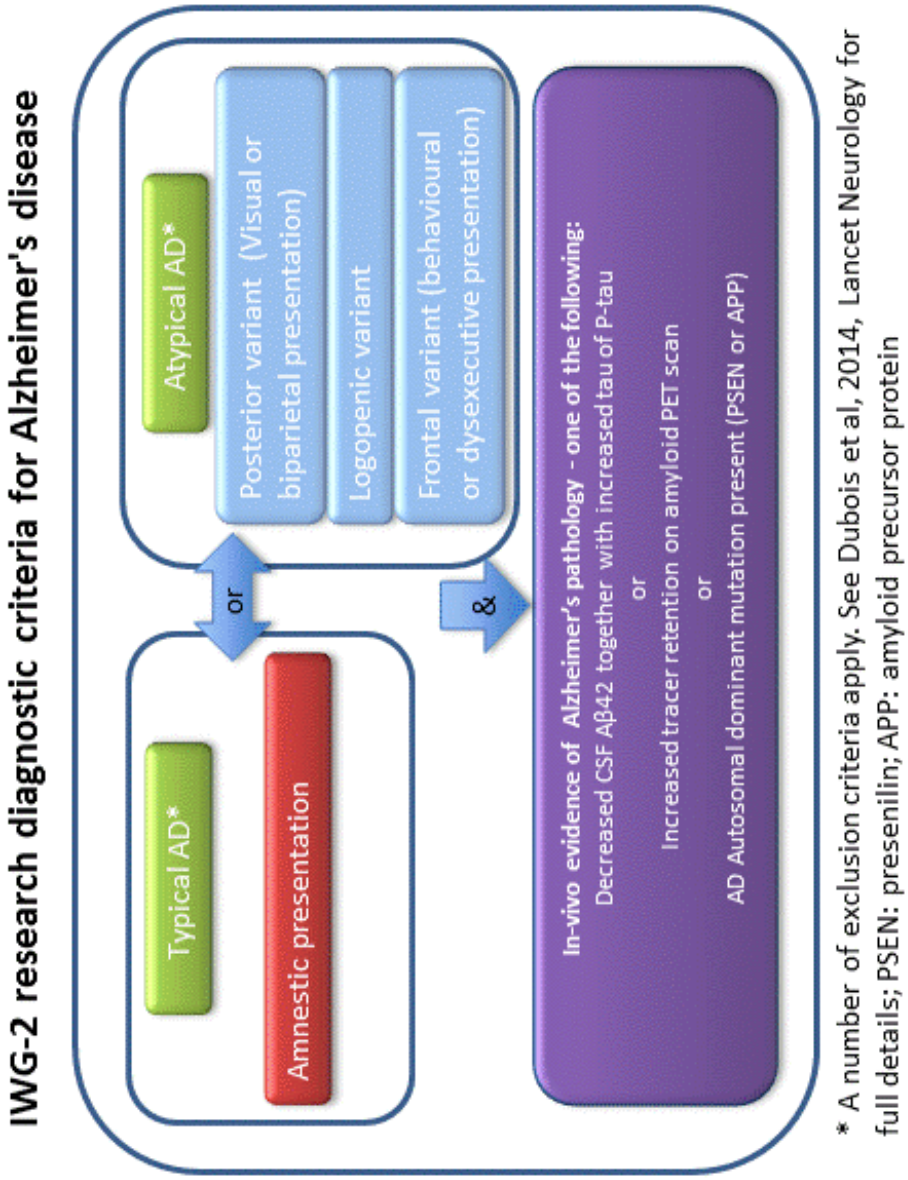


Figure 7.1 Summary of the IWG-2 criteria for AD.

Individuals were retrospectively classified as having typical (amnestic) Alzheimer's disease or atypical Alzheimer's disease according to IWG-2 criteria, and the IWG-2 atypical AD group were further sub-classified into those fulfilling clinical[318] criteria for posterior cortical atrophy (PCA)[32] or criteria for Logopenic Aphasia (LPA). In the absence of published criteria for frontal variant Alzheimer's disease (fvAD), the author examined the notes of all individuals with atypical Alzheimer's disease not

fulfilling PCA or LPA criteria, determining that all had early behavioural features (see table 7.4), thus fulfilling IWG-2 criteria for fvAD ("early, predominant, and progressive behavioural changes including association of primary apathy or behavioural disinhibition") [4]. The nearest mini-mental state examination (MMSE) score to the date of the lumbar puncture was recorded, as was estimated disease duration (in months) from first symptom to LP, based on recorded information from patients and informants. The rate of cognitive decline was estimated using the following formula: (30-MMSE at time of LP)/disease duration in months.

The majority of patients were only seen in routine clinical practice and had not been assessed using a single standardised neuropsychology battery. A proportion (n=22; 22.7%) had been assessed on a research neuropsychology battery, which included Recognition Memory Tests (RMT)[319, 320], and tests of posterior cortical functions including the VOSP Object Decision[321] or space perception (VOSP Number Location test) tests, the Graded Difficulty Arithmetic test[322], and Graded Difficulty Spelling test[323]. Those individuals fulfilling clinical criteria for PCA[318] who also performed above the 5th percentile on a memory test and below the 5th percentile on at least two of the four tests of posterior functions were additionally defined as fulfilling neuropsychology criteria for PCA (n-PCA)[313]. Those with impairment on both memory tests were defined as fulfilling research neuropsychology criteria for typical AD (n-tAD).

7.3.3 Cerebrospinal fluid Collection and Biomarker Analysis

CSF fluid collection and analysis was carried out according to the methods described in Chapter 2.

7.3.4 Statistical Analysis

Demographics and CSF biomarker levels were compared between groups using t-tests when there were no clear departures from a normal distribution, and Wilcoxon rank-sum tests for skewed or truncated data. Demographics and CSF biomarkers were compared across individuals with PCA, LPA and fvAD using one-way ANOVA when the distribution was approximately normal and Kruskal-Wallis rank test for skewed or truncated data, or Chi-squared tests for categorical variables. Post-hoc pairwise comparisons between pairs of groups were made when the initial test across all groups was statistically significant. Linear regression was used to explore the relationship between diagnosis and biomarker incorporating nuisance variables (age, sex, cognitive decline and MMSE) as covariates; non-normally distributed variables were log transformed for linear regression analysis. All statistical analyses used STATA Version 12.1 (Stata corporation, College Station, TX, USA).

7.4 Results

7.4.1 Comparison of IWG2 typical and atypical Alzheimer's disease

61 patients fulfilled criteria for typical AD and 36 for atypical AD (Table 7.1). The groups were similar in terms of age (62.5±6.6 vs. 62.3±7.4) and MMSE (20.6±6.4 vs 19.1±7.5) at the time of LP or estimated rates of cognitive decline (median=2.5 vs 2.8 MMSE points/yr); there was a non-significant trend for more women in the typical AD group (73.8% vs 55.6%).

The CSF biomarker profiles of typical and atypical Alzheimer's disease are shown in Table 7.1. There were no significant differences for any biomarker except for NFL,

which was significantly higher in the atypical Alzheimer's disease group (p=0.03). In a regression model incorporating age, sex, MMSE and rate of decline included in the model, this difference remained significant (p<0.05).

	Typical Alzheimer's disease	Atypical Alzheimer's disease	Typical vs Atypical Alzheimer's
	n=61	n=36	
Age at LP	62.5 ± 6.6	62.3 ± 7.4	0.90
Sex (% male)	26.2	44.4	0.08
MMSE	20.6 ± 6.4	19.1 ± 7.5	0.54
Months to LP	43.3 ± 25.2	48.0 ± 28.6	0.42
Estimated rate of decline (MMSE/yr)*	2.5 (1.3-4.6)	2.8 (1.4-5.3)	0.60
Aβ1-42 (pg/ml)	276.8 ± 100.8	293.3 ± 104.6	0.45
T-tau (pg/ml)*	694.9 (415.0-892.1)	642.6 (520.4-878.5)	0.63
P-tau (pg/L)	96.2 ± 44.7	96.0 ± 34.9	0.98
tau/Aβ1-42 ratio*	2.5 (1.8-3.9)	2.5 (1.7-4.1)	0.92
NFL (ng/L)*	1125 (737-1400)	1235 (1070-1610)	0.03^
YKL-40 (ng/L)	0.169 ± 0.06	0.181 ± 0.07	0.46
AβX-38 (ng/L)	1698 ± 813	1560 ± 515	0.32
AβX-40 (ng/L)	3954 ± 1622	3825 ± 1215	0.66
AβX-42 (ng/L)	165.3 ± 74.7	172.7 ± 74.7	0.64
AβX-40/X-42 ratio	25.4 ± 6.5	24.1 ± 6.5	0.33
APPα(ng/mL)*	349.2 (266.4-542.3)	353.8 (268.8-516.4)	0.81
APPβ(ng/mL)*	199.9 (152.4-337.4)	201.9 (161.3-282.6)	0.88

Table 7.1 Demographics and CSF profiles of individuals fulfilling IWG-2 criteria for typical/atypical Alzheimer's disease. Samples are from the Retrospective cohort. Data are shown as Mean ± SD unless stated * log transformed for regression analyses, values quoted as median (interquartile range); ^In a regression model including age, sex, MMSE and rate of decline, p-value remains significant.

7.4.2 Comparing atypical Alzheimer's disease subtypes

Of the 36 patients with atypical AD, 17 patients fulfilled criteria for PCA, 11 for LPA, with the remaining eight were classified as having fvAD. Demographics and CSF results are shown in Table 7.2. There were no significant differences in age or MMSE, but there were significant differences between the estimated rates of cognitive decline between the groups, with the fvAD cases declining significantly faster (median 5.3 MMSE points/yr) than either the LPA (3 points/yr) or PCA groups (1.9 points/yr). Rate of decline remained significantly higher in the frontal variant group compared with typical Alzheimer's disease even after adjusting for nuisance variables ($p=0.01$).

	PCA (n=17)	LPA (n=11)	fvAD (n=8)	Comparing phenotypes ANOVA (p-value)	Post hoc differences
Age at LP	62.7 ± 8.6	62.4 ± 6.80	61.5 ± 6.4	0.93	
Sex (% male)	35.3	63.6	37.5	0.31	
MMSE	20.7 ± 7.4	17.4 ± 8.9	17.4 ± 6.1	0.3	
Months to LP	53.9 ± 27.3	53.9 ± 31.5	27.3 ± 18.4	0.06	
Rate of decline (MMSE/yr)*	1.9 (0.75-4.25)	3.0 (1.8-3.8)	5.3 (4.0-19.5)	0.03	PCA, FV (p=0.018) LPA, FV (p=0.044)
Aβ1-42 (pg/ml)	311.7 ± 112.8	314.7 ± 91.1	224.6 ± 82.5	0.1	
T-tau (pg/ml)*	604.4 (436.8-675.8)	842.0 (591.8-890.5)	1185.4 (591.7-1329.3)	0.03	PCA, FV (p=0.036) PCA, LPA (p=0.036)
P-tau (pg/L)	79.8 ± 21.8	106.2 ± 34.2	116.4 ± 45.4	0.02	PCA, FV (p=0.012) PCA, LPA (p=0.040)
tau/Aβ1-42 ratio*	2.3 (1.4-2.6)	2.4 (1.7-4.3)	5.2 (3.3-6.9)	0.008	PCA, FV (p<0.01) LPA, FV (p=0.026)
NFL (ng/L)*	1138 (981-1416)	1220 (1130-1663)	1474 (1197 - 1838)	0.3	
YKL-40 (ng/L)	0.158 ± 0.04	0.190 ± 0.07	0.213 ± 0.01	0.39	
AβX-38 (ng/L)	1575 ± 387	1670 ± 729	1394 ± 442	0.54	
AβX-40(ng/L)	3898 ± 803	4246 ± 1698	3152 ± 1044	0.16	
AβX-42 (ng/L)	191.3 ± 75.2	188.2 ± 74.9	116.2 ± 47.0	0.04	PCA, FV (p=0.047)
AβX-40/X-42 ratio	22.1 ± 5.8	23.3 ± 5.2	27.9 ± 7.5	0.047	PCA, FV (p=0.016)
APPα(ng/mL)*	392.5 (336.3-517.2)	292.6 (258.4-558.9)	314.5 (263.3-437.6)	0.33	
APPβ (ng/mL)*	235.0 (178.6-309.2)	178.3 (152.6-367.3)	168.5 (140.9-233.6)	0.27	

Table 7.2 Demographics and CSF profiles of individuals fulfilling IWG-2 criteria for atypical Alzheimer's disease, sub-classified according to clinical syndrome. Subjects are from the Retrospective cohort. Data are shown as Mean ± SD unless stated; * Median (IQR)

Comparing the CSF profiles between the three subgroups (Table 7.2), significant differences were seen in T-tau, P-tau, T-tau/Aβ1-42 ratio, AβX-42 (measured using the MSD Abeta Triplex method), and AβX-40/X-42 ratio. Both T-tau and P-tau were lowest in the PCA group, intermediate in the LPA group and highest in the fvAD group with significant differences between PCA and each of the other groups. T-tau/Aβ1-42 ratio was significantly higher in the fvAD group than the PCA and LPA groups. There was a non-significant trend for Aβ1-42 measured using the Innotest ELISA to be lower in the fvAD group, and this was significant for AβX-42 measured using the MSD Abeta Triplex assay ($p<0.05$). AβX-42 was lowest in the frontal variant subgroup and highest in PCA. AβX-40/X-42 ratio was significantly higher in the fvAD group than the PCA group.

7.4.3 Comparing atypical Alzheimer's disease subgroups to amnestic Alzheimer's disease

Compared to typical AD, the fvAD group had significantly faster rates of MMSE decline ($p=0.01$), and significantly higher T-tau/Aβ1-42 ratio (0.01), NFL (<0.048) and AβX-42 levels measured using the triplex assay ($p=0.02$), and borderline lower AβX-40 levels ($p=0.08$). The LPA group were significantly more likely to be male ($p=0.03$), but there were no differences in any of the CSF profiles. The PCA group as whole had significantly lower levels of P-tau ($p=0.04$), and borderline lower AβX-40/X-42 ratios ($p=0.06$). Of the 22 individuals with detailed neuropsychology, 14, all previously classified as having IWG-2 typical AD, fulfilled criteria for n-tAD. Eight, all of whom fulfilled criteria for IWG-2 atypical AD and Tang-Wai criteria for PCA, also fulfilled criteria for n-PCA. Comparing these groups (Table 7.3), the n-PCA

group had lower T-tau (p=0.048), lower P-tau (p=0.048), and lower AβX-40/X-42 ratio (p=0.01) than the n-tAD group. In a regression model including age, sex, MMSE and rate of cognitive decline as covariates, AβX-40/X-42 ratio remained significantly different between the groups. The neuropsychology results also demonstrate significant group differences in recall memory test for words and for the visual object space and perception battery object decision task.

	Typical AD (n-tAD)	Atypical AD: PCA	Typical AD Vs PCA (p-value)
	n=14	n=8	
Age at LP	62 ± 6.6	64.9 ± 6.0	0.29
Sex (% male)	50	37.5	0.68
MMSE	24.5 ± 4.1	23.0 ± 6.2	0.55
Months to LP	39.1 ± 15.0	58.0 ± 33.0	0.28
Rate of decline (MMSE/yr)*	1.48 (0.5-2.5)	1.3 (0.6- 3.6)	0.62
Aβ1-42 (pg/ml)	265.4 ± 99.9	304.9 ± 102.1	0.39
T-tau (pg/ml)*	777.4 (674.6-1054.4)	639.3 (449.4-	0.048
P-tau (pg/L)	118.7 ± 50.9	85.8 ± 21.3	0.048
tau/Aβ1-42 ratio*	3.0 (2.4-4.1)	2.4 (1.7-2.6)	0.13
AβX-40/X-42 ratio	29.3 ± 8.3	22.5 ± 3.3	0.01^
NFL (ng/L)*	1161 (639-1409)	1271 (1083-1589)	0.33
YKL-40 (ng/L)	190918.7 ± 80475.3	164876.5 ±	0.65
AβX-38 (ng/L)	1961 ± 871	1678 ± 456	0.34
AβX-40 (ng/L)	4370 ± 1927	4085 ± 854	0.64
AβX-42 (ng/L)	165.5 ± 86.6	189.0 ± 55.8	0.46
APPα (ng/mL)*	454.6 (244.3-578.9)	401.8 (335.2-	0.60
APPβ (ng/mL)*	250.8 (155.2-411.9)	243.1 (165.0-	0.82
RMT – Faces (z score)	-1.42 (1.17)	-1.33 (1.54)	0.88
RMT – Words (z score)	-1.79 (1.5)	0.12 (2.77)	0.05
GNT (z score; mean±SD)	-0.16 (1.29)	-1.19 (1.24)	0.08
VOSP (object decision task); % subjects failing	14%	63%	0.05
Arithmetic (z score)	-1.66 (1.17)	-1.89 (0.98)	0.63

Table 7.3 Demographics and psychology profiles of a subgroup of typical and PCA subjects with detailed research neuropsychology who fulfilled research criteria for PCA done around time of LP. Subjects are from the retrospective cohort. * Median (IQR); values log transformed for regression analysis; ^In a regression model including age, sex, MMSE and rate of cognitive decline, p-value remains significant.

7.4.4 Behavioural (frontal variant) AD cases

Individuals classified as having FvAD and are described in Table 7.4.

Case	Sex	Onset Age	Leading symptom(s)	Disease Duration (months)	Psychiatric features	Neurological features	MRI Atrophy	MRI White matter disease
1 (MW)	M	55	Behavioural	48	N	Orofacial and limb dyspraxia increased tone	Parietal, hippocampal	-
2 (WS)	F	55	Behavioural Dyscalculia	36	Depression	Limb dyspraxia	Temporo-parietal	-
3 (CS)	M	60	Behavioural Hesitant speech	8	N	Limb dyspraxia Seizures	Hippocampal	Mild/moderate Periventicular
4 (PC)	F	61	Behavioural	24	Anxiety Depression	Normal	Hippocampal	Mild/moderate Periventicular
5 (BR)	M	74	Behavioural	6	Delusions	Postural tremor; brisk reflexes	None	Subcortical and Periventivcular
6 (JM) *	F	58	Behavioural	24	Anxiety depression	Normal	Hippocampal	Mild Periventicular and deep subcortical
7 (PA) *‡	F	49	Behavioural Psychiatric	48	Anxiety	Limb dyspraxia; myoclonus	Parietal	-
8 (PW)	F	63	Behavioural Word finding difficulty	18	N	Normal	None	Mild subcortical

AD: Alzheimer's disease; Y: yes; N: no; M: male; F: female; CSF: cerebrospinal; NFL: neurofilament light; * Negative Presenilin 1,2,APP genetics; ‡ Negative Progranulin genetics

Table 7.4 Clinical characteristics of 8 individuals from the Retrospective cohort with atypical Alzheimer's disease not fulfilling criteria for PCA or LPA, and defined as behavioural variant AD (fvAD) AD: Alzheimer's disease; Y: yes; N: no; M: male; F: female; CSF: cerebrospinal; NFL: neurofilament light; * Negative Presenilin 1,2,APP genetics; ‡ Negative Progranulin genetics

7.5 Discussion

In this study, an extended CSF panel was used to investigate the syndromic variants of Alzheimer's disease. The key findings are that the CSF profiles of IWG-2 typical and atypical AD are remarkably similar, bar elevation of NFL in the atypical group. However when carefully sub-classified there are significant differences between the various Alzheimer's disease subtypes. Notably PCA emerges as the phenotype associated with lower concentrations of T-tau and P-tau and AβX-40/X-42 ratio, and with a more indolent course; and that we define a small Alzheimer's disease subgroup (fvAD) with prominent behavioural/frontal features higher concentrations of the neurodegeneration markers T-tau, P-tau and NFL, lower concentrations of the amyloidogenic form of Aβ, Aβ1-42, and more aggressive disease.

As a group the cohort had an average age at onset of 62 yrs, with 52% fulfilling criteria for young onset AD (onset <65 years). Whilst atypical for AD *per se*, this reflects both the focus of the clinical service studied, and that patients with younger onset disease are those more likely to be offered a CSF examination as part of the diagnostic work-up[68, 99, 324]. In keeping with previous studies [36] that have shown an over-representation of atypical presentations in younger onset cohorts, this work found that a relatively high proportion (~40%) of the cohort had a non-amnestic presentation. Of those with atypical Alzheimer's disease, when defined using established criteria, 47% had PCA and 30% LPA. The remaining eight individuals (9% of the total sample and 23% of the atypical group) all had early and prominent behavioural features, in keeping with the IWG-2 classification, which divides atypical cases into posterior, logopenic, and frontal variants.

On a group level, the typical and atypical Alzheimer's disease groups were found to be well-matched for gender, age, and severity, and estimated rate of decline, which at ~2.5 MMSE points/year was as expected for individuals with mild-moderate disease[325]. On a group level, the CSF profiles were also very similar, with no significant differences in markers of neurodegeneration, amyloid deposition, amyloid processing, or neuroinflammation. The only difference between the groups was a significant elevation of NFL in the atypical group. NFL, a marker of degeneration of large calibre axons, that has previously been shown to be elevated in vascular dementia, frontotemporal dementia while only slightly elevated in Alzheimer's disease compared to healthy controls [213, 294]. Possible explanations for our findings are either that elevated NFL might be a marker of atypical Alzheimer's disease *per se*; or that the atypical Alzheimer's disease group is heterogeneous, with some individuals having very elevated NFL levels. Subsequent analyses of the atypical group suggest the latter to be the most likely explanation, with the NFL increase in the atypical Alzheimer's disease group being driven by those with fvAD. It is possible that this group had a greater burden of vascular disease; future work might compare vascular burden between AD subtypes using an imaging marker of white matter burden and explore how that might influence rate of clinical disease progression.

Despite the broad similarities to typical Alzheimer's disease on a group level, a more detailed assessment of the atypical Alzheimer's disease group revealed further differences between its constituent subtypes. Although severity was not significantly different at the time of LP, the PCA group had the lowest levels of T-tau and P-tau, the lowest AβX-40/AβX-42 ratios and the slowest rates of estimated cognitive decline. There were significant differences seen in all of these levels between PCA

and fvAD; in P-tau and AβX-40/AβX-42 ratio comparing all PCA cases with all those with typical Alzheimer's disease; and in T-tau, P-tau and AβX-40/X-42 ratio in the subgroup of individuals with more stringently neuropsychologically defined n-PCA and n-tAD. The existing literature examining CSF T-tau and P-tau levels in PCA has shown conflicting results. Several studies have reported levels to be similar between PCA and t-AD[236, 241, 310] although a recent study of 12 PCA patients also found T-tau and P-tau to be reduced in PCA compared to patients with LPA and typical Alzheimer's disease[242]. Whilst the biological significance of CSF T-tau and P-tau needs further study, both are thought to reflect ongoing neuronal degeneration[326]. High CSF T-tau is believed to reflect the intensity of neurodegeneration[127], and is not specific for Alzheimer's disease; highest levels are found in rapidly progressing disorders such as Creutzfeldt-Jakob disease, in encephalitis and after stroke[327]. By contrast, P-tau elevation is thought to be more specific to Alzheimer's disease related neurodegeneration[326], with prior studies suggesting that CSF P-tau correlates well with post-mortem cortical neurofibrillary tangle (NFT) burden[202, 328]. Imaging and pathological studies of PCA have consistently shown similar levels and distribution of amyloid pathology[310-312], but differences in the distribution of cortical tau pathology[34, 318, 329-331] and pattern of atrophy[309, 313, 332]. The lower levels of both T-tau and P-tau in CSF with similar levels of Aβ may therefore reflect differences in the focality of neurodegeneration in this variant of Alzheimer's disease. Another possible explanation might relate to the rate of neurodegeneration, given that as well as the reduced levels of T-tau and P-tau I found estimated rate of progression to be lower in the PCA group than in the other atypical phenotypes. This is however in contrast to another study (Teng *et al)* which found no differences in severity or disease duration in PCA compared to other subtypes. Still an alternate possibility is that there may be pathological

heterogeneity within the PCA group: whilst the vast majority of PCA patients have Alzheimer's disease pathology at autopsy, other pathological diagnoses include dementia with Lewy bodies, corticobasal degeneration, and very rarely prion disease[27]. Whilst it is possible that a few cases had alternative pathology, the very similar levels of Aβ1-42 and the fact that differences were seen not only in comparison with t-AD but other atypical forms of Alzheimer's disease which are perhaps equally if not more likely to be misdiagnosed during life, in our view make this unlikely to be the explanation.

Whilst Aβ1-42, the major component of the Alzheimer's disease amyloid plaque, is reduced in CSF in Alzheimer's disease, AβX-40 is thought to relate more to amyloid angiopathy and less to plaque pathology[333], and is relatively unchanged in Alzheimer's disease[334, 335]. Elevated AβX-40/X-42 ratio is reported as improving diagnostic accuracy in early Alzheimer's disease[336-338] and unlike Aβ42 level alone, to correlate with the extent of tau pathology[165]. The latter is consistent with our finding of both rather lower AβX-40/X-42 ratio and lower levels of P-tau in the PCA group.

In marked contrast to the PCA cases, the fvAD subjects had the highest rates of cognitive decline, together with high T-tau and P-tau levels, and AβX-40/AβX-42 ratio. Additionally, this group also had the highest levels of CSF NFL and T-tau/Aβ1-42 ratio, and the lowest levels of Aβ1-42. There were significant differences between rate of decline, T-tau, P-tau, T-tau/Aβ1-42 and AβX-40/X-42 ratios and AβX-42 (measured using the MSD platform) levels compared to PCA; and rate of cognitive decline, T-tau/Aβ1-42 ratio, AβX-42 and NFL levels compared

to typical Alzheimer's disease. An *a priori* classification system was not used to define the fvAD cases, which were those individuals fulfilling CSF criteria for Alzheimer's disease that did not fulfil criteria for the other Alzheimer's disease variants, and who on review of the case notes and as predicted by IWG2 criteria I found to have early behavioural features. Prior studies have suggested that fvAD (or behavioural variant Alzheimer's disease) is a rare phenotypic variant of Alzheimer's disease that can be clinically indistinguishable from behavioural variant frontotemporal dementia[339-341] and is often but not always associated with young onset[68]. In the few published pathological studies Alzheimer's disease pathology preferentially affected the frontal lobes[35]. Our finding of higher levels of T-tau and P-tau, lower levels of AβX-40/X-42 and more aggressive decline in these cases is the opposite to what was observed in PCA, and consistent with a relationship between these different pathological processes and rate of progression. The marked differences in CSF profile between these two Alzheimer's disease variants suggests that aside from having affecting different brain regions, there may well be fundamental differences in the underlying disease biology, reflected by alterations in amyloid processing and neurodegeneration. The increased NFL levels observed in these cases – and as discussed previously likely to be driving the differences between the typical and atypical Alzheimer's disease groups – is likely to be a further reflection of the more aggressive disease course in these individuals. Alternative explanations are that the elevated NFL level may be influenced by those cases with additional vascular changes on MRI. Whilst all individuals were diagnosed clinically with Alzheimer's disease, and all fulfilled CSF biomarker criteria for Alzheimer's disease, in the absence of post-mortem confirmation it isn't possible to be certain that a proportion of cases did not have non-Alzheimer's disease pathology, or mixed disease.

The LPA cases were biologically very similar to those with typical Alzheimer's disease, and indeed aside from a significant over-representation of males in the LPA group (64%) there were no differences between the LPA and tAD groups. Compared to the other variants, the estimated rate of decline was intermediate and significantly different to both the PCA and fvAD groups; and CSF levels of P-tau were significantly higher than in PCA, and lower than, but not significantly different from, the fvAD group. By contrast, the T-tau/Aβ1-42 ratios were very similar to PCA, but significantly lower than those in fvAD. Whilst a previous study found that T-tau levels were higher in LPA cases than in other forms of Alzheimer's disease[342], others have not found differences between LPA and other Alzheimer's disease subtypes[242]. The fact that some features of LPA were very similar to PCA but different to fvAD (e.g. Aβ1-42 and T-tau/Aβ1-42 ratio), others similar to fvAD and different to PCA (P-tau), with many intermediate between the two provide further evidence for a complex relationship between the various pathological processes underlying the development of Alzheimer's disease and its clinical manifestations.

Despite a number of biological differences between the various Alzheimer's disease subtypes, I did not find any differences in YKL-40. There is growing evidence that neuroinflammation plays a role in the pathogenesis of Alzheimer's disease[343], and with the caveat that the neuroinflammatory process is very complex and YKL-40 is only one of a number of potential biomarkers of neuroinflammation[344], this study did not find evidence for differences in the inflammatory process thought to be a major driver of phenotype. Similarly, whilst there were non-specific trends for both APPα and APPβ to be somewhat higher in the PCA group compared to the others, this

study found no evidence for major differences in APP metabolism between the different clinical phenotypes.

This study has a number of strengths, including a relatively high proportion of well-matched atypical cases allowing for meaningful comparisons with typical Alzheimer's disease. Established criteria were used for defining PCA and LPA cases, and an unbiased approach for determining fvAD. Using a CSF biomarker panel extending beyond conventional measures of Aβ and tau allows for other aspects of Alzheimer's disease biology to be assessed. Weaknesses include the relatively small number of cases in each of the atypical syndromic variants, making this work preliminary, although these numbers are favourable when compared to other studies. The author acknowledges that the biomarker comparisons between atypical variants involve very small sample sizes and should therefore be considered as exploratory. The study was retrospective, and so samples were not always collected under ideal research conditions, limited prospective psychology was available, and genetic information including ApoE4 status were not available. Rates of cognitive decline were estimated, and based on the MMSE which, being heavily weighted towards the deficits associated with typical amnestic Alzheimer's disease, may not accurately capture decline in the atypical phenotypes. As with any study using CSF measures to define Alzheimer's disease, cutpoints to define cases are inevitably a balance between sensitivity and specificity[246]; however, combining a T-tau/Aβ1-42 ratio of >0.52 has been shown to have reasonable sensitivity (>90%) and specificity (>80%) for Alzheimer's disease[235], which is likely to be further enhanced by, as required to fulfil IWG-2 criteria, also incorporating evidence for amyloid deposition (Aβ1-42<550pg/ml). Finally, whilst all cases fulfilled research criteria for Alzheimer's disease employing biomarkers, in the absence of pathology I cannot be certain that

all had underlying Alzheimer's disease, or that individuals with Alzheimer's disease did not have additional pathologies.

7.6 Conclusion

In summary, whilst ostensibly similar to typical Alzheimer's disease these data demonstrate that IWG-2 defined atypical Alzheimer's disease is not a homogeneous entity, with significant differences between PCA, LPA and fvAD; and between typical Alzheimer's disease and both PCA and fvAD. These differences are mainly focused on differential levels of tau and P-tau, and ratio of AβX-40/X-42 ratio and likely rates of clinical progression, suggesting that subtle differences in amyloid processing and neurodegenerative mechanisms may underpin at least some of the phenotypic diversity in Alzheimer's disease. As well as providing biological insights, these results have practical implications when it comes to interpreting CSF results in atypical variant, perhaps most importantly in PCA where levels of T-tau and P-tau are significantly lower, and may fall within normal ranges. Future studies with large number of cases, and exploring the spectrum of deficits rather than classifying individuals into syndromic groups may be necessary to elucidate further the perhaps subtle biological differences underpinning the diversity of Alzheimer's disease. It is also worth acknowledging that the biomarker panel used is still relatively limited and focused around specific aspects of AD pathology such as amyloid processing and neuroinflammation and does not include, for example, markers of synaptic function, which should be the subject of further study. More generally, these results demonstrate that using CSF biomarkers to study rarer forms of the disease may provide important insights into the pathogenesis of more typical amnestic Alzheimer's disease.

7.7 Publications arising from this chapter

RW Paterson, J Toombs, CF Slattery, JM Nicholas, U Andreasson, NK Magdalinou, K Blennow, JD Warren, CJ Mummery, MN Rossor, MP Lunn, SJ Crutch, NC Fox, H Zetterberg, JM Schott. Dissecting IWG-2 typical and atypical Alzheimer's Disease: insights from cerebrospinal fluid analysis. Journal of Neurology. 2015 Dec;262(12):2722

Chapter 8. CSF biomarkers of disease progression

8.1 Introduction

Although Alzheimer's disease (AD) is a relentless progressive condition there is considerable variation in the rate of progression between individuals[316]. Clinical progression can be difficult to accurately measure and compare between individuals; measures of an individuals' functional decline, such as the clinical dementia rating scale (CDR) (Appendix C) or the mini-mental examination score (MMSE) (Appendix A) are designed for individuals fulfilling criteria for memory led AD and don't necessarily accurately reflect clinical progression in atypical AD[345]. Furthermore these scores are expected to change on average ~2 points per year for MMSE and less for CDR, therefore while they might be valuable for measuring functional change in large groups, they are less likely to be of value for the individual or small group sizes particularly over a period of less than 3 years[346]. An alternative to measuring functional or cognitive decline is to measure brain atrophy rates. Rates of atrophy, either of brain or brain substructures can be measured with a high degree of precision from serially acquired MRI and provide a robust measure of progression which correlates with cognitive decline[347]. Atrophy rates are therefore an attractive surrogate marker of disease progression with which to use compare some of the more novel CSF markers.

Previous studies have suggested that atrophy rates may be affected by the age of onset[348], disease severity[349], by the concurrence of other pathologies including vascular disease[350] and TDP43 burden[351]. However, the majority of the variance in rates of atrophy, and therefore variance in clinical progression, between individuals

remains unaccounted for[352]. A more detailed understanding of factors influencing this variability could allow for prognostication for patients, and aid in clinical trial design or interpretation where interindividual variance in atrophy rate increases required sample sizes[352]; and provide insights into the underlying biology of AD, in turn leading to the discovery of new targets for disease prevention strategies.

Biomarkers offer a potential means of a) quantifying the rate of disease progression and b) exploring its influences. As outlined in the introductory chapter, none of the currently available biomarkers are closely correlated with cognitive function, however structural brain imaging changes, occur earlier in AD pathogenesis, and have so far been shown to be at least loosely correlated with levels of CSF Tau[194]. Commercially available CSF biomarker panels assess neuronal, synaptic, inflammatory, and other proteins involved, or potentially involved, in AD pathogenesis. The aim of this chapter is to determine which CSF markers best reflect rates of neuronal damage or loss in AD—and therefore may be useful predictors of clinical disease progression. A previous exploratory pilot study of CSF biomarkers in healthy elderly with amyloid pathology identified a number of analytes that may predict atrophy in specific brain regions[353]. In this study the aim was to assess whether any analytes in a large panel of CSF biomarkers were associated with increased rates of atrophy across the Alzheimer spectrum.

As the majority of individuals in the CSF study cohorts studied earlier in this thesis did not yet have good quality structural MRI scans performed at suitably long intervals to measure atrophy rates, the author accessed the publically available ADNI dataset where large numbers of individuals had both serial imaging as well as detailed CSF analyses.

8.2 Contributions and collaborations

All CSF sample collection and analysis and imaging analysis was carried out by ADNI. The author designed the study and carried out the statistical analysis with the support of Jonathan Bartlett, statistician at the London School of Hygiene and tropical Medicine and Jonathan Schott.

8.3 Methods

8.3.1 Subjects

The author investigated subjects from the Alzheimer's Disease Neuroimaging Initiative (ADNI) (adni.loni.ucla.edu), a multicenter publicly/privately funded longitudinal study of individuals with AD, amnestic mild cognitive impairment (MCI) and normal cognition. Institutional review boards approved the study and subjects gave written consent. Subjects underwent baseline and periodic clinical and neuropsychological assessment and serial MRI. Approximately 60% had CSF. A selected group had additional CSF analysis for the ADNI Biomarkers Consortium project "Use of Targeted Multiplex Proteomic Strategies to Identify Novel Cerebrospinal Fluid (CSF) Biomarkers in AD" as described on the ADNI website. The author downloaded data from LONI (http://adni.loni.ucla.edu) that included all subjects with this supplementary CSF multiplex data. An overview of subject selection is shown in Figure 8.1. As the aim was to explore factors influencing atrophy rates in individuals with AD pathology the author dichotomised subjects using a baseline CSF Aβ1-42 level of 192pg/mL, a level shown in a separate study using the same methodology, to distinguish individuals with autopsy confirmed AD pathology and controls with ~96% sensitivity and ~77% specificity[73]. Subjects who

did not have useable 1.5T MRI scans at baseline and one year were excluded, as was one subject without a defined diagnosis. ApoE status and genotype were recorded at the rs7280100 SNP which has been identified a candidate locus for TFF3[354], as well as Mini Mental State Examination (MMSE) at baseline and 12 months.

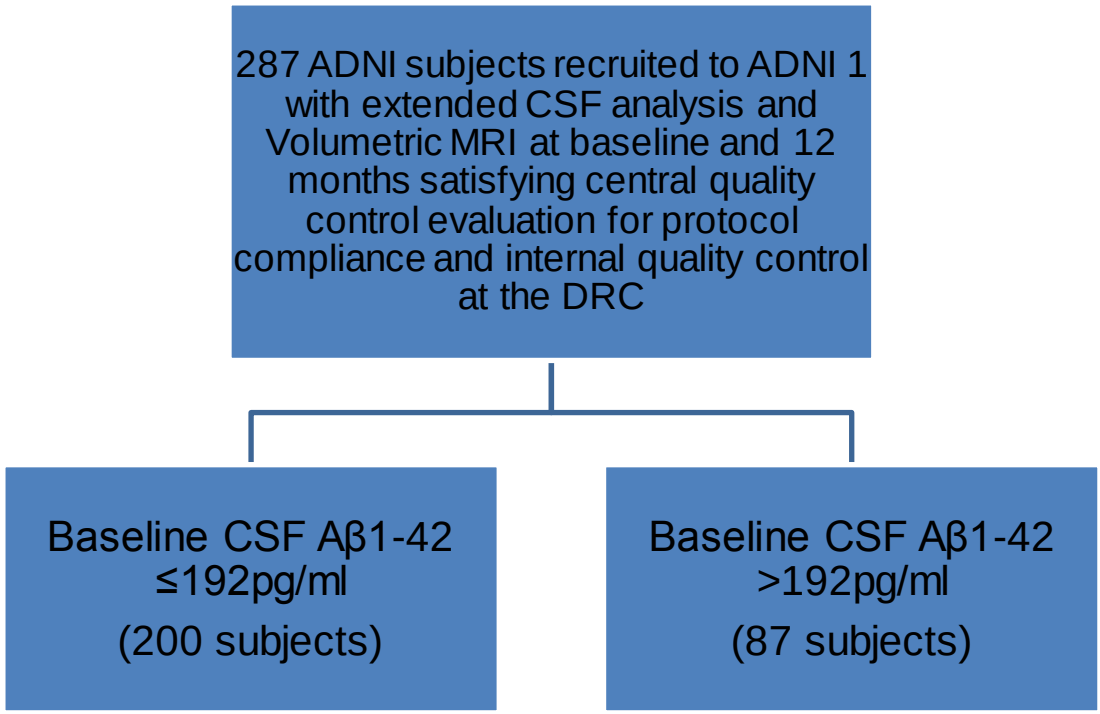


Figure 8.1 Flowchart outlining subjects eligible for inclusion

8.3.2 Cerebrospinal fluid

CSF collection, processing and storage procedures have previously been described[73]. Processing, aliquoting and storage was carried out according to the ADNI Biomarker Core Laboratory Standard Operating Procedures (SOP) (http://adni-info.org/Scientists/Pdfs/adniproceduresmanual12.pdf). Samples were analysed using a multiplex-based immunoassay panel based upon Luminex immunoassay technology developed by Rules Based Medicine (MyriadRBM, TX). CSF Aβ1-42,

total tau (T-tau) and phosphorylated tau (P-tau) and a panel of 159 analytes including inflammatory, metabolic, lipid and other disease relevant analytes were tested. Data were prepared for analysis according to the biomarkers consortium statistical analysis plan (http://adni.loni.ucla.edu/wp-content/uploads/2012/01/2011Dec28-Biomarkers-Consortium-Data-Primer-FINAL1.pdf), and as previously described[355]. Of 159 analytes, 76 had greater than 10% of quality control data missing, leaving 83 available for analysis. For each analyte, the normality of data was assessed by the ADNI Biomarkers Consortium: non-normal data were transformed using the Box and Cox technique[356].

8.3.3 Image acquisition

Details of the MRI methodology have previously been described[357]. T1 weighted, inversion-recovery (IR) prepared structural images were acquired at baseline and 12 months on 1.5T MRI units using standardized protocols. Corrections for distortion due to gradient nonlinearity and for image intensity non-uniformity and scalings were made based on phantom measures. Images underwent central quality control evaluation for protocol compliance and internal quality control at the Dementia Research Centre.

8.3.4 Volume loss measurement

Image analysis was performed using in-house MIDAS software[112]. Whole brain and lateral ventricles were delineated semi-automatically and hippocampal volumes were measured using the automated HMAPS method[113]: Volume loss (ml) between scans was obtained using the boundary shift integral (BSI) following a 9-degrees-of-

freedom registration and differential bias correction of the follow-up to baseline scans. For lateral ventricles, and hippocampi change over time was quantified using the ventricular (VBSI)[358] and hippocampal BSI (HBSI)[113] respectively. Rates of volume loss were annualized using the inter-scan interval.

8.3.5 Statistical Analysis

To assess the relationship between CSF analytes and rates of brain volume change, separate regression models were fitted for rates of ventricular expansion, brain and hippocampal atrophy for each CSF variable, including baseline volume (brain, ventricular and hippocampal volume, respectively) and tau as covariates in both the amyloid positive and negative groups. Subsequent analyses in the amyloid positive group alone were repeated including age, gender, APOE4 status and phospho-tau (P-tau) as additional covariates and finally also adjusting for baseline diagnosis (AD, MCI, control). Implementation of the false discovery rate (FDR) procedure[272] with control at the 5% level to correct for multiple comparisons was used, and the adjusted regression coefficients between each atrophy rate and those CSF variables showing FDR significant relationships reported. Three exploratory reverse stepwise regression analyses were performed to identify combinations of CSF analytes independently predicting increased rates of change for each measure, using FDR significant CSF variables identified in the preceding step and the three sets of adjustment variables. In each stepwise analysis, the corresponding adjustment variables were forced to be included in regression models. Finally, I assessed whether CSF analytes were associated with cognitive function. It was first established whether there was a decline in MMSE between baseline and 12 months that was significantly different from zero. It was then determined if atrophy rates

and decline in MMSE scores at 12 months were correlated using separate regression models for rates of ventricular expansion, brain and hippocampal atrophy. Separate regression models were then fitted for annualised change in MMSE score and each CSF variable found to be (after FDR control) associated with at least one atrophy measure. The FDR procedure used was derived assuming independence between test statistics[272], however, it has been shown that the procedure is valid under certain types of dependence[359], and in a simulation study (results not shown) matching the analysis used here, the procedure correctly controlled the FDR at 5%.

To quantify the unadjusted group discrimination ability of the analytes that were FDR significant adjusted for baseline brain volumes, sex, age, APOE4 status, tau and P-tau the area under the ROC curve for detecting between AD and control groups was estimated.

8.4 Results

8.4.1 Subject demographics

The demographics, genetic characteristics, cognitive scores and atrophy measures of the 287 subjects included in this analysis are described in Table 8.1.

The mean±SD age of this group was 74.9±6.9yrs, 21.6% had a clinical diagnosis of AD, 48.1% MCI and 30.3% were controls. 83 CSF analytes as well as CSF tau, Aβ1-42 and P-tau were available for analysis (Appendix E).

	Subjects with Ab1-42≤192pg/ml (n=200)			
	Combined	**Controls (n=33)**	**MCI (n=108)**	**AD (n=59)**
Age at CSF exam (years)	74.8 ± 6.7	76.5 ± 5.5	74.4 ± 7.0	74.5 ± 7.4
Gender (% male)	59.4	54.5	63.9	52.5
APOE4 positive (%)	65.5	51.5	69.0	78.0
MMSE (mean)	26.1 ± 2.5	29.1 ± 0.1	26.7 ± 1.8	23.5 ± 1.8
Modified ADAS-cog	13.1 ± 6.2	7.1 ± 3.4	12.1 ± 4.6	18.5 ± 6.1
Ab1-42 (pg/ml)	137.2 ± 22.7	146.5 ± 25.5	136.5 ± 29.0	133.1 ± 23.2
t-tau (pg/ml)	114.4 ± 55.3	82.5 ± 30.7	115.3 ± 56.0	130.7 ± 57.7
p-tau (pg/ml)	39.5 ± 17.5	31.2 ± 17.4	39.8 ±15.4	43.6 ± 19.8
KBSI (ml/yr)	13.79 ± 8.65	8.9 ± 7.3	14.2 ± 9	15.7 ± 7.8
VBSI (ml/yr)	3.82 ± 3.08	2.1 ± 2.0	3.8 ± 3.1	4.9 ± 3.1
HBSI (ml/yr)	0.15 ± 0.10	0.08 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
MMSE decline from baseline to 12 months(points/yr) (n=199)	1.7 ± 3.5	0.4 ± 1.7	1.4 ± 2.7	3.0 ± 4.8
% individuals with minor allele rs7280100	12.2	12.5	10.9	14.6
	Subjects with Ab1-42>192pg/ml (n=87)			
	Combined	**Controls (n=54)**	**MCI (n=30)**	**AD (n=3)**
Age at CSF exam (years)	75.4 ± 6.3	74.9 ± 5.2	75.5 ± 8.0	81.4 ± 6
Gender (% male)	62.1	50	80	100
APOE4 positive (%)	89.6	90.7	86.7	100.0
MMSE (mean)	28.3 ± 1.7	29.0 ± 1.0	27.2 ± 1.9	25.3 ± 1.2
Modified ADAS-cog	7.6 ± 1.4	5.7 ± 2.5	10.5 ± 3.8	14.4 ± 6.8
Ab1-42 (pg/ml)	244.4 ± 26.1	243.1 ± 25.6	246.5 ± 26.0	247.0 ± 45.7
t-tau (pg/ml)	62.2 ± 20.9	60.8 ± 21.4	64.3 ± 20.2	66 ± 22.3
p-tau (pg/ml)	21.0 ± 8.0	20.6 ± 7.9	21.8 ± 8.5	21.3 ± 7.5
KBSI (ml/yr)	6.0 ± 6.7	4.7 ± 5.4	7.5 ± 8.0	12.6 ± 10.5
VBSI (ml/yr)	1.4 ± 1.6	1.1 ± 1.1	1.7 ± 2.1	2.5 ± 2.5
HBSI (ml/yr)	0.05 ± 0.1	0.03 ± 0.09	0.07 ± 0.1	0.1 ± 0.06
MMSE decline from baseline to 12 months(points/yr) (n=199)	0.1 ± 1.6	0.06 ± 1.3	0.2 ± 2	0 ± 2
% individuals with minor allele rs7280100	12.0	13.2	11.1	0

Table 8.1. Baseline demographics, ApoE genotype, cognitive profiles, CSF profiles, brain volumes and 1 year atrophy rates of 200 subjects with Ab1-42≤192pg/ml and 87 subjects with Ab1-42>192pg/ml. Subjects are from the ADNI cohort; MCI: mild cognitive impairment; AD: Alzheimer's disease; CSF: cerebrospinal fluid; MMSE: mini mental state examination; ADAS-cog: Alzheimer's Disease Assessment Scale-cognitive subscale; T-tau: total tau; P-tau: phosphorylated tau 181; KBSI: Whole brain boundary shift integral; VBSI: ventricular boundary shift integral; HBSI: hippocampal boundary shift integral. Mean ± SD provided unless stated.

8.4.2 Analytes predicting atrophy

In subjects without evidence for significant amyloid deposition, i.e. those with baseline CSF Aβ1-42 >192pg/mL, after adjusting for baseline brain volumes and CSF T-tau and with FDR correction to control for multiple comparisons, none of the CSF analytes were significantly associated with any of the atrophy measures.

In subjects with CSF Aβ1-42 ≤192pg/mL, after adjusting for baseline brain volumes and T-tau and with FDR correction to control for multiple comparisons, 10/83 analytes were associated with whole brain atrophy rate, 45/83 analytes with ventricular expansion rate, and 4/83 with hippocampal atrophy rate (Table 8.2). After additionally adjusting for P-tau, age, ApoE status and sex and with FDR correction to control for multiple comparisons, 4/83 analytes were associated with whole brain atrophy rate, 1/83 analyte with ventricular expansion rate, and 2/83 with hippocampal atrophy rate (Table 8.2). These relationships are illustrated using scatter plots in Figure 8.2. After additionally adjusting for baseline diagnosis, 2/83 analytes were associated with hippocampal atrophy, and none with brain atrophy or ventricular expansion (Table 8.2).

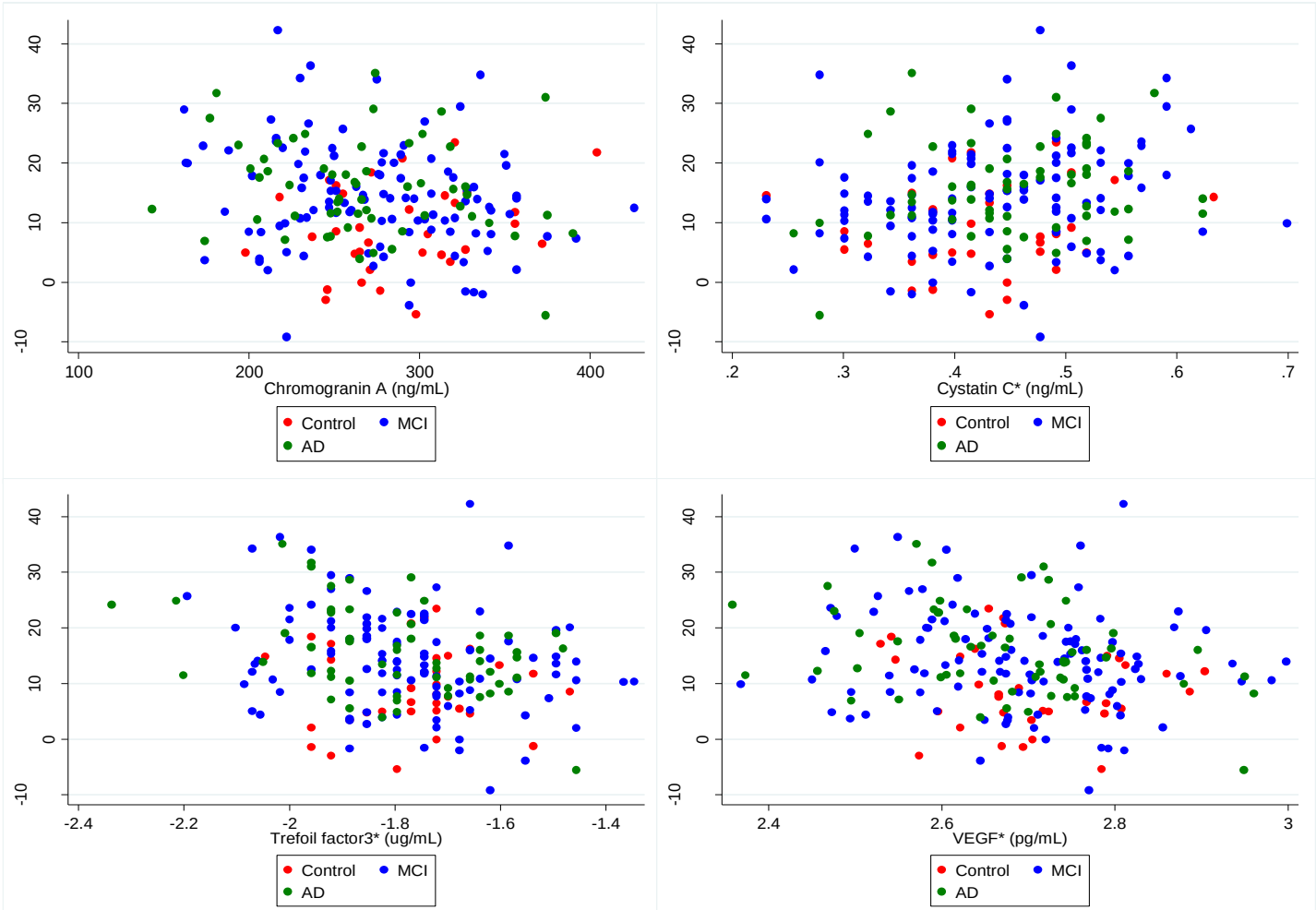


Figure 8.2. Scatter plots of annualized atrophy (BSI—whole brain atrophy) against analytes for those found to be associated (after FDR correction) with rates of volume change after adjusting for baseline volume, sex, age, APOE4 status, T-tau and P-tau. AD, Alzheimer's disease; MCI, mild cognitive impairment. Where data have been transformed (TFF3, CysC, VEGF), the units relate to data before transformation.

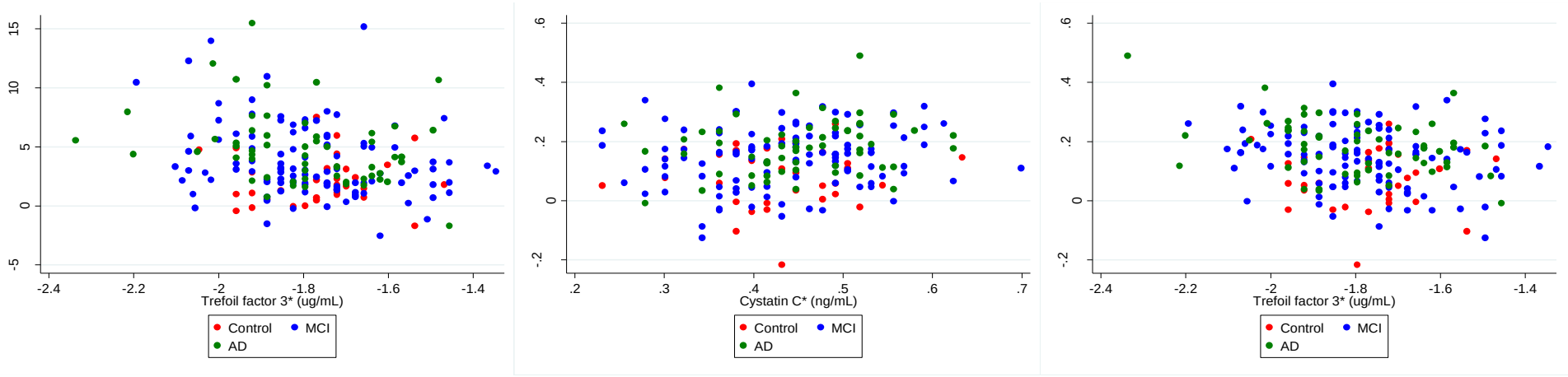


Figure 8.2 (continued). Scatter plots of annualized atrophy (HBSI—hippocampal atrophy, VSBI—ventricular expansion) against analytes for those found to be associated (after FDR correction) with rates of volume change after adjusting for baseline volume, sex, age, APOE4 status, T-tau and P-tau; *: transformed data. Where data have been transformed (TFF3, CysC, VEGF), the units relate to data before transformation. AD, Alzheimer's disease; CSF, cerebrospinal fluid; BSI, boundary shift integral; FDR, false discovery rate; MCI, mild cognitive impairment; P-tau, phosphorylated tau; T-tau, total tau; TFF3, trefoil factor 3.

Lower levels of trefoil factor 3 (TFF3) were consistently associated with greater ventricular expansion ($p<0.001$), hippocampal atrophy rate ($p<0.001$) and whole brain atrophy rate ($p<0.001$) even after adjusting for baseline brain volumes, T-tau, P-tau, age, APOE status and sex. After additionally adjusting for baseline diagnosis lower levels of TFF3 were still associated with higher hippocampal atrophy ($p=0.007$). Higher levels of Cystatin C (CysC) were positively associated with all three atrophy measures after adjusting for baseline brain volumes and T-tau and remained predictive of higher whole brain ($p=0.009$) and hippocampal atrophy ($p=0.034$) after adjusting for P-tau, age, ApoE status and sex. Lower levels of vascular endothelial growth factor (VEGF) were positively associated with all three atrophy measures after adjusting for baseline brain volumes and T-tau and remained associated with higher whole brain atrophy ($p=0.023$) after adjusting for P-tau, age, APOE status and sex . Lower levels of Chromogranin A (CgA) were associated with higher whole brain atrophy (0.008) and ventricular expansion (0.009) after adjusting for baseline volumes and T-tau and predicts higher whole brain atrophy ($p=0.009$) after additionally adjusting for P-tau, age, ApoE status and sex.

	Brain Atrophy		Ventricular Expansion		Hippocampal Atrophy	
	Regression coefficient	*P*	Regression coefficient	*P*	Regression coefficient	*P*
Adjusting for baseline brain volumes and t-tau						
Alpha-1-Microglobulin*(ug/mL)			-3.17	0.002		
Alpha-2-Macroglobulin*(mg/mL)			-4.31	0.006		
Alpha-1-Antitrypsin*(mg/mL)			-0.19	0.018		
Angiotensin-Converting Enzyme*(ng/mL)			-3.17	0.027		
Angiopoietin-2*(ng/mL)			-3.24	0.013		
Apolipoprotein A-I*(mg/mL)			-2.39	0.018		
Apolipoprotein C-III*(ug/mL)			-2.02	0.042		
Apolipoprotein D* (ug/mL)			-3.54	0.003		
Apolipoprotein E* (ug/mL)	-14.71	0.017	-0.26	0.002		
Apolipoprotein H* (ug/mL)			-2.99	0.004		
AXL Receptor Tyrosine Kinase (ng/mL)	-1.59	0.01	-0.40	0.018		
Beta-2-Microglobulin* (ug/mL)			-3.87	0.018		
Complement C3*(mg/mL)			-3.80	0.003		
CD 40 antigen* (ng/mL)			-5.20	0.004		
Chromogranin-A (ng/mL)	-0.05	0.008	-0.013	0.009		
Clusterin*(ug/ml)	-11.90	0.023	-4.20	0.002		
Cystatin-C*(ng/mL)	32.89	0.003	10.77	<0.001	0.34	0.019
Fibroblast Growth Factor 4*(pg/mL)			3.45	0.022		
Fibrinogen*(mg/mL)			-0.28	0.001		
Heparin-Binding EGF-Like Growth Factor* (pg/mL)			-4.08	0.048		
Hepatocyte Growth Factor* (ng/mL)			-2.59	0.048		
Immunoglobulin A* (mg/mL)			-1.28	0.048		
Interleukin-3* (ng/mL)	-5.66	0.036	-2.02	0.003		
Insulin-like Growth Factor-Binding Protein (ng/mL)			-0.017	0.004		
Interferon gamma Induced Protein 10* (pg/mL)			-0.17	0.034		
Lectin-Like Oxidized LDL Receptor 1 (ng/mL)			-0.30	0.006		

Table 8.2. Regression coefficients for dependence of atrophy measures on CSF with control for the False Discovery Rate in subjects with low CSF Aβ1-42 (≤192pg/mL)

Macrophage Colony-Stimulating Factor 1* (ng/mL)	-15.02	0.023	-5.89	<0.001		
Monokine Induced by Gamma Interferon*(pg/mL)			-1.66	0.009		
Neutrophil Gelatinase-Associated Lipocalin*(ng/mL)			-2.49	0.018		
N-terminal prohormone of brain natriuretic peptide*(pg/mL)			-3.91	0.006	-0.15	0.035
Placenta Growth Factor* (pg/mL)			-2.34	0.043		
Pancreatic Polypeptide* (pg/mL)			-1.31	0.038		
Serum Amyloid P-Component* (ug/mL)			-1.56	0.03		
Stem Cell Factor* (pg/mL)			-0.18	0.027		
Sex Hormone-Binding Globulin*(nmol/L)			-2.23	0.018		
Thyroxine-Binding Globulin* (ug/mL)			-0.18	0.023		
Tissue Factor* (ug/mL)	-11.79	0.017	-3.73	0.003		
Trefoil Factor 3* (ug/mL)	-14.69	0.003	-6.19	<0.001	-0.178	0.002
Tissue Inhibitor of Metalloproteinases 1* (ng/mL)			-3.88	0.015		
Thrombomodulin* (ng/mL)			-2.52	0.045		
Tumor Necrosis Factor Receptor 2*(ng/mL)			-4.05	0.007		
TNF-Related Apoptosis-Inducing Ligand Receptor 3*(ng/mL)			-4.05	0.004		
Vascular Cell Adhesion Molecule-1* (ng/mL)			3.46	0.018		
Vascular Endothelial Growth Factor* (pg/mL)	-21.32	0.004	-6.86	<0.001	-0.21	0.034
von Willebrand Factor* (ug/mL)			-4.18	0.003		
Adjusting for baseline brain volumes, sex, age, APOE4 status, tau and p-tau						
Chromogranin-A (ng/mL)	-0.05	0.009				
Cystatin-C*(ng/mL)	32.58	0.009			0.35	0.034
Trefoil Factor 3* (ug/mL)	-16.43	0.009	-4.46	0.03	-0.23	0.001
Vascular Endothelial Growth Factor* (pg/mL)	-20.09	0.023				
Adjusting for baseline brain volumes, sex, age, APOE4 status, tau, p-tau and baseline diagnosis						
Trefoil Factor 3*(ug/mL)					-0.21	0.007
N-terminal prohormone of brain natriuretic peptide*(pg/mL)					-0.16	0.05

Table 8.2 (continued). Regression coefficients for dependence of atrophy measures on CSF with control for the False Discovery Rate in subjects with low CSF Aβ1-42 (≤192pg/mL): adjusted for baseline brain volumes and tau; adjusted for baseline brain volumes, sex, age, APOE4 status, T-tau and P-tau; adjusted for baseline brain volumes, sex, age, APOE4 status, T-tau, P-tau and baseline diagnosis. Regression coefficients are shown for those measures showing FDR significant (5% level) associations. P-values are FDR (5% level) corrected. *: transformed data. The statistics are presented for the transformed values (see methods). Where data have been transformed the units relate to data before transformation. None of the analytes in Appendix E was FDR significant except those shown in this table. Subjects are from the ADNI cohort

In exploratory reverse stepwise models that included only those variables showing FDR significant associations in the initial analyses (Table 8.3), lower levels of TFF3 were associated with higher rates of whole brain atrophy, ventricular expansion and hippocampal atrophy, even when, P-tau, age, APOE status and sex were included in the model. Lower levels of CgA were associated with higher whole brain atrophy and ventricular expansion when T-tau and baseline volume were included in the model; and with higher whole brain atrophy when P-tau, age, APOE status and sex were also included as covariates. Additionally adjusting for baseline diagnosis, TFF3 was the only analyte independently associated with hippocampal atrophy rate; and no analytes were (independently) associated with whole brain atrophy or ventricular expansion.

Brain atrophy			Ventricular Expansion			Hippocampal atrophy		
Analyte	*Adjusted regression coefficient*	*p=*	Analyte	*Adjusted regression coefficient*	*p=*	Analyte	*Adjusted regression coefficient*	*p=*
Adjusted for t-tau and baseline volume								
TFF3* (ug/mL)	-12.3	0.001	TFF3* (ug/mL)	-4.7	<0.001	TFF3* (ug/mL)	-0.18	<0.001
Chromogranin-A (ng/mL)	-0.04	0.006	Fibrinogen* (mg/mL)	-1.4	0.002			
			Angiotensin-Converting Enzyme*(ng/mL)	4.3	0.012			
			Macrophage Colony-Stimulating Factor 1* (ng/mL)	-4.2	0.030			
			Chromogranin-A (ng/mL)	-0.01	0.032			
Adjusted for baseline volume, sex, age, APOE4 status, t-tau and p-tau								
TFF3 *(ug/mL)	-13.2	0.004	TFF3 *(ug/mL)	-4.5	<0.001	TFF3 *(ug/mL)	-0.23	<0.001
Chromogranin-A (ng/mL)	-0.04	0.004						
Adjusted for baseline volume, sex, age, APOE4 status, t-tau and p-tau and baseline diagnosis								
						TFF3* (ug/mL)	-0.23	<0.001

Table 8.3. Exploratory reverse stepwise regression analysis of CSF analytes with an FDR significant association with brain atrophy measurement in subjects with low CSF Aβ1-42 (≤192pg/mL): when adjusted for T-tau and baseline volume; when adjusted for baseline volume, sex, age, APOE4 status, T-tau and P-tau; when adjusted for baseline volume, sex, age, APOE4 status, T-tau and P-tau and baseline diagnosis. P-values shown here do *not* account for multiple comparisons. *:transformed data. The statistics are presented for the transformed values (see methods). Where data have been transformed the units relate to data before transformation. Subjects are from the ADNI cohort.

Only 23 of this cohort had a minor allele at the rs7280100 locus (predicted to reduce CSF TFF3). These individuals had 18% higher rates of ventricular expansion, 14% higher rates of brain atrophy, and 30% higher rates of hippocampal atrophy

compared to non-carriers, the latter reaching borderline significance after adjustment for baseline diagnosis, (p=0.07).

8.3.4 Analytes predicting cognitive decline

In subjects with CSF Aβ1-42 >192pg/mL MMSE data at 12 months was available for all 87 subjects. The average decline was not statistically significantly different from zero and therefore no further regression analyses were conducted in this group.

In subjects with CSF Aβ1-42 <192pg/mL, serial MMSE data at 12 months was available for 199 subjects, who declined on average by 1.7±3.5 points/yr. Change in MMSE score at 12 months was strongly associated with change in whole brain atrophy rate (regression coefficient -0.15, p<0.001), ventricular expansion (-0.49, p<0.001), and hippocampal atrophy rate. (-11.16, p<0.001) Baseline levels of 11 CSF markers – AXL, Apo E, CD-40 antigen, CgA, cystatin C, M-CSF, matrix metalloproteinase-2 (MMP-2), pregnancy associated plasma protein, tissue factor, TFF3 and VEGF – were significantly (without FDR correction) associated with decline in MMSE at 12 months.

8.3.5 Predictive Value

For those analytes that were FDR significant in Table 8.2, the area under the ROC curve for detecting between AD and control groups were 0.59 (95% CI 0.50, 0.69) (CgA), 0.55 (0.45, 0.64) (CysC), 0.55 (0.45, 0.65) (TFF3) and 0.61 (0.52, 0.70) (VEGF). For reference, the corresponding estimated values for T-tau and P-tau were 0.85 and 0.83 respectively.

8.4 Discussion

Current models of AD pathogenesis suggest that deposition of brain Aβ is a very early feature of AD, probably occurring prior to the onset of AD-related neuronal loss (i.e. neurodegeneration)[78]. Rates of atrophy are significantly increased in individuals with established AD,[360] mild cognitive impairment due to AD[361], and in asymptomatic brain amyloidosis[362], and correlate more closely with cognitive decline and disease progression than amyloid burden or rate of accumulation[95]. In this study, using a panel of analytes selected on the basis of relevance to a range of different diseases including cancer and autoimmune disorders as well as AD I have identified a number of CSF biomarkers associated with increased rates of neurodegeneration. In particular, our results suggest that in individuals with evidence for brain amyloid deposition, CSF TFF3 level is associated both with rate of cognitive decline and with rates of brain and hippocampal atrophy and ventricular expansion.

Whilst I failed to find an association between any analyte and rate of atrophy in the amyloid negative group, after allowance for multiple comparisons, in the amyloid positive group in which I adjusted for baseline brain volume and T-tau I found that 45 analytes predicted increased ventricular expansion, and ten predicted rate of whole brain atrophy. Ventricular expansion and brain atrophy are closely correlated[332], and as expected, all ten factors predicting increased rates of whole brain atrophy also predicted increased ventricular expansion. The higher precision with which rate of ventricular change can be quantified[358] is likely to explain the larger number of analytes associated with ventricular expansion compared to whole brain loss.

When I additionally adjusted for P-tau, ApoE status and sex, adjusting for multiple comparisons, the number of factors associated with rates of neurodegeneration in the amyloid positive group alone was considerably reduced, with only four analytes showing an association with excess neurodegeneration. Lower levels of TFF3, VEGF, and CrA and higher levels of CysC were associated with increased rates of brain atrophy; lower levels of TFF3 with increased ventricular expansion; and lower levels of TFF3 and higher levels of CysC with increased rates of hippocampal atrophy. In exploratory reverse stepwise analyses TFF3 was significantly associated with rate of decline in all three measures (Table 8.3), with an effect both independent from and as least as great as CSF T-tau, which as expected also (unadjusted) predicted all three measures of neurodegeneration[363]. The effect of TFF3 persisted even once baseline P-tau, age, sex and APOE4 status had been accounted for (Table 8.3), and was still associated with rate of hippocampal atrophy even once clinical diagnosis (e.g. control/MCI/AD) had been accounted for. These results therefore support an association between CSF TFF3 and increased rates of neurodegeneration independent of established CSF biomarkers in individuals with amyloid deposition, suggesting that CSF TFF3 may be a novel and valuable biomarker of decline across the spectrum of AD.

8.4.1 Trefoil Factor 3 (TFF3)

Encoded by the TFF3 gene on chromosome 21, TFF3 is a protein expressed by secretory epithelial cells principally in the gastrointestinal tract, but also in human hypothalamus and pituitary[364], and in the hippocampi, temporal cortices and cerebellum of mice[365]. Its function in the central nervous system is unknown[365], although TFF3 administration to mice has been reported to improve memory[366]. In

the periphery, TFF3 has important roles in NOTCH processing, and measurement of TFF3 in blood/urine/faeces has been patented[367] and used[368] as a means of assessing NOTCH-related side-effects in trials of gamma-secretase inhibitors for the treatment of AD. To our knowledge, this is the first study to implicate TFF3 as a marker for neurodegeneration across the AD spectrum, and furthermore to show that this is independent of T-tau and P-tau: whilst there are few data on which to suggest mechanisms, one intriguing possibility is that this effect might in some way be mediated by alterations in gamma-secretase processing. Whilst numbers with a minor allele were too small for meaningful comparisons, the observation that genotype at the rs7280100, a candidate locus associated with TFF3, is intriguing, and if replicated in independent samples, suggests that CSF TFF3 and/or the rs7280100 genotype may both help predict rate of neurodegeneration in individuals with amyloid pathology; and that elucidating the function of TFF3 in the central nervous system may provide insights into mechanisms influencing neurodegeneration in the presence of brain amyloidosis.

8.4.2 Cystatin C (CysC), Vascular Endothelial Growth Factor (VEGF), and Chromogranin A (CgA)

Of the other three biomarkers emerging prominently from our analyses, CysC co-localizes with β amyloid in amyloid plaques, amyloid-laden vascular walls in cerebral amyloid angiopathy and in Down's syndrome and is typically reduced in AD CSF, with multiple lines of evidence suggesting that it has *protective* roles in AD principally due to influences on amyloid processing and deposition[369]. Conversely, increased CysC immunoreactivity is seen in specific neuronal population in AD suggesting a role in neurodegeneration[369]; and in dopaminergic neurons, CysC has

been shown to play a role in neuronal injury mediated microglial activation and neurotoxicity[370]. Our finding of a positive relationship between rates of atrophy and CysC, in individuals in whom amyloid deposition has already occurred, could therefore be explained in terms of a harmful neuroinflammatory response, which results in neuronal damage. VEGF, abundantly expressed in the CNS, has roles in modulation of angiogenesis, vascular remodelling, repair, permeability and inflammation[371], and is involved in microglial chemotaxis perhaps reflecting an early response to amyloid deposition[372]. Our finding of increased atrophy with lower levels of CSF VEGF is consistent with VEGF having a protective role in AD, and in keeping with reports that transgenic AD mice with increased neuronal expression of VEGF have a functional improvement in memory[371], suggests that up-regulation of VEGF may be a useful therapeutic strategy for AD. Increased levels of CSF VEGF has been seen in individuals with AD and vascular dementia compared with controls[373]– this could also represent a protective response although VEGF levels were not correlated with rate of atrophy or rate of cognitive decline. The neuroendocrine secretory protein CgA is the major protein of large dense-core synaptic vesicles and may be a marker of synaptic dysfunction[206]. In one study lower CSF levels of CgA were reported in the CSF of subjects with early onset sporadic or familial Alzheimer's disease[374], potentially in keeping with our finding of inverse relationship between CgA level and increased rates of brain atrophy and ventricular expansion.

In a previous study using this same panel of analytes, ten CSF measures (ACE, CgA, AXL, TNF-related apoptosis-inducing ligand receptor, CD40, M-CSF, beta-2-microglobulin, stem cell factor, CLU and IL-3) were shown to predict increased rates of amyloid deposition in cognitively normal elderly individuals[355]. When

comparing these results aiming at identifying markers predicting rate of amyloid accumulation in healthy controls with ours (assessing rate of neurodegeneration in individuals with likely amyloid pathology) it is notable that in our initial analysis I identified five CSF analytes common to both: AXL, CgA, CLU, IL-3, and M-CSF; and including more stringent covariates that CgA remained a consistent finding. Whilst this could reflect that rates of amyloid deposition and brain atrophy are highly correlated – as would be predicted in the mid-phase of AD pathogenesis – this would also be consistent with common mechanisms linking amyloid deposition to subsequent neurodegeneration. The fact however that TFF3, CysC and VEGF were not identified in previous analyses may suggest that these analytes may be exerting their effects on neurodegeneration independent of amyloid deposition.

This study has a number of limitations. The number of subjects is relatively small making the findings preliminary, particularly relative to the number of analytes, and these findings thus require replication in other, larger cohorts. However, I have used a statistical procedure to control for multiple comparisons, indicating that the evidence for associations is moderately strong. Whilst the use of reverse stepwise analysis must be considered exploratory, the consistency with which TFF3 emerges as a strong independent predictor of atrophy is striking. However, that the same analytes often predict all three atrophy measures is perhaps less surprising, given that the atrophy measures are mutually correlated. A relatively small percentage of the variance in atrophy rates is explained by these findings suggesting that other factors and other biomarkers reflecting other independent pathways have yet to be identified, noting that a certain proportion of variance may also be due to measurement error. Including all individuals with low Aß1-42 in our analysis assumes that all patients with brain amyloidosis are on the same neuropathological spectrum. Whilst larger, more homogeneous samples are required to assess whether

the relationships I show are driven by individuals with asymptomatic amyloidosis, MCI, or established AD, it is notable that the relationship between TFF3 and hippocampal atrophy remains even after adjusting for clinical diagnosis – which in this study probably simply reflects different stages of disease.

8.5 Conclusions

This present work identifies a number of CSF markers that may be associated with rate of neurodegeneration in individuals with amyloid deposition. These candidate biomarkers warrant further investigation, potentially providing prognostic information for patients; covariates for clinical trials; and insights into AD biology. Whilst several of the CSF biomarkers hint at immune mediated links between responses to amyloid deposition and brain volume loss the function of TFF3 which was found to be the single strongest predictor of neurodegeneration across the spectrum of brain amyloidosis is unknown. Further studies to replicate these findings and investigate the role of TFF3 in the pathogeneses of AD are required.

8.6 Publication arising from this chapter

RW Paterson, JW Bartlett, K Blennow, NC Fox; Alzheimer's Disease Neuroimaging Initiative, LM Shaw, JQ Trojanowski, H Zetterberg, JM Schott. Cerebrospinal fluid markers including trefoil factor 3 are associated with neurodegeneration in amyloid-positive individuals. Translational Psychiatry. 2014 Jul 29;4.

Chapter 9. Exploring New AD biomarkers

9.1 Introduction

9.1.1 Limitations of current biomarkers

In this present work molecular biomarkers currently used in clinical research diagnostic criteria for AD have been discussed including amyloid PET imaging and cerebrospinal fluid (CSF) tau and β-amyloid 1-42, which reflect key pathological hallmarks of AD pathology, *i.e.*, amyloid plaques and neurofibrillary tangles[3, 78]. Although these biomarkers can distinguish AD pathology from non-AD pathology with good sensitivity and specificity[73] there remains a need for new biomarkers[206].These include biomarkers that can detect pathological changes prior to overt neuronal death; correlate with the progression of neurodegeneration for clinical trials; explain phenotypic diversity[315]; and allow for accurate prognostication.

9.1.2 CSF Biomarker Discovery

A number of hypothesis generating methods have been used to identify potential candidate biomarkers for Alzheimer's disease. These include i) Large scale epidemiological studies and the use of 'Big Data' to identify AD risk factors and dysfunction of specific biological pathways; ii) Genetic studies to identify genetic risk factors and potential gene products that can be measured; iii) Pathological studies; iv) Proteomic and metabolomic studies of AD blood, CSF or other body fluids or tissue.

Over recent years a large number of candidate biomarkers have been identified, particularly in CSF, that reflect a range of pathophysiological processes including cholesterol metabolism, neuroinflammation and amyloid processing[206]. However, to date few if any have been adopted in clinical practice. One contributing factor is the rate-limiting step in biomarker validation. Two realistic means of validating candidate biomarkers are: i) multiplex CSF ELISAs and ii) targeted mass spectrometry. Multiplex ELISAs such as the one used in ADNI (see Chapter 8) developed by Rules Based medicine (Tx, USA) allow for many biomarkers to be validated simultaneously, however, they take significant time to develop and are costly. The development of ultrasensitive platforms which make use of single molecule array technology (SIMOA, RBM, Tx, USA) are potentially customizable and could lead to more rapid biomarker validation, however the platforms are costly and there are currently none available for use in the UK. Targeted mass spectrometry has been in use for a number of a years (reviewed by Kroksveen[375] & Brinkmalm[376]) and can measure a large number of potential biomarkers concurrently and therefore has considerable promise for use in clinical practice. A number of mass spectrometers are avaiable for research purposes in the UK, including one at the Institute of Child Health, department of Translational omics, where this work was carried out.

Most studies to date focused on biomarkers for which there is already an immunoassay[377]; and whilst mass spectrometry has considerable potential clinical utility, its use has been limited in part due to the lack of a streamlined, cost effective pipeline that can be used to rapidly test large numbers of potential biomarkers concurrently[260].

9.1.3 Existing targeted mass spectrometry assays

Recently the Omics centre at UCL and other groups have used targeted proteomic MRM LC-MS/MS assays to multiplex scores of peptides in a single rapid CSF assay which has low technical variability[377] and relatively low cost. This has been applied to clinical cohorts of patients with Parkinson's disease and Dementia with Lewy Bodies[260, 378], and a previous study has used similar technology to assess CSF biomarkers of progression in a small number of AD subjects longitudinally[379]. Assays of this kind have potential utility in facilitating the rapid validation of biomarkers in clinical cohorts thus overcoming a bottleneck in biomarker development allowing for precise quantification of proteins[380] and excellent reproducibility[381].

The aims of this study were to (a) evaluate the feasibility of this rapid 'one pot' targeted mass spectrometry multiplex assay to measure biomarkers of interest in clinical cohorts of individuals with AD, other degenerative diseases and healthy controls; and (b) explore differences in biomarker concentrations between individuals with AD and non-AD classified according to CSF biomarker criteria.

9.2 Contributions and Collaborations

The targeted mass spectrometry panel tested in the chapter was developed by Dr Wendy Heywood and Dr Kevin Mills, based at the Institute of Child Health, University College London. Subject recruitment and sample collection was carried out by the author, Dr Nadia Magdalinou and the Swedish samples were collected as per the General Methods section. The author carried out all the lab work, data analysis and interpretation relating to this chapter under the direct supervision of

Dr Amanda Heslegrave. The SIMCA analysis was carried out with the support of Dr Ulf Andreasson at the University of Gothenburg.

9.3 Methods

9.3.1 Subjects and Cerebrospinal Fluid Collection

9.3.1.1 Cohort 1 (The 'Swedish' Cohort)

This cohort is fully described in Chapter 2 and was collected by Dr Johansson and Svensson. In brief it included 107 individuals, 88 undergoing investigation for cognitive concerns and 19 healthy age matched controls without cognitive concerns. The majority of subjects were from a single memory centre at Skaraborg hospital in Sweden (n=78) and this cohort has previously been described in detail[258]. Healthy control participants had LP for research purposes only; they were asymptomatic spouses of affected individuals or healthy controls without subjective cognitive concerns. A further 29 CSF samples from individuals with cognitive concerns from another single memory centre in Sweden were included.

9.3.1.2 Cohort 2 (Retrospective, prospective and healthy control cohorts)

This cohort was composed of individuals from the UCL retrospective and prospective cohorts as fully described in chapter 2. In brief, the cohort included 92 individuals assessed at the Specialist Cognitive Disorders Service at Queen Square, London UK between 2011 and 2014. All subjects had a clinical CSF examination as part of diagnostic work-up. 26 asymptomatic controls (spouses of research participants) were also included; these individuals were collected by Dr Nadia Magdalinou as part of her prospective study into atypical Parkinsonism, had no cognitive concerns and had lumbar punctures for research purposes only. For the patient group, we recorded the nearest mini-mental state examination (MMSE)

score to the date of the lumbar puncture. Rate of cognitive decline was estimated using the formula (30-MMSE at time of LP/duration of cognitive symptoms in months). *APOE* genotype was determined by measuring peptides corresponding to apoE2, apoE3 and apoE4 in CSF using the MRM-based triple quadrupole MS assay as previously described[382]. Individuals were classified as *APOE* ε4 positive or negative.

9.3.2 Cerebrospinal fluid Collection and Routine Biomarker Analysis

For all subjects, CSF was collected by lumbar puncture as described in chapter 2.

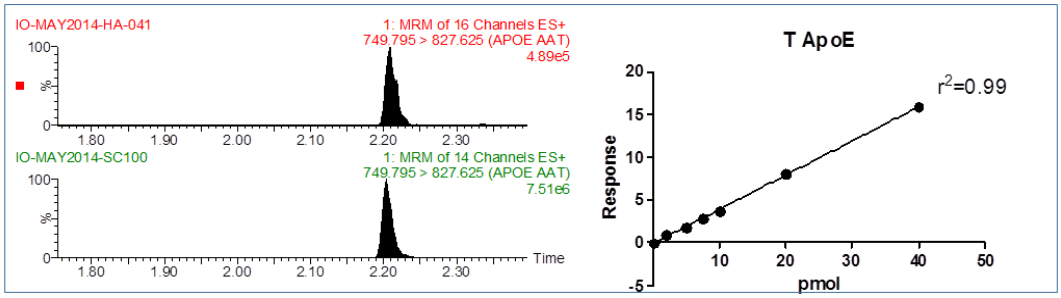
9.3.3 Neurochemical Classification

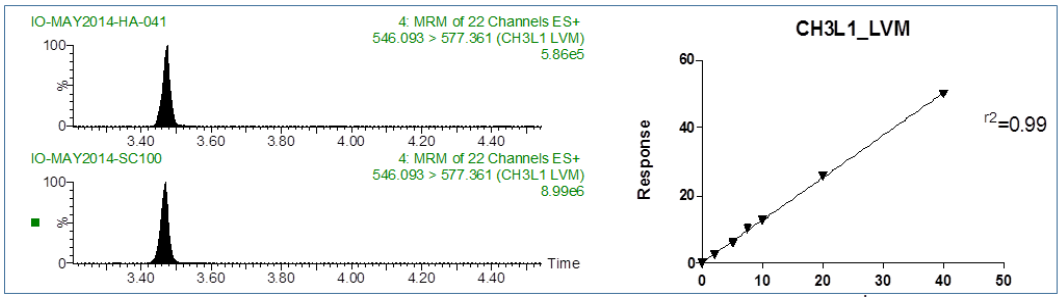
To enrich the samples as far as possible for AD/non-AD pathology respectively, we classified each individual independent of clinical diagnosis on the basis of CSF profile. A positive Alzheimer's signature CSF profile was defined as: tau/β-amyloid (1-42) ratio>1 & P-tau >63; a negative Alzheimer's signature CSF profile was defined by Tau/β-amyloid (1-42) ratio<0.52 & P-tau<63. This Tau/β-amyloid (1-42) ratio gives a sensitivity of ~93% and specificity of ~93% for AD[235] and according to manufacturer's guidelines, a P-tau of >63 gives a sensitivity of 74% and specificity of 85% for AD compared to other neurodegenerative diseases[383]. As the purpose of this study was to determine biomarkers that differentiate between established AD and healthy controls, individuals with 'grey zone' CSF profiles were excluded.

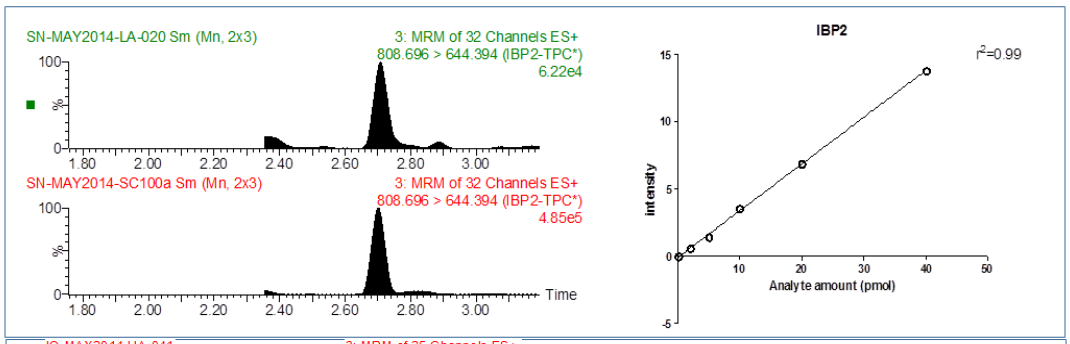
9.3.4 Mass Spectrometry

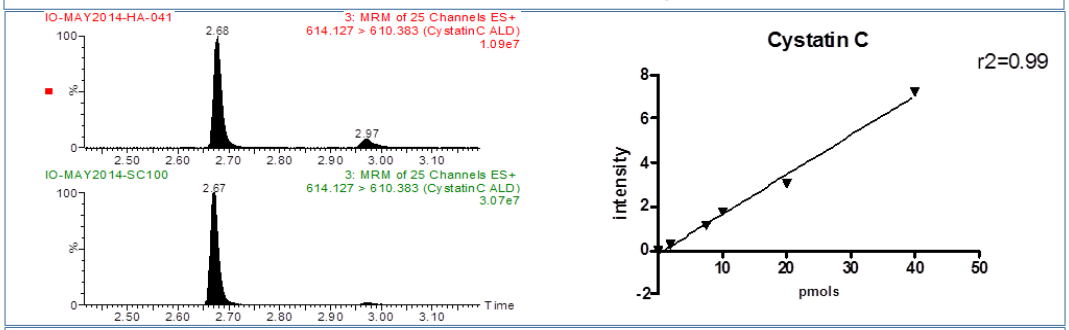
9.3.4.1 Targeted proteomics: MRM-based triple quadrupole mass spectral assay

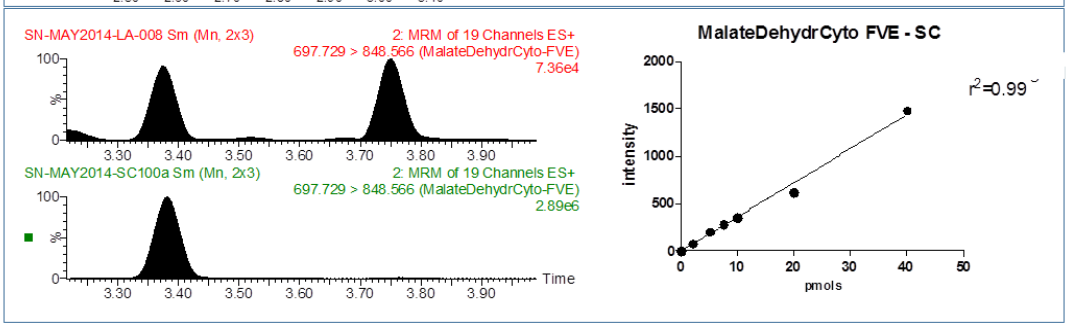
A multiplexed 10 minute, targeted proteomic triple quadrupole, peptide MRM-based assay was used to detect a panel of 54 biomarkers as described previously[260]. The panel consisted of proteins were identified from a literature review by Dr Wendy Heywood (see Appendix F) and new markers identified from proteomic profiling described previously including four novel markers previously found to be elevated in AD and Dementia with Lewy Bodies compared to controls: malate dehydrogenase; serum amyloid A4; GM_2-activator protein and prosaposin[260]. A standard curve 0 - 40pmols / 100 µl CSF of each peptide was analysed in duplicate at the end of the run for quantitation and performance standardisation (CV <10% was considered acceptable).


IO-MAY2014-HA-041
1: MRM of 16 Channels ES+
749.795 > 827.625 (APOE AAT)
4.89e5
IO-MAY2014-SC100
1: MRM of 14 Channels ES+
749.795 > 827.625 (APOE AAT)
7.51e6
Time
T ApoE
r2=0.99
Response
pmol


IO-MAY2014-HA-041
4: MRM of 22 Channels ES+
546.093 > 577.361 (CH3L1 LVM)
5.86e5
IO-MAY2014-SC100
4: MRM of 22 Channels ES+
546.093 > 577.361 (CH3L1 LVM)
8.99e6
Time
CH3L1_LVM
r2=0.99
Response


SN-MAY2014-LA-020 Sm (Mn, 2x3)
3: MRM of 32 Channels ES+
808.696 > 644.394 (IBP2-TPC*)
6.22e4
SN-MAY2014-SC100a Sm (Mn, 2x3)
3: MRM of 32 Channels ES+
808.696 > 644.394 (IBP2-TPC*)
4.85e5
Time
IBP2
r2=0.99
intensity
Analyte amount (pmol)


IO-MAY2014-HA-041
3: MRM of 25 Channels ES+
614.127 > 610.383 (CystatinC ALD)
1.09e7
IO-MAY2014-SC100
3: MRM of 25 Channels ES+
614.127 > 610.383 (CystatinC ALD)
3.07e7
Time
Cystatin C
r2=0.99
intensity
pmols


SN-MAY2014-LA-008 Sm (Mn, 2x3)
2: MRM of 19 Channels ES+
697.729 > 848.566 (MalateDehydrCyto-FVE)
7.36e4
SN-MAY2014-SC100a Sm (Mn, 2x3)
2: MRM of 19 Channels ES+
697.729 > 848.566 (MalateDehydrCyto-FVE)
2.89e6
Time
MalateDehydrCyto FVE - SC
r2=0.99
intensity
pmols

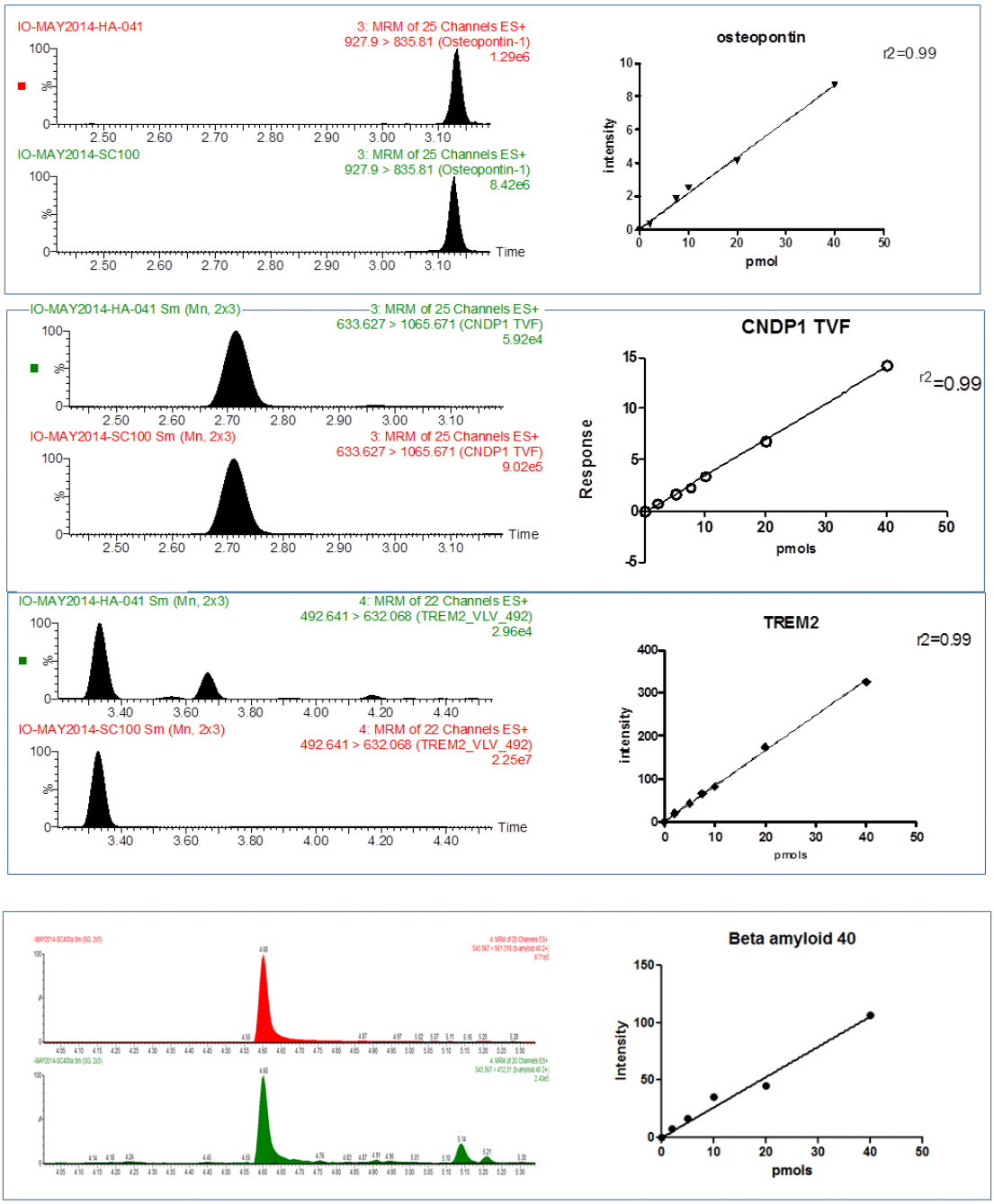


Figure 9.1 Chromatograms and standard curves of biomarkers surviving FDR correction in the univariate analysis comparing AD and non-AD CSF in Cohort 1. Top chromatograms are from a patients CSF (single subject) and the bottom chromatograms are from CSF spiked with peptides.

9.3.4.2 Experimental design

The experimental design of this study is summarised in figure 9.2. A panel of markers was assessed using the targeted proteomics multiplex panel in cohort 1. Significant markers from this initial analysis were then further validated in cohort 2.

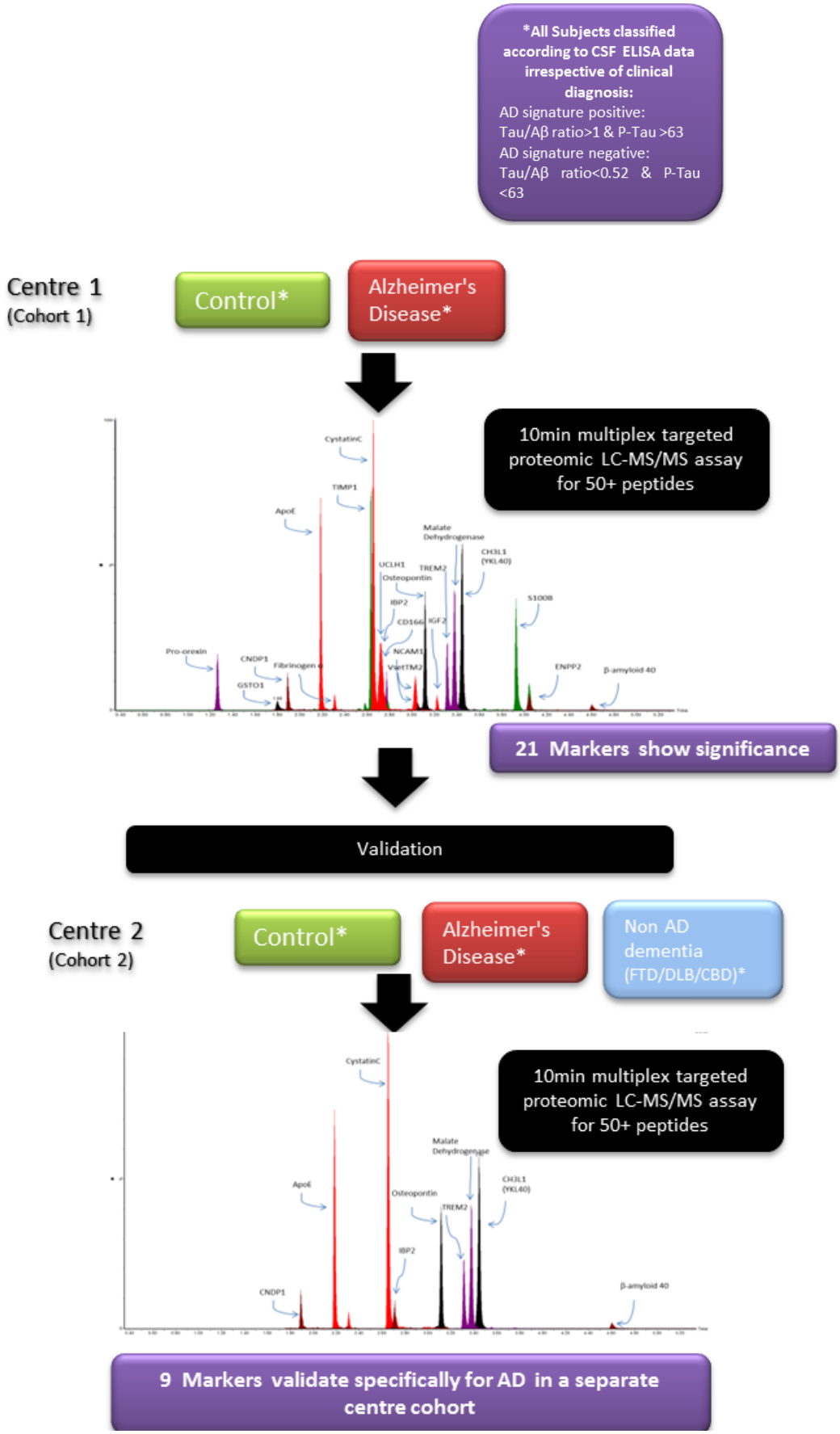


Figure 9.2 Study design outline (univariate analysis)

9.3.5 Statistical Analysis

9.3.5.1 Univariate analysis of proteins of interest

A univariate analysis of all proteins of interest was performed. It was determined which individuals in cohort 1 were Alzheimer-positive and Alzheimer-negative based on their CSF neurochemical profile, and compared levels of proteins determined using targeted mass spectrometry using t-tests between the positive/negative groups when there were no clear departures from a normal distribution, and Wilcoxon rank-sum tests for skewed or truncated data. Proteins showing statistically significant differences between AD positive/negative groups in cohort 1 were then tested in cohort 2 as a validation set. All analyses were carried out at a significance level of $p<0.05$; to control for the risk of Type 1 error for multiple biomarker comparisons results were also controlled using the False Discovery Rate (FDR) at 5%. "Validated biomarkers" were those found to separate neurochemically defined AD/non-AD in both datasets at an FDR-corrected significance level of $p<0.05$.

9.3.5.2 Unbiased analysis using SIMCA

Independent of the biomarkers discovered in step 1, we carried out an unbiased analysis of the entire targeted mass spectrometry data set to determine which markers were the greatest determinants of variance in each cohort. To do this an orthogonal projection to discriminant analysis (OPLS-DA) was used using soft independent modelling of class analogies software (SIMCA, Umetrics, Sweden)

software as previously described[384], classifying subjects on the basis of their AD signature (positive/negative) CSF. Cohort 2 was used as a training set since this group contained the greatest number of subjects; the analysis was then repeated using cohort 1 as a validation set. Non-normally distributed data (as determined by SIMCA) were log transformed.

Receiver operating characteristic (ROC) curves were used to determine the diagnostic utility of the 'validated' biomarkers from step 1 using the 'roctab' command in STATA Version 12.1 (Stata corporation, College Station, TX, USA) using the healthy control subjects with a non-AD neurochemical profile as the control group. Finally, the relationship between each of the Validated Biomarkers and the established CSF biomarkers Tau, P-tau, and rate of cognitive decline was explored by fitting separate regression models for each of the 'Validated' Biomarkers including all subjects with AD or non-AD CSF in the model, except when exploring the relationship with cognitive function when only individuals with AD CSF were included. Unless otherwise stated, all analyses were carried out using STATA. Linear regression was used to explore the relationship between novel biomarkers and T-tau, P-tau, β-amyloid (1-42), MMSE and rate of cognitive decline. Graphs were created using GraphPad prism V5 (GraphPad Software, La Jolla, CA, USA). The correlation matrix was created using Microsoft Excel (Microsoft, Redmond, WA, USA).

9.4 Results

9.4.1 Comparing Neurochemical AD and Non-AD subjects

In cohort 1, 35 individuals fulfilled CSF neurochemical criteria for AD, and 31 had a non-AD CSF profile. The remaining 43 had an intermediate profile and were not included in further analyses. As expected there were significantly more *APOE* ε4 carriers in the AD group (Table 9.1A). Groups were well matched for sex; the neurochemical AD group were significantly older than the non-AD group.

In cohort 2, 46 individuals fulfilled neurochemical CSF criteria for AD, 44/46 of whom had a clinical diagnosis of AD and thus fulfilled contemporary (IWG-2[4] and NIA[3]) criteria for AD; the remaining two were controls. Of the 36 subjects with non-AD CSF, 22 were healthy controls; of the other 14, 7 had subjective cognitive concerns, and the others were diagnosed with other non-AD neurodegenerative dementias including semantic dementia, behavioural variant frontotemporal dementia and Lewy Body dementia. Groups were well matched for age and sex. As expected there were significant differences in MMSE and *APOE* status. CSF ELISA biomarker data are given in Table 9.1B.

	Neurochemical AD	Neurochemical Non-AD	AD vs Non-AD (p-value)
	N=35	N=31	
Age at LP	74.9 ± 5.2	70.7 ± 7.8	P=0.02
Sex (% male)	42.9	64.5	0.09
% APOE ε4 positive	68.6	25.8	P<0.001
Aβ1-42 (pg/ml)	452.5 ± 147.4	906.9 ± 220.6	P<0.001
T-tau (pg/ml)*	654 (505 – 969)	255 (210-294)	P<0.001
P-tau (pg/ml)	119. 7 ± 72.4	44.5 ± 12.0	P<0.001
tau/Aβ1-42 ratio*	1.51 (1.25-2.06)	0.25 (0.22- 0.34)	P<0.001

Table 9.1a Demographics and CSF profiles of individuals from cohort 1 (composed of subjects from the Swedish cohort). Data are shown as Mean ± SD unless stated; * log transformed for regression analyses, values quoted as median (interquartile range)

	Neurochemical AD	Neurochemical Non-AD	AD vs Non-AD
	n=46	n=36	
Age at LP	62.9 ± 8.0	58.5 ± 8.8	0.2
Sex (% male)	39.1	44.4	0.5
MMSE	20.6 ± 5.6	26.7 ± 6.9	P<0.001
Duration of cognitive symptoms	36.4 ± 17.4	NA	NA
Rate of cognitive decline (MMSE)	0.36 ± 0.42	NA	NA
% individuals fulfilling McKhann	95.7	0	P<0.001
% APOE ε4 positive	67.4	33.4	P<0.001
Aβ1-42 (pg/ml)	408.4 ± 168.4	960.2 ± 290.9	P<0.001
T-tau (pg/ml)*	947 (760-1196)	234.5 (174.5-315.5)	P<0.001
P-tau (pg/ml)	107.5 ± 38.12	35.5 ± 13.2	P<0.001
tau/Aβ1-42 ratio*	2.5 (1.8-4.1)	0.25 (0.19 -0.33)	P<0.001

Table 9.1b Demographics and CSF profiles of individuals from cohort 2 (composed of subjects from the retrospective, prospective and healthy control cohorts). Data are shown as Mean ± SD unless stated * log transformed for regression analyses, values quoted as median (interquartile range)

9.4.2 Univariate Analysis: comparing Neurochemical AD and Non-AD subjects

Comparing the neurochemically defined AD and non-AD groups in cohort 1, there were significant differences in measured biomarker concentrations in 21 markers, of which 15 survived FDR correction (Table 9.2A). Taking these 15 proteins forward to the validation cohort (cohort 2), 9 markers (Total apoE, β-amyloid40, Carnosine Dipeptidase 1 (CNDP1), cystatin C, Insulin-like growth factor-binding protein 2 (IBP-2), malate dehydrogenase, osteopontin, Triggering receptor expressed on myeloid cells 2 (TREM2) and YKL-40) were significantly elevated in the patients with both clinically and neurochemically defined AD (Figure 9.2). Five biomarkers (Total apoE, cystatin C, malate dehydrogenase, osteopontin & YKL-40) survived FDR correction in both cohort 1 and cohort 2 and were defined as "validated biomarkers". The values of these five biomarkers in the AD +/- groups in cohort 1 are illustrated in Figure 9.3.

The AD (CSF +ve) and non-AD (CSF –ve) dementias in cohort 2 were compared, including individuals with suspected non-AD neurodegeneration but excluding healthy control subjects. A similar list of 16 markers was significantly different between the 2 groups, with only malate dehydrogenase surviving FDR correction.

	P value (Cohort 1)	*P* value (Cohort 2)	Fold change in cohort 2
Malate Dehydrogenase^	0.005*	<0.001*	2.12
Total APOE^	<0.001*	0.005*	1.55
Chitinase-3-like protein 1(YKL-40)^	<0.001*	<0.001*	1.52
Osteopontin^	<0.001*	<0.001*	1.50
Neural cell adhesion molecule 1(NCAM1)	0.03	0.38	1.40
Ubiquitin carboxyl-terminal esterase 1 (UCLH1)	0.003*	0.88	1.30
Cystatin C^	0.008*	0.003*	1.28
Beta-amyloid 40	<0.001*	0.01	1.28
Carnosine dipeptidase 1 (CNDP1)	0.01*	0.03	1.26
V-Set and transmembrane domain containing protein	0.03	0.06	1.25
Fibrinogen A	0.03*	0.83	1.24
Insulin-like growth factor binding protein 2 (IBP-2)	0.007*	0.04	1.20
S100B	<0.001*	0.06	1.20
TREM2	0.001*	0.05	1.18
Serum amyloid p-component	0.007*	0.33	1.14
CD166	0.03	0.25	1.12
Pro-orexin	<0.001	0.22	1.11
TIMP metallopeptidase inhibitor 1	0.03	0.5	1.05
IGF2	0.005*	0.72	0.97
Glutathione-S-transferase Omega-1	0.006*	0.75	0.91
ENPP2	0.05	0.11	0.89

Table 9.2a Univariate analysis comparing neurochemical AD subjects with non-AD subjects. Dark grey indicates a biomarker that differentiated neurochemical AD from non-AD; significant after FDR correction in cohorts 1 and 2. Light grey indicates a biomarker that differentiated neurochemical AD from non-AD; significant after FDR correction in cohort 1 only. ^denotes biomarkers also identified using OPLS-DA analysis where subjects were classified neurochemically.

	P value (Cohort 2)	Fold change
Malate Dehydrogenase	<0.001	1.85
V-Set and transmembrane domain containing protein 2A	0.001	1.71
LSAMP	0.003	1.65
Total APOE	<0.001	1.61
S100B	0.004	1.48
Chitinase-3-like protein 1(YKL-40)	<0.001	1.47
Cystatin C	0.003	1.44
Osteopontin	0.03	1.43
LAMP1	0.008	1.42
CD166	0.02	1.40
Pro-orexin	<0.001	1.30
Beta-amyloid 40	<0.001	1.38
Carnosine dipeptidase 1 (CNDP1)	<0.001	1.38
Carboxypeptidase E	0.004	1.37
GM2	0.04	1.35
Neural cell adhesion molecule 1(NCAM1)	0.03	1.25

Table 9.2b Univariate analysis comparing neurochemical AD subjects with non-AD subjects, excluding healthy controls. Grey indicates a biomarker that differentiated neurochemical AD from non-AD; significant after FDR correction.

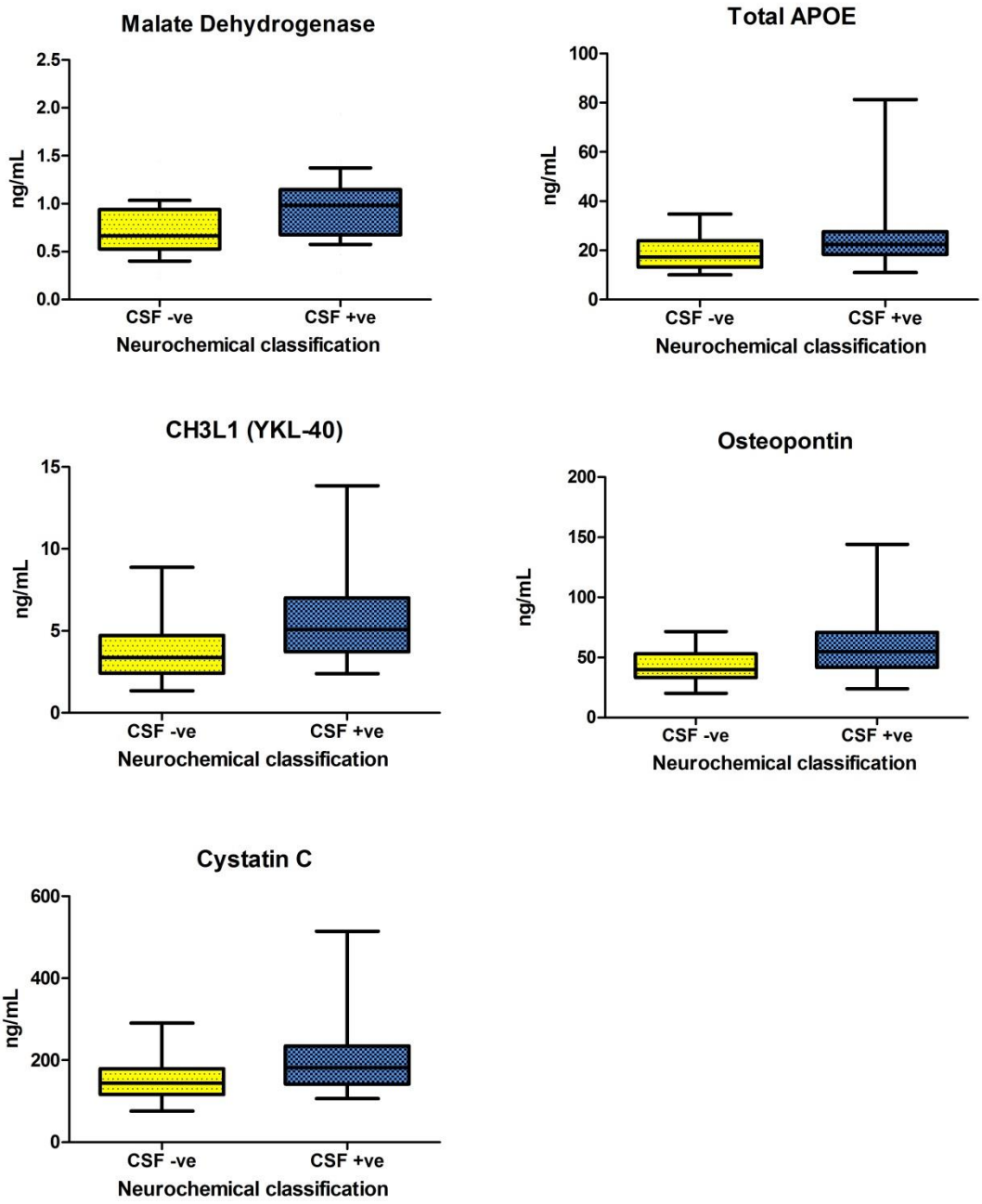


Figure 9.3 Boxplots and whiskers (representing 10th and 90th percentiles) comparing AD and non-AD CSF concentrations of 'validated' proteins.

9.4.3 Multivariate analysis classified according to clinical diagnosis and according to neurochemical diagnosis

Results of the OPLS-DA analysis using cohort 2 as the training set and cohort 1 as the test cohort are shown in Figure 9.3. Peptides corresponding to the following biomarkers were identified as the seven strongest predictors of group membership when separating the groups on neurochemical diagnosis (AD profile positive; AD profile negative): osteopontin, YKL-40 (also known as Chitinase-3-like protein 1; CHI3L1), malate dehydrogenase, vitronectin, total apoE, limbic system-associated membrane protein (LSAMP) and cystatin C.

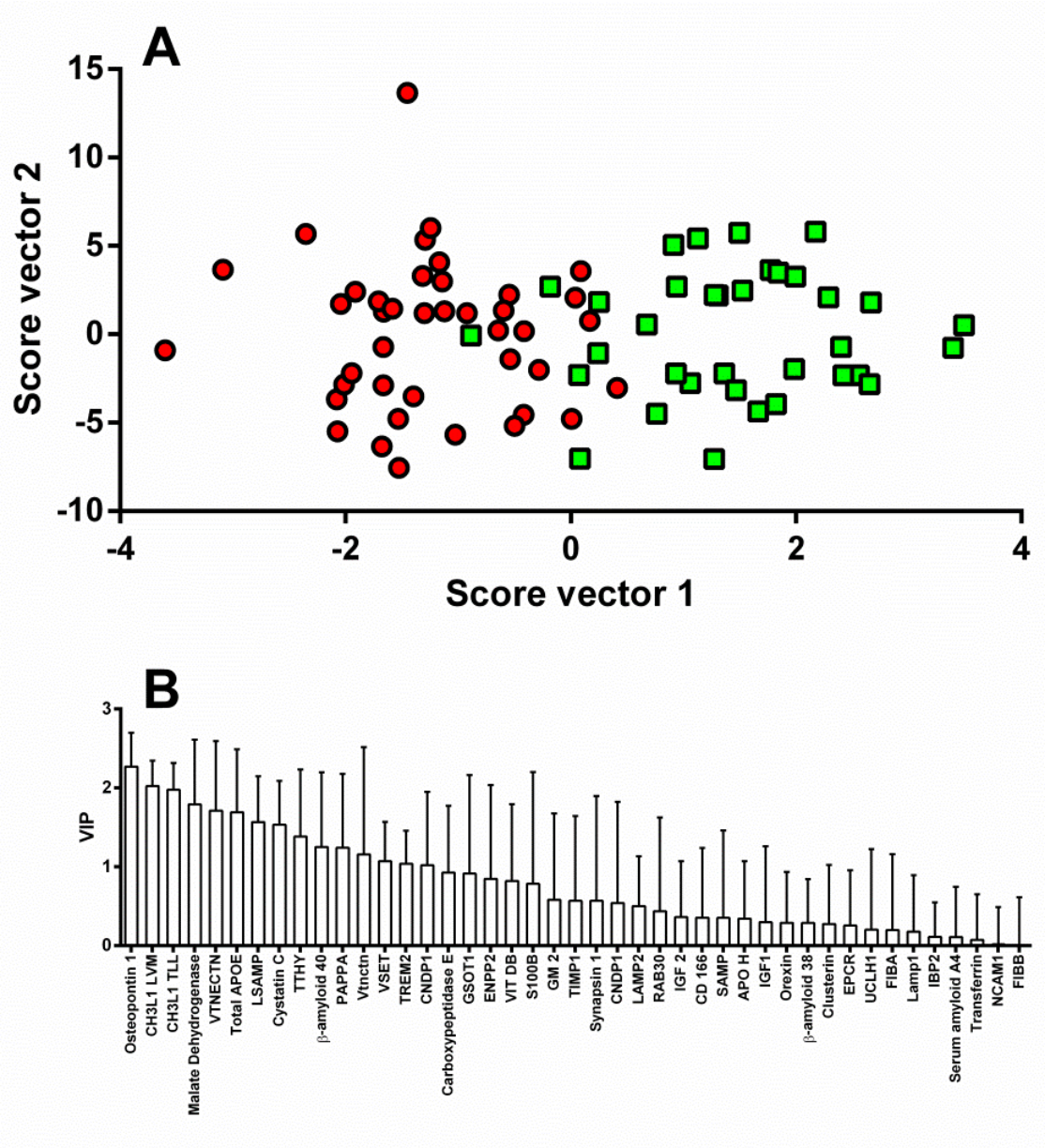


Figure 9.4a OPLS-DA analysis using data from cohort 2. Subjects are colour coded according to neurochemical status: red circles=AD; green squares=non-AD.
Figure 9.4b Variable importance on projection plot corresponding to the score plot in Figure 9.4a.

9.4.5 Diagnostic utility

When applied to cohort 2 for whom full clinical data were available, the five "Validated Biomarkers" could individually differentiate AD from non-AD healthy

control CSF with areas under the curve as follows: Total apoE=0.62; cystatin C=0.62; malate dehydrogenase=0.67; osteopontin=0.79; YKL-40=0.75. In a multivariate logistic regression analysis including all of these variables, the combination could differentiate AD from non-AD healthy control CSF with an area under the curve of 0.88.

9.4.6 Correlation of proteins with each other and existing CSF biomarkers

To explore the relationship between established CSF biomarkers measured using ELISA and the proteins measured using this targeted proteomics assay regression analyses were carried out between each of the five Validated Biomarkers and β-amyloid 1-42, T-tau and P-tau including all subjects in the analysis irrespective of neurochemical status. None was significantly correlated with age or β-amyloid 1-42. Cystatin C, malate dehydrogenase, osteopontin and YKL-40 were each correlated with both T–tau and P-tau (Figure 9.5a and 9.5b). A correlation map shows which of the proteins from Table 9.2 were correlated with one another. (Figure 9.6)

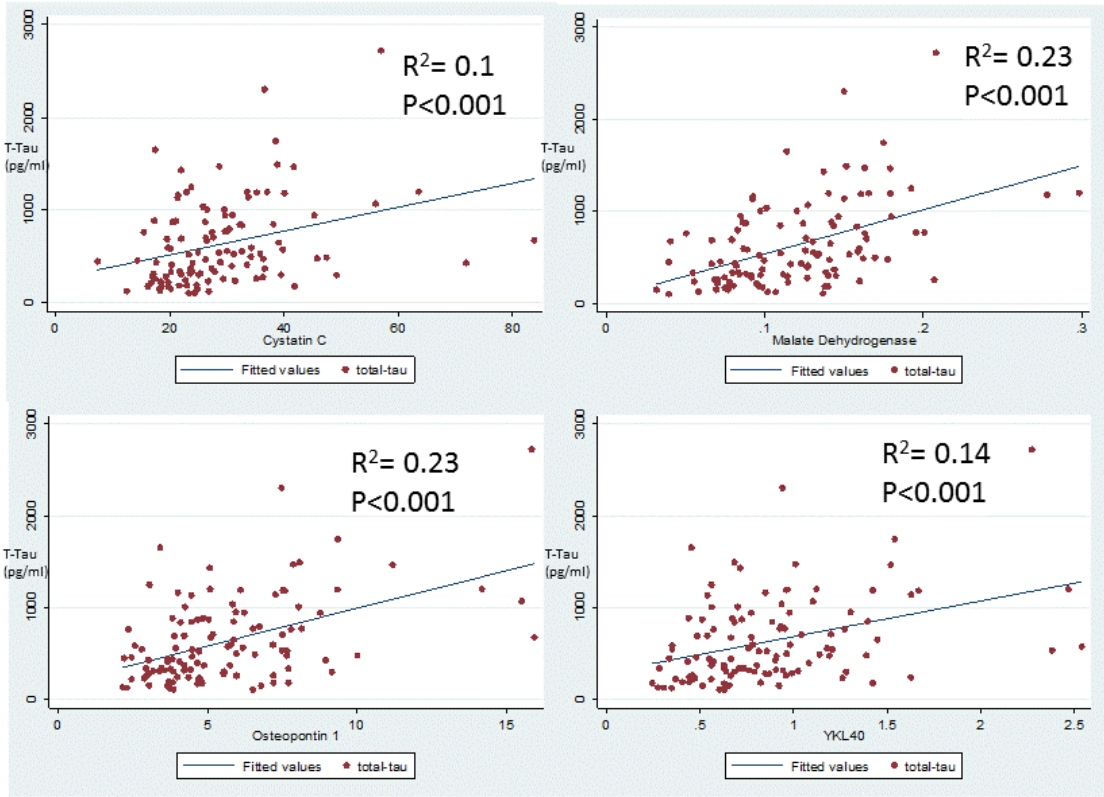


Figure 9.5a: Scatter plots showing correlations between CSF T-tau (ELISA) and "validated biomarkers" measured using targeted proteomics using subjects in cohort 2.

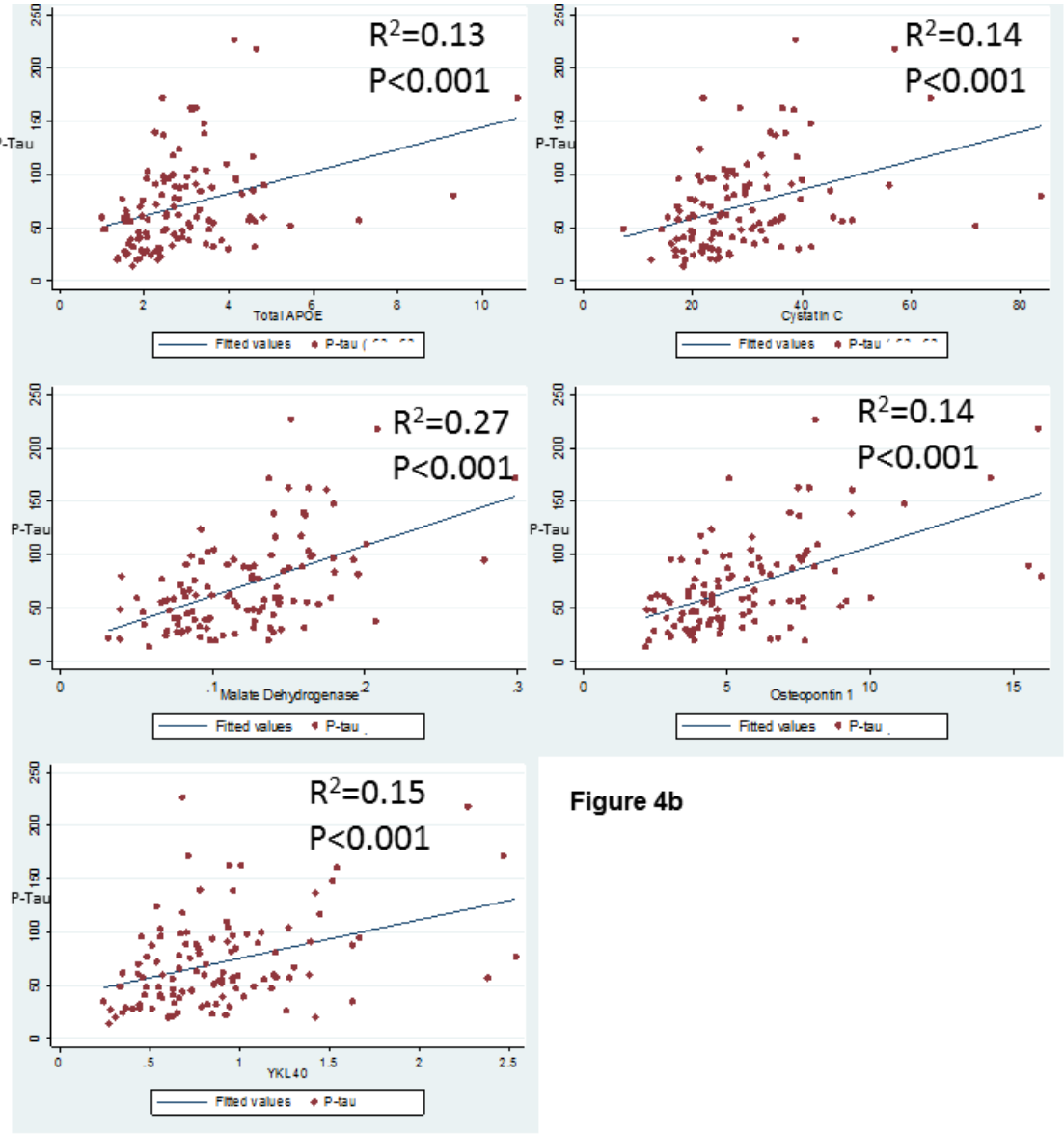


Figure 4b

Figure 9.5b: Scatter plots showing correlations between CSF P-tau (ELISA, pg/mL) and "validated biomarkers" measured using targeted proteomics using subjects in cohort 2.

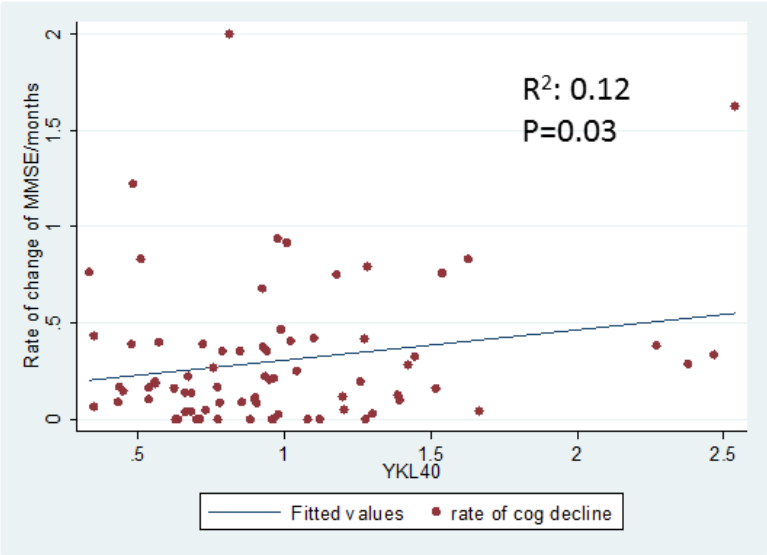


Figure 9.5c Scatter plots showing correlations between rate of cognitive decline (30-MMSE score/duration of cognitive symptoms in months) and "validated biomarkers" measured using targeted proteomics using subjects in cohort 2

	LSAMP	TIMP1	CD166	Osteop	VSETTM	TREM2	CH3L1	ENPP2	Carboxy	IGF2	GM2	CNDP1	Cystatin	Total A	Orexin	GSOT1	S100B	Malate	SAMP	NCAM1	IBP2	UCLH1	FIBA	Ab40	Age at	Ab142	Ttau	Ptau
LSAMP	1.00																											
TIMP1	0.67	1.00																										
CD166	0.29	0.32	1.00																									
Osteopontin	0.68	0.52	0.31	1.00																								
VSETTM2	0.47	0.29	0.53	0.64	1.00																							
TREM2	0.62	0.50	0.30	0.76	0.46	1.00																						
CH3L1	0.37	0.47	0.35	0.61	0.53	0.38	1.00																					
ENPP2	0.30	0.50	0.57	0.15	0.13	0.23	0.19	1.00																				
CarboxyE	0.76	0.56	0.44	0.71	0.52	0.65	0.27	0.39	1.00																			
IGF2	-0.03	0.17	0.74	0.09	0.32	0.12	0.23	0.53	0.19	1.00																		
GM2	0.36	0.36	0.62	0.53	0.64	0.45	0.50	0.45	0.55	0.52	1.00																	
CNDP1	0.61	0.53	0.72	0.67	0.68	0.60	0.55	0.51	0.68	0.51	0.73	1.00																
CystatinC	0.83	0.59	0.35	0.85	0.68	0.70	0.59	0.18	0.77	0.08	0.52	0.68	1.00															
TotalAPOE	0.82	0.65	0.50	0.75	0.65	0.66	0.53	0.43	0.82	0.24	0.66	0.81	0.87	1.00														
Orexin	0.18	0.19	0.61	0.26	0.55	0.19	0.38	0.37	0.26	0.64	0.59	0.56	0.24	0.38	1.00													
GSOT1	0.28	0.35	0.65	0.36	0.49	0.34	0.36	0.41	0.45	0.67	0.67	0.58	0.40	0.50	0.63	1.00												
S100B	0.31	0.20	0.54	0.35	0.50	0.31	0.36	0.39	0.37	0.47	0.63	0.60	0.32	0.54	0.64	0.67	1.00											
Malate	0.19	0.10	0.53	0.42	0.63	0.26	0.49	0.17	0.37	0.45	0.65	0.55	0.40	0.49	0.63	0.67	0.72	1.00										
SAMP	0.07	0.12	0.48	-0.03	0.00	0.10	0.02	0.25	0.09	0.20	0.16	0.15	-0.02	0.11	0.08	0.25	0.17	0.20	1.00									
NCAM1	0.24	0.23	0.36	0.35	0.43	0.15	0.59	0.08	0.20	0.31	0.29	0.47	0.45	0.37	0.34	0.41	0.27	0.42	0.02	1.00								
IBP2	0.22	0.31	0.63	0.50	0.50	0.50	0.39	0.34	0.38	0.69	0.63	0.64	0.41	0.49	0.65	0.66	0.56	0.54	0.13	0.29	1.00							
UCLH1	0.29	0.28	0.69	0.26	0.51	0.22	0.26	0.49	0.46	0.67	0.68	0.66	0.32	0.50	0.64	0.71	0.64	0.65	0.22	0.39	0.56	1.00						
FIBA	0.06	0.18	0.02	0.08	-0.02	0.15	0.03	0.02	0.10	-0.13	0.03	0.01	0.10	0.10	-0.14	0.06	0.04	0.07	0.45	0.07	-0.03	0.06	1.00					
Ab40	0.12	-0.01	0.06	0.11	0.29	0.06	0.11	-0.13	0.12	0.15	0.16	0.09	0.19	0.22	0.14	0.24	0.25	0.34	-0.07	0.11	0.20	0.20	-0.08	1.00				
Age at LP	0.19	0.13	0.03	0.26	0.17	0.29	0.33	-0.24	0.06	0.01	0.23	0.13	0.26	0.18	0.16	0.19	0.21	0.13	-0.05	0.04	0.24	0.02	-0.13	0.16	1.00			
Ab142 (ELISA)	-0.12	-0.04	-0.05	-0.27	0.06	-0.04	-0.28	0.03	-0.01	0.04	-0.01	-0.06	-0.07	-0.09	-0.03	0.10	-0.18	-0.24	-0.11	-0.09	-0.14	0.00	-0.10	-0.10	-0.12	1.00		
total tau (ELISA)	0.21	0.02	0.10	0.52	0.45	0.29	0.44	-0.20	0.21	0.00	0.30	0.28	0.39	0.32	0.16	0.01	0.19	0.51	-0.06	0.10	0.29	0.06	-0.04	0.19	0.20	-0.50	1.00	
Ptau (ELISA)	0.27	0.07	0.18	0.56	0.51	0.32	0.47	-0.16	0.24	0.02	0.37	0.35	0.45	0.41	0.20	0.05	0.31	0.54	-0.07	0.13	0.33	0.09	-0.05	0.25	0.21	-0.49	0.90	1.00

Figure 9.6 Correlation matrix including all biomarkers listed in Table 9.2 and ELISA data for β-amyloid 1-42, T-tau and P-tau. Red highlight: Correlation coefficient ≥0.6 or ≤-0.6; green highlight: correlation coefficient 0.4 to 0.6 or -0.4- to -0.6. Malate: malate dehydrogenase; CarboxyE: carboxypeptide E

In a regression analysis including age, sex and APOE status in the model, there was a weak association between YKL-40 and rate of cognitive decline in the AD cohort. (Figure 9.5C) There were no other significant associations between proteins measured using this targeted proteomics assay and rate of cognitive decline.

9.5 Discussion

This study demonstrates that this targeted fully-quantitative multiplexed assay can measure a panel of 54 proteins of potential interest in AD. It is a 'one-pot' test, which requires a small volume of CSF (100 microliters) and can be run rapidly, over 10 minutes, and can be used to rapidly validate biomarkers of potential interest in clinical cohorts.

Moreover this study has been able to validate 5 biomarkers that differentiate neurochemical AD from non-AD in 2 independent clinical populations from different centres, all of which were also identified as those measures contributing most to the variance in an independent multivariate model differentiating by neurochemical AD/non-AD. These include markers of neuroinflammation, i.e. YKL-40, cystatin C and osteopontin; total apoE, the best recognized genetic risk factors for AD; and malate dehydrogenase, a key enzyme in brain glucose metabolism. When comparing AD CSF with other suspected non-AD neurodegenerative subjects and although with the caveat that sample sizes are small, malate dehydrogenase was also significantly higher in the AD cohort suggesting that it could be specific to AD neurodegeneration. While the majority of these biomarkers are unlikely to have diagnostic utility individually, malate dehydrogenase, YKL-40 and osteopontin are individually capable of differentiating AD from non-AD CSF with AUC≥0.75; and

collectively all five of the "validated" biomarkers could distinguish individuals with AD positive/negative CSF with AUC=0.88.

9.5.1 Malate dehydrogenase

The biomarkers identified all have potentially important roles in AD pathogenesis. MDH is one of eight mitochondrial enzymes involved in the tricarboxylic acid (TCA) cycle, the main pathway for oxidation of glucose in the brain. It is found in increased concentrations in the cortex of AD brains of humans and mice at autopsy compared with healthy controls[385, 386] while other enzymes in the cycle are reduced or unchanged[385]. MDH has been previously identified in other biomarker discovery studies in AD CSF[387]. The mechanism for increased CSF MDH is unclear but from studies of other pathological brain conditions (ischaemia and hypoglycaemia), anabolic catabolism of glucose may occur as an alternative mitochondrial energy generating pathway[388]. Glucose hypometabolism measured using FDG PET predates cognitive symptoms and is closely correlated with cognitive function in AD. Future studies are required to assess if malate dehydrogenase concentration correlates with FDG-PET measures of hypometabolism; if so CSF measurement of malate dehydrogenase may have potential utility for early diagnosis, staging and could be a meaningful measure of therapeutic engagement in drug trials.

9.5.2 Cystatin C, osteopontin and YKL-40

Cystatin C (CysC) colocalises with amyloid and is involved in microglial activitation[369]. CSF CysC predicts rate of brain atrophy, a surrogate marker of neurodegeneration, in AD[389]. Several previous biomarker discovery studies have

identified differences in CysC in AD CSF using ELISA[387, 390] and it has been identified using mass spectrometry in a biomarker discovery studies of AD CSF[391]. Furthermore it predicts rates of brain atrophy in the CSF of amyloid positive individuals[389]. Osteopontin is a cytokine expressed by cytotoxic T cells and is involved in macrophage recruitment and activation. It is increased in pyramidal neurons in AD[392] in AD transgenic mouse models[393], and is elevated in human AD CSF[394,395] and is elevated in the CSF of familial AD mutation carrying individuals[396]. YKL-40 is expressed by microglia and astrocytes in the brain and is implicated in the neuroinflammatory response to β-amyloid deposition[229]. Elevated CSF YKL-40 is seen in a number of neurodegenerative diseases including prodromal AD[229], as well as in stroke and multiple sclerosis. It was previously identified as a potential AD biomarker in an unbiased LC-MS biomarker discovery study comparing CSF from individuals with AD to controls[232] and was higher in AD CSF in another targeted proteomics study[379]. Although there are commercially available immunoassays for YKL-40 and it is unlikely to be specific for AD, it could prove a useful marker in the context of a multiplexed panel of CSF markers of neuroinflammation, which might improve diagnostic accuracy or help predict rate of disease progression.

9.5.3 ApoE isoforms

As previously described this type of assay can measure peptides corresponding to apoE isoforms E3, E4 and E2 accurately in order to determine APOE genotype[397], an added benefit which could have significant practical & financial benefits. However the utility of CSF Total apoE concentration is less well established, with previous studies showing no clear difference in concentration between AD and control CSF[397, 398].

9.5.4 Strengths and Limitations

This study has a number of strengths, notably the use of two independent cohorts allowing for discovery/replication and conservative statistical approaches correcting for multiple comparison; and two independent techniques for assessing biomarker differences between groups. Subjects were prospectively recruited and samples were collected according to a standard operating protocol[399] to minimise the influence of pre-analytical factors on biomarker profile. While detailed clinical data were available for some of cohort 1, as previously described[258], cohort 2 were extremely well characterised and matched for age and sex, were from a single centre, with information about MMSE and duration of symptoms available. Individuals in the AD group were relatively young reflecting our clinical focus and that younger individuals are more likely to be referred for diagnostic lumbar puncture[368]. As the design of this study was to determine whether the assay could differentiate between AD and non-AD pathology, groups were defined by CSF neurochemical status rather than clinical diagnosis since clinical diagnostic accuracy can be variable, even in specialist centres[400] while a combination of CSF tau and β-amyloid can predict diagnosis with a sensitivity and specificity of around 90%[73] in individuals whose brains were subsequently examined post-mortem. The neurochemical non-AD group was mixed; 61% were controls, whilst the other 39% were concerned about their cognition and may have had another neurodegenerative disease. This study is therefore likely to identify biomarker associated with amyloidosis and may not be capable of detecting other markers of neurodegeneration which may also be altered in the non-AD CSF group. Further studies are needed to identify and validate biomarkers in pre-symptomatic or very early AD, which this study is not capable of doing since only a very small number of individuals were in the presymptomatic

phase of AD (asymptomatic with an AD like CSF profile). To date a large number of candidate CSF proteins have been suggested as potential biomarkers for presymptomatic AD based on biomarker discovery experiments in asymptomatic individuals carrying an autosomal dominant mutation for AD[396] and blood based biomarkers identified from twin studies[401] where some individuals subsequently develop cognitive impairment. This type of MRM assay could potentially validate those biomarkers of clinical importance in months rather than years that it might take to develop an ELISA based assay and the reagent costs, which might be substantial for a novel immunoassay, are negligible[402]. Finally, although highly selective and specific[403], and with a wide dynamic range[404, 405] MRM is still likely to be less sensitive than some of the most advanced ELISA techniques which remain the gold standard for protein detection[406].

A previous study of AD, mild cognitive impairment (MCI) and control CSF[159] used a similar pipeline to validate a panel of biomarkers in a single cohort with longitudinal CSF samples, and found four biomarkers that differentiated clinical AD from healthy controls, including YKL-40, Complement component C3, transthyretin and amyloid A4 protein. YKL-40, identified in both studies, was significantly elevated in AD and MCI CSF and was identified in our OPLS-DA analysis and univariate analysis comparing neurochemical AD to non-AD. Similarly transthyretin was identified in AD and MCI CSF[159] and in our OPLS-DA analysis; serum amyloid A4 protein contributed to variance in our OPLS-DA analysis; complement component C3 was not included on our panel. This present work uses a larger panel of biomarkers and has some methodological advantages: the time taken to run the assay is significantly shorter, samples do not require to be aliquoted into multiple small volumes and can be analysed from one single pot, and therefore lends itself extremely well to

multiplexing large numbers of peptides concurrently and cost effectively. One limitation of this study is that the list of potential biomarkers was derived from biomarker discovery experiments using a different population of subjects with a range of neurodegenerative diagnoses and from AD related literature searches rather than carrying out biomarker discovery experiments as part of the pipeline. Consequently, this panel of biomarkers is not intended to be exhaustive.

9.6 Conclusions

This work describes a streamlined and efficient mass spectrometry technique for measuring multiple CSF biomarkers concurrently, and using this methodology validate a number of biomarkers including markers of neuroinflammation and glucose metabolism that collectively have clinical diagnostic utility for AD diagnosis. This highly specific method offers the opportunity to validate large numbers of candidate biomarkers in very small volumes of CSF with negligible reagent costs, and is ideally suited both for biomarker discovery, and for translation into a rapid and cost-effective clinical test.

9.7 Publications relating to this chapter

WE Heywood, D Galimberti, E Bliss, E Sirka, **RW Paterson**, NK Magdalinou, M Carecchio, E Reid, A Heslegrave, C Fenoglio, E Scarpini, JM Schott, NC Fox, J Hardy, K Bhatia, S Heales, NJ Sebire, H Zetterberg, K Mills. Identification of novel CSF biomarkers for neurodegeneration and their validation by a high-throughput multiplexed targeted proteomic assay. Molecular Neurodegeneration. 2015 Dec 1;10:64.

RW Paterson*, WE Heywood* *et al.* Targeted Proteomic Multiplex CSF Assay identifies increased malate dehydrogenase and other neurodegenerative markers in individuals with AD pathology. Accepted for publication in Translational Psychiatry, Aug 2016. *joint first authors

Chapter 10. Conclusions and Further Work

10.1 Unmet needs in AD biomarker research

In the introduction, a description of the clinical features of Alzheimer's disease, its impact on individuals and carers and the stark realities of rising AD incidence make plain the pressing need for disease modifying therapies. This chapter goes on to describe the neuropathology of AD, disease causing genetic mutations and genetic risk factors and the sequence of events that lead from amyloid deposition, to neurodegeneration, to atrophy and ultimately dementia. The amyloid cascade hypothesis remains the pre-eminent hypothesis for AD pathogenesis but the author sets out the details of an increasingly complicated model of AD biology; evidence implicating several biological pathways is given. It is proposed that AD will need to be diagnosed earlier, the complexities of AD pathobiology more completely understood and the facility to track disease and monitor therapeutic engagement established. The introduction explains why biomarkers are key to meeting all of those aims.

An overview of currently available biomarkers, their limitations and biomarkers in development across all modalities is provided and a case made for the development of novel CSF derived biomarkers.

10.2 Do CSF prehandling methods affect measured biomarker concentrations?

The work in chapter 3 advances knowledge of the preanalytical confounding variables in the measurement of CSF analytes, specifically Aβ1-42. Building on knowledge that a number of preanalytical factors are known to influence CSF Aβ1-42 concentration, the hypothesis that aliquot storage volume and CSF volume to surface area could influence amyloid adsorption and measured Aβ1-42 concentration was tested. A two-fold difference in measured Aβ1-42 concentration was identified between the smallest and largest aliquot volumes. This confounding variable has the potential to significantly impact the validity of a laboratory's assay and could mean that individuals are misdiagnosed. Whilst adding a buffer detergent such as Tween seems to correct this problem to some extent, its effect on amyloid kinetics and conversion rates to oligomers and fibrils in vitro is unknown. A more pragmatic approach might be to suggest that volumes of CSF aliquots stored are standardized within and between laboratories.

It was also hypothesized that handling methods used in clinical practice to transfer samples from the bedside to the laboratory might also adversely influence measured biomarker concentrations. Since most research studies of CSF biomarkers have used samples collected according to strict research protocols it was important to determine whether the conclusions were applicable to clinically acquired samples. Importantly no significant differences were found between samples transferred according to gold standard research protocols compared to those 'mishandled' by

standard hospital portering services. This provides reassurance that research CSF can be acquired from non-specialist centres, and that research CSF findings and reference ranges are applicable to clinical cohorts, provided a standard operating procedure is followed.

10.3 Can amyloid PET be used to determine cutpoints for existing biomarkers of amyloid pathology, in the absence of sufficient pathology proven cases?

There are very few large studies of individuals who have given research CSF and who have also subsequently died and received a pathological diagnosis. However, amyloid PET has now been pathologically validated in several studies. The aims of this chapter were to determine whether comparing CSF molecular biomarkers and amyloid PET might provide a useful means of determining clinical CSF cutpoints in a local population of individuals with suspected AD, individuals anticipated to be healthy controls, or suspected to have other neurodegenerative diseases. As expected, there was a strong correlation between amyloid PET status and the 'core' biomarkers of AD pathology. In cases where the two methods of PET interpretation were concordant, CSF biomarker concentrations lay clearly on one side of the cutpoints determined in other large multi-centre studies, and our local cutpoints for distinguishing AD from healthy controls and most other neurodegenerative diseases. However, in discordant cases, CSF concentrations tended to lie in a range around those cutpoints, suggesting a diagnostic 'grey-zone'. This is a helpful and important concept for the interpretation of clinical CSF biomarker concentration values in clinical practice.

10.4 Can an extended panel of cross-sectional quantitative CSF biomarkers be used to improve clinical decision making in the differential diagnosis of AD?

Although core biomarkers of AD pathology are already in use in clinical practice for differentiating AD from healthy controls, their utility in differentiating AD from other neurodegenerative diseases is less well established. The second major problem is that it is a challenge to blind clinicians to the CSF results which will inevitably influence the clinical diagnosis. The author sought to overcome this problem by establishing and independently verifying *pre-LP* diagnosis.

Based on meta-analysis data, it has now been established that T-tau, Aβ1-42, P-tau and NFL have greatest diagnostic utility in distinguishing AD from healthy controls. In this study the same biomarkers emerge as having maximum utility for distinguishing AD from other neurodegenerative diseases.

Single biomarkers, or simple ratios (Tau/ Aβ1-42 or AβX-40/X-42) can differentiate AD from SD or bvFTD with high sensitivity and specificity without the need for multiple biomarkers or complex formulae.

By contrast none of the biomarkers were able to distinguish AD from either PNFA or DLB. This is likely to be due to these clinical syndromes being due to either mixed pathology (DLB) or being underpinned by one a number of pathologies (PNFA). Furthermore combining multiple biomarkers into a diagnostic model does not significantly improve diagnostic utility.

Incorporating biomarkers that are more specific to the underlying pathology of these diseases may improve diagnostic specificity.

10.5 Can an extended panel of cross-sectional quantitative CSF biomarkers help explain clinical heterogeneity within AD?

In this chapter an extended panel of CSF biomarkers was used to explore phenotypic diversity within AD. Typical memory led AD and the atypical variants, posterior cortical atrophy, logopenic aphasia and frontal variant AD are underpinned by the same neurodegenerative pathology, yet there seem to be important biological differences; it was hypothesized that differences in amyloid processing, neuroinflammation or large fibre myelinated axon involvement might, in part, explain some of this clinical heterogeneity. The aim of the study was to determine if an extended panel of biomarkers measured cross-sectionally could detect group differences. Typical and atypical AD groups were well matched demographically and had no significant differences in either markers of amyloid processing or neuroinflammation, however levels of CSF NFL were higher in the atypical group, suggesting greater involvement of large fibre myelinated axons. Within atypical AD, levels of T-tau and P-tau were lowest in the PCA group, which also had lowest rates of cognitive decline suggesting a more indolent course. Conversely, the frontal AD group had fastest rates of cognitive decline, highest levels of T-tau and lowest levels of Aβ1-42 and AβX-40/42 ratio, suggesting a more aggressive disease course.

Taken together these findings suggest there may be subtle differences in amyloid deposition, processing and rates of neurodegeneration between clinical subtypes. This work builds on previous observations demonstrating differences in brain Tau

deposition and genetic variability between AD subtypes and begins to unpick some of the biological heterogeneity within AD.

In future, wider biomarker panels probing different biological pathways are likely to further elucidate clinical heterogeneity. This work should a) be targeted towards known biological pathways discussed in the introductory chapters, with particular attention to pathways related to the genetic risk factors identified for PCA and LPA and b) involve further hypothesis generating experiments using CSF from well phenotyped atypical cases, early in their disease course.

10.6 Are cross-sectional biomarkers able to predict rate of AD clinical progression?

The author postulated that CSF markers that correlate with atrophy rates could be used to predict rates of neurodegeneration and clinical disease progression. It had previously been demonstrated that rates of hippocampal and whole brain atrophy correlate well with clinical disease progression but few studies to date had explored the relationship between CSF biomarkers and atrophy rates. Although the clinical cohorts collected by the author had insufficient longitudinal data to explore atrophy rates for this thesis, these will be the subject of future work. In the meantime the established and publically accessible ADNI cohort was used to explore this relationship instead. The ADNI sub-cohort studied had CSF data that had been analysed using a large panel of ELISA based assays. Since the author had no prior knowledge of the biomarkers included in the multiplexed assay it was largely an unbiased study with a large number of participants, but methodologically this work would have been considerably strengthened by validation using an independent

cohort. As expected, none of the biomarker in this panel of biomarkers significantly correlated with atrophy rates in individuals with negative CSF amyloid profiles, however it was demonstrated that a number of biomarkers were correlated with brain atrophy in the amyloid positive group, even after correction for nuisance variables and for multiple comparisons. Chromogranin A and Cystatin C, markers of amyloid processing and neuroinflammation respectively, had previously been implicated in AD pathogenesis and emerged as strong predictors of atrophy. Notably Trefoil factor 3 was correlated with whole brain atrophy, ventricular expansion rate and hippocampal atrophy in a regression models controlling stringently for nuisance variables and also emerged as a predictor of atrophy using an exploratory reverse stepwise analysis. Trefoil factor 3 has not previously been identified as an AD biomarker in humans but has been patented as a potential marker of NOTCH activity and is therefore of considerable interest as a marker of disease progression and potentially of drug target engagement.

In summary, this chapter identifies a number of possible CSF biomarker candidates of clinical disease progression in individuals with amyloid pathology, which require to be validated in an independent cohort of individuals with clinical AD. The role of trefoil factor 3 in AD pathogenesis should also be the subject of further study.

10.7 Could mass spectrometry be used to rapidly identify and validate new biomarkers for AD?

In the introduction of this thesis a case was made for the development of new biomarkers, and in earlier chapters the limitations of biomarkers to aid decision making in clinical diagnosis, explain phenotypic diversity and explain rate of disease

progression was outlined in detail. As efforts to develop disease modifying drugs for AD have been redoubled, the number of candidate biomarkers identified in GWAS studies and exploratory hypothesis generating experiments has increased. Means of rapidly developing assays capable of testing hypotheses using human biofluids has become a priority in the field and the time taken to develop effective ELISA assays has been a bottleneck in this process. Using a targeted mass spectrometry approach, with assays developed 'in house' by Dr Kevin Mills' lab this present work has demonstrated that this type of assay can measure novel markers, not necessarily possible to measure using ELISA, and that they can be rapidly developed and tested on human CSF within weeks. Furthermore, using an existing panel of biomarkers, several were significantly different in AD and non-AD CSF in two independent clinical cohorts. This work shows that this technique, widely used in other diseases, has potential to rapidly validate CSF biomarkers in AD and other neurodegenerative diseases in a fast, high throughput cost effective multiplexed assay.

Further work might include using this technique to develop a bespoke panel of biomarkers for AD, and to explore whether they can also be detected in other more accessible biofluids such as blood, urine, or even saliva where protein concentrations are likely to be lower. As genetic risk factors that might explain variance in disease progression or clinical phenotype are identified, this technique has the potential to rapidly develop assays that might be used to measure gene products of interest or probe related biological pathways.

10.8 Final Comments

There are two major conclusions from this thesis with implications for changing clinical practice. First, the aliquot storage volume of CSF samples is key to accurate

and reproducible measurement of amyloid moieties. Second, samples can be transferred to the laboratory in a timeframe that is compatible with normal prehandling clinical practices, even in regional neurology centres. The author envisages that CSF biomarkers will become more widely used in routine clinical assessment of cognitive impairment in memory clinics outside major centres in the UK, particularly if disease-modifying treatments become available. There will be a place for amyloid and tau PET scans in clinical diagnosis, but these imaging modalities are likely to be expensive and expose individuals to radiation, and capacity will be limited. As new biomarkers become available, PET tracers are unlikely to be developed in a timely fashion to keep up with clinical demand. By contrast CSF biomarkers can be more rapidly developed, validated and translated into clinical practice, as demonstrated in chapter 9, and lumbar punctures can be carried out in most clinical settings with limited training.

Ultimately for accurate diagnosis of AD during life, the field requires biomarkers that are specific to the proteins or pathogenic processes that result in neurodegeneration. For DLB this might be α-synuclein; in FTLD, biomarkers of specific tauopathies or TDP-opathies will be required. The author believes that simple measurements of these proteins alone may be insufficient to detect the earliest signs of pathogenicity, particularly as the field moves towards the presymptomatic diagnosis of neurodegenerative disease. Rather, evidence of dysfunction of protein metabolism will be required. In Alzheimer's disease this might be the ability to measure and quantify reliably the propensity of amyloid to form toxic oligomeric structures or to measure the extent to which tau misfolds and forms tangles. As yet no such 'functional' assays exist and will be a major challenge over coming years. However, the author believes that CSF, which is in direct contact with the brain and

therefore most likely to directly reflect metabolic processes occurring in the brain, is most likely to yield meaningful discoveries.

10.9 Publications arising from this thesis

R.W. Paterson *et al.* Biomarker Modelling of Early Changes in Alzheimer's Disease, Molecular Diagnosis and Therapy (2013)

R.M. Ahmed and **R.W. Paterson** (joint first authors) *et al.* Biomarkers in Dementia: clinical utility and new directions. Journal of Neurology, Neurosurgery and Psychiatry (2014)

J. Toombs and **R.W. Paterson** *et al.* Identification of an important potential confound in CSF AD studies: aliquot volume. Clin Chem Lab Med. (2013) 51(12)

P.S.J. Weston, **R.W. Paterson** *et al.* Using florbetapir positron emission tomography to explore cerebrospinal fluid cut points and gray zones in small sample sizes. Alzheimer's and Dementia: Diagnosis, assessment and disease monitoring, (2015)

R.W. Paterson et al. Dissecting IWG-2 typical and atypical Alzheimer's Disease: insights from cerebrospinal fluid analysis. Journal of Neurology (2015)

R.W. Paterson *et al.* Do cerebrospinal fluid transfer methods affect measured amyloid β42, total tau, and phosphorylated tau in clinical practice? Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring 1 (2015)

R.W. Paterson *et al.* CSF in the differential diagnosis of Alzheimer's Disease: Clinical Utility of an Extended Panel of Biomarkers in a Specialist Cognitive Clinic. Alzheimer's & Dementia (Conference Abstract, Alzheimer's Association International Conference, 2016)

Heywood WE, Galimberti D, Bliss E, Sirka E, **Paterson R.W.**, *et al.* Identification of novel CSF biomarkers for neurodegeneration and their validation by a high-throughput multiplexed targeted proteomic assay. Molecular Neurodegeneration (2015).

RW Paterson*, WE Heywood* *et al.* Targeted Proteomic Multiplex CSF Assay identifies increased malate dehydrogenase and other neurodegenerative markers in individuals with AD pathology. Accepted for publication in Translational Psychiatry, Aug 2016. *joint first authors

R.W. Paterson *et al.* Cerebrospinal fluid markers including trefoil factor 3 are associated with neurodegeneration in amyloid-positive individuals. Translational Psychiatry (2014).

Amanda Heslegrave, Wendy Heywood, **Ross W Paterson**, Nadia Magdalinou, Johan Svensson, Per Johansson, Annika Öhrfelt, Kaj Blennow, John Hardy, Jonathan Schott, Kevin Mills, Henrik Zetterberg_Increased cerebrospinal fluid soluble TREM2 concentration in Alzheimer's disease. Molecular Neurodegeneration (2016)

Appendices

Appendix A: Mini Mental State Examination

Not included due to copyright restrictions

Appendix B. Standard Operating Procedure for Cerebrospinal Fluid Collection and Biobanking (complete version)

UCLH R&D No: 12/0344
Author: Ross Paterson MBChB MRCP
Principal Investigator: Dr Jonathan Schott BSc MD MRCP
Collaborators
Consultant Neurologists at the Dementia Research Centre (Professor Martin Rossor, Professor Nick Fox, Dr Jason Warren, Dr Catherine Mummery)
Cerebrospinal fluid laboratory at the Institute of Neurology (Dr Michael Lunn, Professor Henrik Zetterberg)
Collaborating centres within and outside the European Union

Start date of project: 16-May-2013
End date of project: 16-May-2021
Project Duration: 8 years
Clinical and laboratory staff will follow standard operating procedures (SOPs) for CSF and blood sample collection and handling, and log all sample information in a relational database that is also used to track sample location. This is an outline of those procedures and a description of the basic sample information that will be collected.

Identification of Individuals:

Suitable research subjects will be identified via the specialist cognitive disorders service at the National Hospital for Neurology and Neurosurgery or when they attend the National Hospital Day care unit having been referred for lumbar puncture by a member of the specialist cognitive disorders team. Any patient who is undergoing lumbar puncture for suspected neurodegenerative disease as a part of their usual clinical work-up and who is under the care of a dementia research centre clinician would be eligible.
Subject may be identified at one of 2 time points:
(1)At the specialist cognitive disorders clinic

In the specialist cognitive disorders clinic subjects will be provided with written information, asked to sign the Data Protection Act form and they will be asked to sign the consent form if they feel ready to do so.

(2)At the National Hospital day care unit

The Dementia Research Fellow responsible for managing the CSF database (or deputy) will review the day care unit admission list each Friday afternoon in order to identify potential participants who may or may not have previously been identified in clinic.

The consultant in charge will be informed by email at least 48 hours notice prior to the admission and given the opportunity to opt-out.

The CSF laboratory technician will be informed of prospective participants by email 48 hours prior to admission. The pseudonymised subject code will be emailed by Ross Paterson or his deputy.

Seeking Consent

All patients will have signed the data protection act form and have indicated their willingness to be approached by the research team.

Informed written consent will be sought by a member of the research team which will be either one of the PIs, medical doctors or clinical research nurses working at the dementia research centre. ***This should be somebody other than the consultant neurologist directly responsible for the patient.***

Where a potential participant is deemed to lack the capacity to give informed consent, a consultee will be identified. The personal consultee will usually be the participant's next of kin, spouse, partner or other relative or friend.

In the event that a personal consultee is unable or unwilling to undertake the role of consultee, they may suggest that someone else takes on the role as personal consultee and other appropriate individuals may be approached.

If another personal consultee cannot be identified, the Mental Capacity Act requires that a nominated consultee should be appointed. A nominated consultee can be a professional person involved in the care of the person who lacks capacity. For example, a member of staff working in a care home where the participant resides may be appointed as a nominated consultee. A doctor or healthcare professional may be an appropriate nominated consultee if they are not involved in the research in any capacity.

The consultee will be provided with an information sheet outlining their responsibilities as the consultee. They will also be given a copy of the participant information sheet providing them with detailed information about the research study. The outcome of the discussion with the consultee and their views on the presumed wishes and feelings of the research participant in relation to this research study will be recorded on a record of consultation sheet which will be filed in the patient notes. A copy of the record of consultation will be given to the consultee. If the time between consent and the lumbar puncture **exceeds three months** then the patient will be reconsented. Written consent will be filed in the clinical notes. A copy of the patient information sheet will be given to the patient. A copy of the consent form will be filed in the patients notes. The original consent form will be passed to Suzie Barker and an entry will be signed and dated in the notes indicating the study reference number.

Research pack preparation and sample labelling

A transparent bag containing the following will be prepared in advance by the Clinical research fellow (Ross Paterson or deputy):
2 blood bottles (as shown in Figure 1)
2 polypropylene CSF tubes
1 urine container
5 spare labels
Clinical information sheet (Figure 2)
Lab transfer sheet (Figure 3)
Spare consent form

Each blood, CSF and urine vessel will be labelled with a pseudonymised code printed on paper designed to withstand temperatures of -80 degrees Celsius or less. The clinical information sheet (Figure 2) and lab transfer form (Figure 3) will have a label with the same code attached.

The unique DRC code will be created in the following format:
(Hyphens separate the different components of the ID)

DRC_0001-20120131-1

[SubjectID]-YYYYMMDD-#

DRC_XXXX=[SubjectID]=A number generated by the clinical research fellow. Numbers will be generated as sequential integers in the order that subjects are recruited.
YYYY= year sample is collected
MM=month sample is collected
DD=day sample is collected
#= number of occasions this subject has donated CSF or blood for research in this project.

The code will be generated on Microsoft excel software.
A barcode will be generated using *Barcode* appropriate software
Labels displaying the code and barcode will be produced in 2 sizes (12.70 x 25.40mm and 66.68 x 25.4mm) on a Brady BBP11 Thermal Transfer printer on nylon cloth (Brady material 499).

The subject-specific code stickers would be attached to the front of the clinical notes at the point at which consent is obtained. Clinical details (outlined in table 1) would be collected using a brief questionnaire by a member of the cognitive disorders team and logged and stored along with this code in a secure clinical database by Suzie Barker at the Dementia Research Centre.

Appendix B, Table 1 Information to be collected in the questionnaire at the time of lumbar puncture

Issue	Clinical information	Comments
1	Name	
2	Hospital Number	
3	Date of birth	
4	Date of first symptom	
5	First symptom	
6	Pre-LP diagnosis	
7	Consultant	
8	Date and time of lumbar puncture	

9	Age at lumbar puncture	
10	Manometer used	Yes/No

Lumbar puncture

This will be performed by the doctor in the patient's usual clinical team. The procedure would be undertaken in the usual clinical setting.

The CSF sample must be collected between 8am and 12pm and samples may be collected up to 4pm in exceptional circumstances.

In addition to samples taken for routine clinical analysis, CSF will be collected in two screw-top polypropylene tubes (Sarstedt 62.610.018). Guidelines for CSF collection are outlined in Table 2.

These should be the last tubes filled

Pressure readings using a manometer should be avoided unless there is a compelling clinical reason to measure it.

Blood collection [255]

Venous blood will be collected using the vacutainer system at the same time as blood samples are taken for clinical analysis.

The following vacutainer tubes will be collected:

1 or 2 x EDTA tubes (lavender top), total 10ml

1x Clot activator tubes (gold top), total 8.5ml

The optimal time from withdrawal to spinning and freezing is less than 60minutes.

Appendix B, Figure 1 Blood bottles to be filled with venous blood

Urine Sampling

Urine should be collected in a 100ml polypropylene screw-top container (Starstedt, product code : 75.1354.001)

A mid stream spot collection (taken at any time of the day) is adequate

At least 25ml is required.

Transferring samples to the laboratory

All research samples including blood, CSF and urine will be stored transferred and processed together and should not be separated.

Clinical samples should be handled and transported separately.

Samples should be accompanied by the lab transfer sheet and no other clinical information.

The CSF laboratory technician will be contacted by the doctor performing the lumbar puncture or a member of the research team by telephone call 02034484204 (or on the CSF laboratory technician's personal mobile if this is known to the clinician) immediately after the sample has been successfully collected.

Samples (blood, CSF and urine) will be left in the designated metal box on the daycare unit desk for collection.

The CSF technician will personally collect samples and deliver them to the CSF laboratory within 30 minutes.

If the CSF technician or his deputy is unavailable then he will inform the DRC research fellow or his deputy in advance. In this situation a member of the specialist cognitive disorders team will collect the samples and deliver them to the lab manager who will freeze the CSF and urine at -80 Celsius, and store the blood at ambient temperature until the CSF technician or his deputy can process them at a later date.

Appendix B Table 2 Overview of the procedure

Issue		Ideal Situation
1	Preferred Volume	In addition to CSF collected for routine clinical examination (e.g. cell count, oligoclonal bands, cytology etc.) a further 15ml should be collected between two polypropylene screw-top containers (Sarstedt 62.610.018).
2	Time of collection	Ideally between 8-12am to avoid potential for diurnal variation in CSF biomarkers. Always before 4pm.
3	Other samples that should be collected simultaneously	Blood for storage Urine for storage
4	Local anaesthesia	As per usual clinical guidelines
5	If bloody	To be sent to CSF laboratory regardless
6	Storage conditions	Room temperature before, during and after spinning.
7	Transfer to laboratory	Within 30 minutes. CSF technician to be contacted by telephone 02034484204 or on the CSF laboratory technician's personal mobile if this is known to the clinician
8	Post lumbar puncture advice	As per usual clinical guidelines
9	Manometer	Avoid unless a good clinical reason as CSF markers of neurodegeneration adhere to it

Laboratory Procedures

The CSF laboratory technician will receive a mobile phone call from the doctor performing the lumbar puncture informing them that the sample has been collected.

They will pick up the CSF sample, blood samples, completed data transfer sheet (Figure 3) and ensure that each of them is properly labelled with the unique DRC code and barcode. The samples will be transferred in the clear plastic bag provided by the research group. They will ensure that they reach the CSF laboratory within 30 minutes.

Upon arrival the samples will be logged in a standard import spreadsheet for the database. This will be done either manually or by using a barcode scanner that will be read using *appropriate* software.

CSF:

The sample will be centrifuged (3000rpm for 10 minutes) and the supernatant fluid aliquoted in volumes of 1.0ml within 1.5ml polypropylene tubes. The tubes will be allocated storage positions using Item tracker software, and the individual aliquots given specific positions on the rack with individually unique identifiers. The location information will be stored in the database.

Unique identifiers for each aliquot generated using *appropriate* software will be printed on nylon labels using a Brady BBP11 Thermal Transfer printer with Ribbon appropriate for storage conditions of up to -100C.

Labels will be fixed to the tubes at ambient temperature.

Date and time of sampling, collector, condition, date and time of arrival, date and time of freezing and storage condition, cell count, sample quality comments (for example, indicating possible contamination) will also be logged in the database.

Samples will be stored in a -80 freezer within one hour of arrival.

The freezers have a monitoring and alarm system for ensuring that sample conditions are maintained, and there is a backup freezer should a freezer fail.

Blood

EDTA Samples (Lavender):

Preparation of Guthrie cards: A spots of blood will be extracted from the EDTA sample with a Pasteur pipette and dropped onto the Guthrie card. These cards will be dried well and stored in an alphabetical filing system at -80C.

After blood has been spotted on card the sample will be discarded..

Clot activator samples (Gold):

The serum fraction produced by the gel separator plug is transferred directly to labelled cryostorage tubes (colour coded) and stored at -80C, again in 2 tubes.

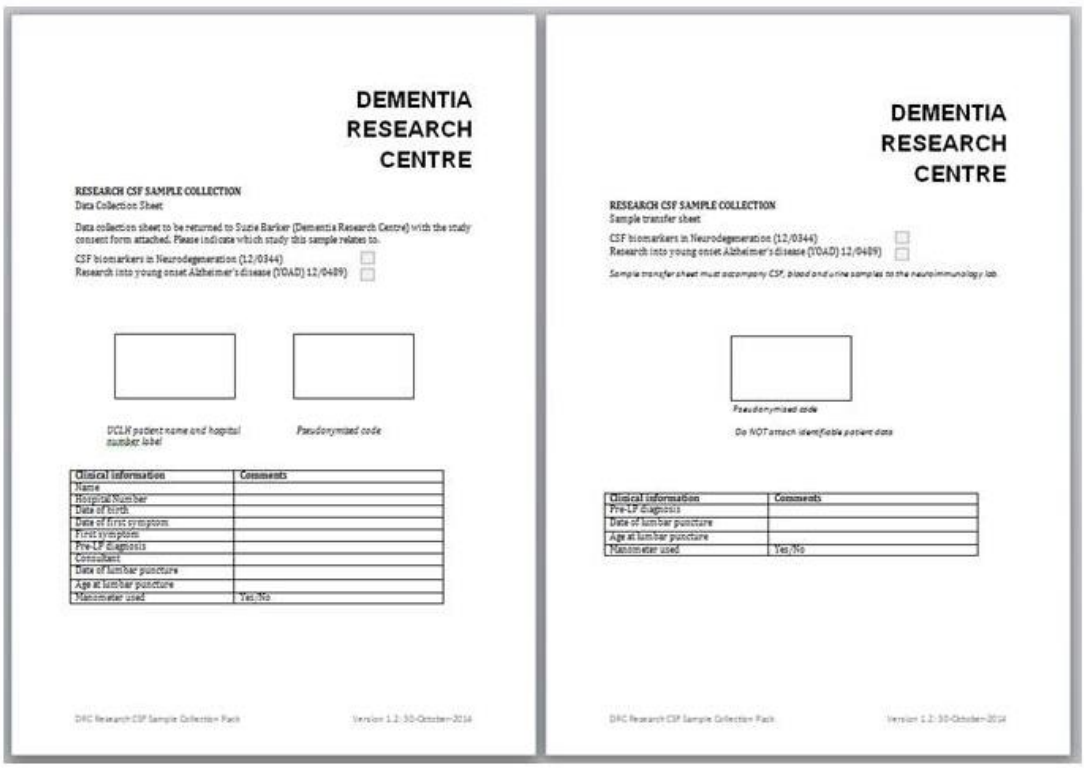
Unique identifiers for each aliquot generated using appropriate software will be printed on nylon labels using a Brady BMP53 printer with Ribbon appropriate for storage conditions of up to -100C.
Labels will be fixed to the tubes at ambient temperature.
Samples will be stored in a -80 freezer within the Institute of Neurology within one hour of arrival.

Urine
Urine will be aliquoted as two samples of 1ml within 1.5ml polypropylene tubes and the remaining volume will be stored as a single aliquot within the original screw top polypropylene container.
Unique identifiers for each aliquot generated using appropriate software will be printed on nylon labels using a Brady BMP53 printer with Ribbon appropriate for storage conditions of up to -100C.
Labels will be fixed to the tubes at ambient temperature.
Samples will be stored in a -80 freezer within the Institute of Neurology within one hour of arrival.

Transferring samples between centres:
Sharing of samples between laboratories will not take place without discussion between the CSF laboratory and the PI or his nominees. Samples will be anonymised. Mechanisms for transfer sample transfer will be the responsibility of the CSF laboratory.

Clinical Follow-up
Subjects will be routinely asked whether or not they experienced a headache after the lumbar puncture.
This information will be documented by a member of the specialist cognitive disorders team during their next clinical visit
The Dementia Research Centre Clinical Research Fellow responsible for managing the CSF database (or deputy) will review the relevant specialist cognitive disorders clinic lists each week to identify subjects who have had a lumbar puncture since their last clinic visit. Their attending clinician will be asked to collect this information which will be documented in the research database.

Appendix B, Figure 2 Data collection sheet and sample transfer sheet



DEMENTIA RESEARCH CENTRE

RESEARCH CSF SAMPLE COLLECTION

Data Collection Sheet

Data collection sheet to be returned to Susie Barker (Dementia Research Centre) with the study consent form attached. Please indicate which study this sample relates to.

CSF biomarkers in Neurodegeneration (12/0344) ☐
Research into young onset Alzheimer's disease (YOAD) 12/0489) ☐

UCLH patient name and hospital number label

Pseudonymised code

Clinical information	Comments
Name	
Hospital Number	
Date of birth	
Date of first symptom	
First symptom	
Pre-LP diagnosis	
Consultant	
Date of lumbar puncture	
Age at lumbar puncture	
Manometer used	Yes/No

DRC Research CSF Sample Collection Pack Version 1.2: 30-October-2014

DEMENTIA RESEARCH CENTRE

RESEARCH CSF SAMPLE COLLECTION

Sample transfer sheet

CSF biomarkers in Neurodegeneration (12/0344) ☐
Research into young onset Alzheimer's disease (YOAD) 12/0489) ☐

Sample transfer sheet must accompany CSF, blood and urine samples to the neuroimmunology lab

Pseudonymised code

Do NOT attach identifiable patient data

Clinical information	Comments
Pre-LP diagnosis	
Date of lumbar puncture	
Age at lumbar puncture	
Manometer used	Yes/No

DRC Research CSF Sample Collection Pack Version 1.2: 30-October-2014

Appendix C. Clinical Dementia Rating scoring sheet

Not included online due to potential copyright restrictions

Appendix D. Overview of Young Onset Alzheimer's Disease (YOAD) study

	Visit 1		Visit 2	Visit 3	
	Day 1	Day 2		Day 1	Day 2
	Screening visit	Continuation into full study			
Neurological assessment	X			X	
Neuropsychology assessment	X			X	
MRI – standard structural	X			X	
MRI – advanced sequences (fMRI and sMRI)		X	X		X
Smell testing	X				
Blood and urine samples	X			X	
Eye tracking		X			
Lumbar puncture (optional)					X
Consent for brain donation				X	

Appendix D. Overview of Young Onset Alzheimer's Disease (YOAD) study design (produced in collaboration with Dr Catherine Slattery and reproduced with her permission). Day 1 of the first visit includes the screening assessments for suitability to continue into the full study. 'X' indicates that the investigation of procedure was carried out at that point.

Appendix E. Mean and standard deviation of all proteins included in the ELISA multiplex panel in Chapter 8

Cerebrospinal fluid analyte	Abbreviation	All Diagnoses (Mean ± SD)	Control (Mean ± SD)	MCI (Mean ± SD)	AD (Mean ± SD)
Alpha-1-Microglobulin (ug/mL)*	A1Micro	-1.3±0.22	-1.4±0.22	-1.3±0.22	-1.3±0.23
Alpha-2-Macroglobulin(mg/mL)*	A2Macro	-2.4±0.13	-2.4±0.11	-2.4±0.13	-2.4±0.14
Alpha-1-Antitrypsin(mg/mL)*	AAT	-2.3±0.15	-2.4±0.15	-2.3±0.14	-2.4 0.17
Angiotensin-Converting Enzyme(ng/mL)*	ACE	0.3±0.16	0.3±0.16	0.3±0.15	0.3±0.16
Adiponectin(ng/mL)*		-2.3±0.18	-2.3±0.19	-2.3±0.17	-2.3±0.19
Agouti Related Protein(pg/mL)	AGRP	56.8±22.37	53.5±23.0	58.1±20.9	58.4±24.4
Angiopoietin-2*(ng/mL)	ANG-2	0.01±0.17	-0.001±0.16	0.03±0.18	0.002±0.18
Apolipoprotein A-I*(mg/mL)	Apo A-I	-3.1±0.22	-3.1±0.23	-3.1±0.21	-3.1±0.20
Apolipoprotein C-III(ug/mL)*	Apo C-III	-1.2±0.22	-1.2 ±0.3	-1.2±0.21	-1.3±0.21
Apolipoprotein D(ug/mL)*	Apo D	0.7±0.18	0.7±0.18	0.7±0.18	0.7±0.18
Apolipoprotein E(ug/mL)*	Apo E	0.8±0.14	0.8±0.13	0.8±0.14	0.8±0.15
Apolipoprotein H(ug/mL)*	Apo H	-0.1±0.20	-0.1±0.19	-0.1±0.20	-0.1±0.22
AXL Receptor Tyrosine Kinase(ng/mL)	AXL	4.2±1.42	4.2±1.37	4.2±1.5	3.9±1.0
Beta-2-Microglobulin(ng/mL)*	B2M	0.1±0.13	0.1±0.12	0.1±0.12	0.1±0.14
Complement C3(mg/mL)*	C3	-2.6±0.17	-2.6±0.15	-2.6±0.17	-2.6±0.17
Cancer Antigen 19-9(U/mL)	CA-19-9	1.1±0.50	1.2±0.5	1.2±0.5	1.0±0.4
Calcitonin(pg/mL) *		1.0±0.41	0.9±0.4	0.9±0.4	1.0±0.4
CD 40 antigen(ng/mL)*	CD40	-0.6±13	-0.6±0.14	-0.6±0.12	-0.7±0.12
Chromogranin-A(ng/mL)	CgA	276.1±54.45	283.0±53.2	275.4±54.9	267.9±54.7
Clusterin(ug/mL)*	CLU	1.4±0.16	1.4±0.16	1.4±0.16	1.4±0.18
Cortisol(ng/ml)		16.1±5.93	15.6±6.3	16.5±5.5	16.0±6.4
C-reactive protein(ug/mL)*	CRP	-2.8±0.56	-2.7±0.5	-2.9±0.5	-2.9±0.7
Cystatin-C(ng/mL)*		0.4±0.084	0.4±0.08	0.4±0.09	0.4±0.08
Endothelin-1(pg/mL)	ET1	12.4±3.69	12.3±4.1	12.7±3.6	12.0±3.4
Fatty Acid-Binding Protein, heart(ng/mL)*	FABP, heart	0.5±0.27	0.4±0.3	0.5±0.3	0.6±0.3
Fas Ligand(pg/mL)	FasL	11.2±4.81	10.9±4.2	11.6±5.5	10.8±3.9
Fibroblast Growth Factor 4(pg/mL)*	FGF-4	1.6±0.15	1.6±0.16	1.7±0.14	1.7±0.15

Appendix E.1 Mean and standard deviation of each measured analyte across all 287 subjects. *: transformed data. The statistics are presented for the transformed values (see methods, chapter 8). Where data have been transformed the units relate to data before transformation. Continued in **Appendix E.2/3**.

Fibrinogen(mg/mL)*		-3.4±0.38	-3.5±0.4	-3.4±0.4	-3.3±0.3
Ferritin(ng/ml)*	FRTN	0.8±0.15	0.8±0.13	0.8±0.16	0.8±0.16
Follicle-Stimulating Hormone(mIU/mL)*	FSH	-0.1±0.34	-0.1±0.4	-0.1±0.3	-0.1±0.3
Heparin-Binding EGF-Like Growth Factor(pg/mL)*	HB-EGF	2.4±0.11	2.4±0.12	2.4±0.10	2.4±0.11
Chemokine CC-4 (ng/ml)*	HCC-4	-1.4±0.19	-1.4±0.19	-1.4±0.2	-1.5±0.18
Hepatocyte Growth Factor(ng/mL)*	HGF	0.4±0.17	0.4±0.16	0.4±0.17	0.5±0.17
T Lymphocyte-Secreted Protein I-309(pg/mL)*	I-309	1.3±0.17	1.3±0.17	1.3±0.16	1.3±0.20
Intercellular Adhesion Molecule 1(ng/mL)	ICAM-1	1.0±0.44	0.9±0.4	1.0±0.5	1.0±0.4
Immunoglobulin A(mg/mL)*	IgA	-2.5±0.31	-2.5±0.3	-2.5±0.3	-2.5±0.3
Interleukin-16(pg/mL)*	IL-16	0.9±0.19	0.9±0.19	0.9±0.18	0.9±0.18
Interleukin-25(pg/mL)	IL-25	9.3±3.54	9.3±3.6	9.4±3.6	9.0±3.3
Interleukin-3(ng/mL)*	IL-3	-2.2±0.33	-2.1±0.3	-2.2±0.3	-2.2±0.3
Interleukin-6 receptor(ng/mL)*	IL-6r	-0.003±0.15	0.005±0.15	-0.0008±0.15	-0.02±0.14
Interleukin-8(pg/mL)*	IL-8	1.7±0.14	1.7±0.13	1.7±0.16	1.7±0.13
Insulin-like Growth Factor-Binding Protein(ng/mL)		108.1±35.01	105.5 ±31.9	107.4 ±30.6	113.3±46.6
Interferon gamma Induced Protein 10(pg/mL)*	IP-10	2.6±0.21	2.6±0.19	2.6±0.23	2.6±0.20
Leptin(ng/mL)*	Leptin	-1.0±0.32	-0.9±0.3	-1.0±0.3	-0.9±0.3
Lectin-Like Oxidized LDL Receptor 1(ng/mL)	LOX-1	6.4±2.24	6.2±2.1	6.5±2.3	6.4±2.3
Apolipoprotein(ug/mL)*	LP(a)	-1.6±0.52	-1.6±0.6	-1.6±0.5	-1.5±0.6
Monocyte Chemotactic Protein 1(pg/mL)*	MCP-1	2.7±0.13	2.7±0.12	2.7±0.13	2.7±0.14
Monocyte Chemotactic Protein 2(pg/mL)*	MCP-2	0.5±0.17	0.6±0.17	0.5±0.18	0.5±0.16
Macrophage Colony-Stimulating Factor 1(ng/mL)*	M-CSF	-0.2±0.14	-0.2±0.14	-0.2±0.14	-0.2±0.13
Macrophage Migration Inhibitory Factor(ng/mL)*	MIF	-0.7±0.33	-0.7±0.3	-0.6±0.3	-0.6±0.3
Monokine Induced by Gamma Interferon(pg/mL)*	MIG	2.4±0.32	2.4±0.3	2.4±0.3	2.4±0.3
Macrophage Inflammatory	MIP-1 beta	1.2±0.19	1.2±0.21	1.2±0.20	1.2±0.15

Appendix E.2 Mean and standard deviation of each measured analyte across all 287 subjects (continued). *: transformed data. The statistics are presented for the transformed values (see methods). Where data have been transformed the units relate to data before transformation.

Protein-1 beta(pg/mL)*					
Matrix Metalloproteinase-2(ng/mL)*	MMP-2	0.9±0.23	0.9±0.22	0.9±0.25	0.8±0.23
Matrix Metalloproteinase-3(ng/mL)*	MMP-3	-0.5±0.20	-0.5±0.18	-0.5±0.20	-0.4±0.19
Myoglobin(ng/mL)*		-0.4±0.4	-0.4±0.4	-.0.4±0.4	-0.4±0.5
Neutrophil Gelatinase-Associated Lipocalin(ng/mL)*	NGAL	0.2±0.20	0.2±0.20	0.3±0.21	0.3±0.18
N-terminal prohormone of brain natriuretic peptide(pg/mL)*	NT proBNP	2.2±0.15	2.1±0.15	2.2±0.15	2.2±0.15
Osteopontin(ng/mL)		33.4±9.8	31.4±10.0	33.9±9.7	35.1±9.0
Plasminogen Activator Inhibitor 1(ng/mL)*	PAI-1	-0.02±0.17	-0.03±0.16	- 0.02±0.17	0.02±0.19
Prostatic Acid Phosphatase(ng/mL)*	PAP	-0.8±0.19	-0.8±0.21	-0.8 0.18	-0.8±0.18
Pregnancy-Associated Plasma Protein A (mIU/mL)*	PAPP-A	-2.0± 0.17	-2.0±0.14	-2.0±0.19	-2.0±0.17
Placenta Growth Factor(pg/mL)*	PLGF	1.8±0.20	1.8±0.21	1.8±0.20	1.8±0.19
Pancreatic Polypeptide(pg/mL)*	PPP	0.4±0.32	0.3±0.3	0.4±0.3	0.5±0.3
Prolactin(ng/mL)*	PRL	0.3±0.13	0.2±0.11	0.3±0.13	0.3±0.15
T-Cell-Specific Protein RANTES(ng/mL)*	RANTES	-2.6±0.3	-2.5±0.4	-2.6±0.24	-2.6±0.24
Resistin(ng/mL)*	Resistin	-1.4±0.3	-1.4±0.3	-1.4±0.3	-1.3±0.3
S100 calcium-binding protein B(ng/mL)*	S100-B	0.4±0.15	0.4±0.2	0.4±0.16	0.5±0.14
Serum Amyloid P-Component(ug/mL)*	SAP	-2.6±0.3	-2.6±0.3	-2.6±0.3	-2.6±0.3
Stem Cell Factor(pg/mL)*	SCF	1.6±0.17	1.6±0.16	1.6±0.17	1.6±0.15
Serum Glutamic Oxaloacetic Transaminase(ug/mL)*	SGOT	0.6±0.11	0.6±0.12	0.6±0.10	0.6±0.11
Sex Hormone-Binding Globulin(nmol/L)*	SHBG	-0.9±0.24	-0.9±0.23	-0.9±0.24	-0.9±0.24
Sortilin(ng/mL)*	Sortilin	0.8±0.11	0.8±0.10	0.8±0.11	0.8±0.11
Thyroxine-Binding Globulin(ug/mL)*	TBG	-0.7±0.21	-0.7±0.18	-0.7±0.22	-0.6±0.23
Tissue Factor(ug/mL)*	TF	0.6±0.21	0.5±0.20	0.6±0.21	0.5±0.20
Trefoil Factor 3(ug/mL)*	TFF3	-1.8±0.18	-1.7±0.18	-1.8±0.17	-1.8±0.20
Transforming Growth Factor alpha(pg/mL)*	TGF-alpha	1.0±0.28	1.1±0.23	1.0±0.3	1.0±0.3
Tissue Inhibitor of Metalloproteinases 1(ng/mL)*	TIMP-1	1.6±0.13	1.6±0.10	1.6±0.13	1.6±0.16
Thrombomodulin (ng/mL)*	TM	-0.9±0.16	-0.9±0.17	-0.9±0.17	-0.9±0.14
Tumor Necrosis Factor Receptor 2 (ng/mL)*	TNFR2	-0.1±0.15	-0.2±0.14	-0.1±0.15	-0.1±0.16
TNF-Related Apoptosis-Inducing Ligand Receptor 3 (ng/mL)*	TRAIL-R3	-0.2±0.16	-0.2±0.14	-0.2±0.16	-0.2±0.16
Vascular Cell Adhesion Molecule-1 (ng/mL)*	VCAM-1	1.2±0.14	1.1±0.12	1.2±0.15	1.2±0.15
Vascular Endothelial Growth Factor (pg/mL)*	VEGF	2.7±0.13	2.7±0.12	2.7±0.13	2.7±0.13
von Willebrand Factor(ug/mL)*	vWF	-1.5±0.17	-1.4±0.17	-1.5±0.16	-1.5±0.17

Appendix E.3 Mean and standard deviation of each measured analyte across 287 subjects (continued). *: transformed data.

Appendix F. Proteins and peptides included in mass spectrometry assay

Name	Protein	Precursor m/z	Product m/z	Peptide sequence	Detectable in 100ul CSF (Cohort 1)?	Detectable in 100ul CSF (Cohort 2)?
Apolipoprotein AII	APOA2 SPE	487.0511	659.4627	SPELQAEAK	Yes	yes
	APOA2 SPE	487.0511	788.5582			
Apolipoprotein E (total)	APOE AAT	749.7949	642.4312	AATVGSLAGQPLQER	Yes	yes
	APOE AAT	749.7949	827.6254			
Apolipoprotein E E2 isoform	ApoE E2 carbo	554.79	345.14	CLAVYQAGAR	Yes	yes
	ApoE E2 carbo	554.79	835.5545			
Apolipoprotein E 112 E3	ApoE E3 112	611.76	491.24	LGADMEDVCGR	Yes	not tested
	ApoE E3 112	611.76	606.27			
Apolipoprotein E 158 E3	APOE E3 158	475.0873	502.3396	LAVYQAGAR	Yes	yes
	APOE E3 158	475.0873	665.4433			
Apolipoprotein E E4 isoform	APOE E4	503.5173	835.4926	LGADMEDVR	Yes	not tested
	APOE E4	503.5173	892.499			
Apolipoprotein E E4 isoform Aqua peptide	APOE E4 AP	506.524	841.5126	AAQARLGADMED[V(13C5;15N)]R	Yes	yes
	APOE E4 AP	506.524	898.519			
Apolipoprotein H (beta2microglobulin)	ApoH non carbo	796.0762	503.325	ATFGCHDGYSLDGPEEIECTK	Yes	yes
	ApoH non carbo	796.0762	531.799			
Carboxypeptidase E	CarboxypepE-ELL	817.2226	356.2624	ELLVIELSDNPGVHEPGEPEFK	Yes	yes
	CarboxypepE-ELL	817.2226	820.165			
CD166	CD166-QIG*	838.2949	397.0361	QIGDALPVSCTISASR	Yes	yes
	CD166-QIG*	838.2949	829.1371			

Chitinase 3 Like protein 1 (YKL40)	CH3L1 LVM	546.0934	439.7986		Yes	yes
	CH3L1 LVM	546.0934	577.3608	LVMGIPTFGR		
Chitinase 3 Like protein 1 (YKL40)	CH3L1 TLL	761.7649	654.4573	TLLSVGGWNFGSQR	Yes	yes
	CH3L1 TLL	761.7649	1008.5999			
Clusterin	CLUS_LFD	625.6611	585.9436	LFDSDPITVTVPVEVSR	Yes	yes
	CLUS_LFD	625.6611	686.5015			
Beta-Ala-His dipeptidase	CNDP1 TVF	633.6272	533.4147	TVFGTEPDMIR	Yes	yes
	CNDP1 TVF	633.6272	1065.6713			
Beta-Ala-His dipeptidase	CNDP1 WNY	506.0911	284.1387	WNYIEGTK	Yes	yes
	CNDP1 WNY	506.0911	497.3354			
Cystatin C	CystatinC ALD	614.1272	300.2003		Yes	yes
	CystatinC ALD	614.1272	610.383	ALDFAVGEYNK		
Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	ENPP2 WWG*	772.4634	929.4495	WWGGQPLWITATK	Yes	yes
	ENPP2 WWG*	772.4634	1171.4946			
Fibrinogen beta chain	FIBB_MGP_3p	846.65	333.18	MGPTELLIEMEDWK	Yes	yes
Ganglioside GM2 activator	GM2_by_KM*	775.6234	769.6363		Yes	yes
	GM2_optimum*	775.6234	213.176	SEFVVPDLELPSWLTTGNYR		
Insulin like Growth Factor 2	IGF2_SCD	906.9619	315.2079	SCDLALLETYCATPAK	Yes	yes
	IGF2_SCD	906.9619	363.0458			
Lysosome-associated membrane glycoprotein 2	LAMP2_GIL_2P_02*	656.91	829.45		Yes	yes
	LAMP2_GIL_2P_03*	656.91	359.21	GILTVDELLAIR		
Limbic system associated membrane protein	LSAMP Doubly	529.9043	521.1044	INSANGLEIK	Yes	yes
	LSAMP	529.9043	831.6159			

	Doubly					
Osteopontin	Osteopontin-1	927.9	511.36	AIPVAQDLNAPSDWDSR	Yes	yes
	Osteopontin-1	927.9	835.81			
Pappalysin-1	PAPPA LDG 3+ ok	488.3037	365.4114	LDGSTHLNIFFAK	yes- not quantifiable due to peak interference	yes
	PAPPA LDG 3+ ok	488.3037	495.5653			
Prosaposin*	SAPA_EIV_2P_02	865.46	215.18	EIVDSYLPVILDIIK	Yes	not tested
	SAPA_EIV_2P_03	865.46	910.53			
Serum Amyloid A4	SerumAmyloidA4-EAL	567.0596	363.2195	EALQGVGDMGR	Yes	yes
	SerumAmyloidA4-EAL	567.0596	535.3104			
Apolipoprotein E Heavy peptide	TIMP1-GFQ	617.5534	404.1179	GFQALGDAADIR	Yes	yes
	TIMP1-GFQ	617.5534	717.3912			
Metalloproteinase inhibitor 1	TREM2_VLV_492	492.6411	632.0681	VLVEVLADPLDHR	Yes	yes
	TREM2_VLV_492	492.6411	688.6233			
Triggering receptor expressed on myeloid cells 2	TRFE_EFQ_3p	426.5	387.21			
	TTHY_AAD_2p	698.13	606.36	AADDTWEPFASGK	Yes	not tested
	TTHY_YTI_3p	787.82	1002.3	YTIAALLSPYSYSTTAVVTNPK	Yes	yes
Transthyretin	Ubqtn_TIT	894.7511	298.1879	TITLEVEPSDTIENVK	Yes	No
Transthyretin	Ubqtn_TIT	894.7511	1002.5528			
Ubitqutin species derived peptide	VITAMIN D BINDING PROTEIN	789.54	657.24	VPTADLEDVLPLAEDITNILSK	Yes	yes
	VITAMIN D BINDING PROTEIN	789.54	1053.93			
VITAMIN D BINDING PROTEIN	Vitrnctn FED*	712.1811	435.3108	FEDGVLDPDYPR	Yes	yes
	Vitrnctn FED*	712.1811	647.4367			

Vitromectin	Vitrnctn SIA*	835.4319	310.2264	SIAQYWL GCPAPG HL	Yes	yes
	Vitrnctn SIA*	835.4319	423.2977			
Vitromectin	VSetTM2-GPE*	1008.031	627.2576	GPEDLDP GAEGAG AQVELLP DR		
	VSetTM2-GPE*	1008.031	1198.2634		Yes	yes
	Yeast Enolase AVD 789	790.1596	661.4872	AVDDFLI SLDGTAN K		
Vset transmembrane domain 2	Yeast Enolase AVD 789	790.1596	805.5706			
Yeast Enolase internal standard protein	Yeast Enolase AVD 789	790.1596	918.6694			
	Yeast Enolase GNP 708	709.0596	377.2864	GNPTVE VELTTEK		
	Yeast Enolase GNP 708	709.0596	451.4681		Yes	yes
	Yeast Enolase GNP 708	709.0596	948.6764			
	Yeast Enolase SIV 614	614.5796	306.2782		Yes	yes
	Yeast Enolase SIV 614	614.5796	514.8752	SIVPSGAS TGVHEA LEMR		
	Yeast Enolase SIV 614	614.5796	547.8778	TLLSVGG WNFGSQ R	Yes	yes
	Yeast Enolase SIV 614	614.5796	821.3544			
b-amyloid 40	b-amyloid 40 2+	543.5673	412.31			
	b-amyloid 40 2+	543.5673	561.3157	GAIIGLM VGGVV	Yes	yes
Triggering receptor expressed on myeloid cells 2	TREM2PEP 1	562.01	937.18	VVSTHNL WLLSFLR	Yes	yes
	TREM2PEP 1	562.01	1050.27			
Neural Cell	NCAM1 FIV	662.6772	532.9085	FIVLSNN	Yes	not tested

Adhesion Molecule 1				YLQI		
	NCAM1 FIV	662.6772	597.0048			
Trefoil 3	trefoil3_IPG	726.8572	715.201	IPGVPWCFKPLQEAECTF	No	not tested
	trefoil3_IPG	726.8572	721.1159			
glucosylceramidase 1	GBA1-2_Doubly	731.16	1100.6	NFVDSPIIVDITK	Yes	no
	GBA1-2_Doubly	731.16	1199.67			
Insulin like Growth Factor 2	IGF1-GPE	770.1011	347.1612	GPETLCGAELVDALQFVCGDR	Yes	yes
	IGF1-GPE	770.1011	606.2991			
Pappalysin-1	PappA VSF	802.0043	786.5862	VSFSSPLVAISGVALR	Yes	yes
	PappA VSF	802.0043	1095.86			
Lysosome-associated membrane glycoprotein 1	LAMP1_FFLQGIQLNTILPDAR	923.9249	458.272	FFLQGIQLNTILPDAR	Yes	yes
	LAMP1_FFLQGIQLNTILPDAR	923.9249	571.3269			
Myelin basic protein	MBP_TQD	487.8949	285.1158	TQDENPVVHFFK	No	not tested
	MBP_TQD	487.8949	616.4953			
Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1-LGF	532.7281	747.3007	LGFEDGSVLK	Yes	no
	UCHL1-LGF	532.7281	894.3056			
DJ1	DJ1_triply	554.2234	674.1549	GLIAAICAGPTALLAHEIGFGSK	Yes	no
	DJ1_triply	554.2234	723.7188			
Serum Amyloid P	SerumAmyloidP	578.9681	508.3865	VGEYSLYIGR	Yes	yes
	SerumAmyloidP	578.9681	708.5315			
Ras-related protein Rab-30	Rab30_QNT3+	585.2757	442.2915	QNTLVNNVSSPLPGEGK	Yes	yes
	Rab30_QNT3+	585.2757	487.2676			
	Yeast Enolase SIV 614	614.5796	306.2782			

	Yeast Enolase SIV 614	614.5796	514.8752			
	Yeast Enolase SIV 614	614.5796	547.8778			
	Yeast Enolase SIV 614	614.5796	821.3544			
Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1-MPF	615.1096	734.5051	MPFPVNHGASSEDTLLK	Yes	no
	UCHL1-MPF	615.1096	856.5666			
Alpha synuclein	A syn quadru	643.7273	339.3462	EQVTNVGGAVVTGVTAVAQK	Yes	no
	A syn quadru	643.7273	346.3015			
Tau protein (MAPT)	T-tau LQT	655.7134	472.3165	LQTAPVPMPDLK	Yes	no
	T-tau LQT	655.7134	896.6035			
Tau protein (MAPT) heavy peptide	T-tau LQT AP	659.2219	479.3165	SRLQTAPVPMPD[L (13C6; 15N)]K		
	T-tau LQT AP	659.2219	903.6035			
	GBA2 ACG	691.522	743.1383			
	GBA2 ACG	691.522	836.2809			
Aldolase B	AldoB-IAD	715.1687	709.1391	IADQCPSSLAIQENANALAR	No	not tested
	AldoB-IAD	715.1687	858.7665			
Synapsin 1	Synapsin 1	727.7049	443.3207	EMLSSTTYPVVVK	Not very well	yes
	Synapsin 1	727.7049	541.5081			
Neural Cell Adhesion Molecule 1	NCAM1 YIF	759.2849	1093.695	YIFSDDSSQLTIK	Yes	yes
	NCAM1 YIF	759.2849	1240.867			
Insulin-like growth factor-binding protein 2	IBP2-TPC*	808.6957	644.3944	TPCQQELDQVLER	Yes	yes
	IBP2-TPC	808.6957	758.3367			
b-amyloid38	b-amyloid38 2+	444.4611	363.1562	GAIIGLMVGG	Yes	yes
	b-amyloid38 2+	444.4611	525.3007			
Endothelial protein C receptor	EPCR-TLA	516.0596	433.2602		Yes	no

	EPCR-TLA	516.0596	816.6147	TLAFPLTIR		
Protein S100B	S100B	569.9873	703.7626	AMVALIDVFHQYSGR	Yes	yes
	S100B	569.9873	753.2795			
Neurofilament light protein	NFL-VLE	578.2011	387.2939	VLEAELLVLR	not very well due to interfering peak	not tested
	NFL-VLE	578.2011	942.7229			
Insulin B	InsB digest 651 4+	651.4043	521.3715	FVNQHLCGSHLVEALYLVCGER	No	not tested
	InsB digest 651 4+	651.4043	797.3206			
	GBA2 NVI	663.5311	514.3973			
	GBA2 NVI	663.5311	888.35			
Malate dehydrogenase	MalateDehydrCyto-FVE	697.7287	546.3637	FVEGLPINDFSR	Yes	yes
	MalateDehydrCyto-FVE	697.7287	848.5662			
	Yeast Enolase AVD 789	790.1596	661.4872			
	Yeast Enolase AVD 789	790.1596	805.5706	AVDDFLISLDGTANK		
	Yeast Enolase AVD 789	790.1596	918.6694			
Lysosome-associated membrane glycoprotein 1	LAMP1_NMTFDLPSDATVVLNR_897	897.3534	364.1983	NMTFDLPSDATVVLNR	Yes	yes
	LAMP1_NMTFDLPSDATVVLNR_897	897.3534	1071.726			
Trefoil 3	Trefoil3_vdc	424.9557	529.736	VDCGYPHVTPKECNNR	No	not tested
	Trefoil3_vdc	424.9557	587.2962			
Pro-orexin	14 OREXIN PROPEP TC	451.4787	512.5109	AGAEPAPRPCLGR	Yes	yes
	14 OREXIN PROPEP TC	451.4787	641.1357			
Progranulin	PRGN_VHC	492.2181	237.1425	VHCCPHGAFCDLVHTR	No	not tested

	PRGN_VHC	492.2181	413.234			
Chitotriosidase	CHITO_ADG_2P_02	501.65	311.2	ADGLYPNPRER	not very well	yes
	CHITO_ADG_2P_01	501.65	646.2			
Fibrinogen A	FIBA_HPD_2p	532.19	621.25	HPDEAAFFDTASTGK	Yes	yes
Glial fibrillary acidic protein	GFAP	589.5873	616.4967	LADVYQAELR	Yes	no
	GFAP	589.5873	779.5966			
GlutathioneS transferase omega	GSTO1-GSA	661.1811	553.3864	GSAPPGPVPEGSIR	Yes	yes
	GSTO1-GSA	661.1811	658.3671			
	Yeast Enolase GNP 708	709.0596	377.2864	R.GNPTVEVELTTEK.G [15, 27]		
	Yeast Enolase GNP 708	709.0596	451.4681			
	Yeast Enolase GNP 708	709.0596	948.6764			
Trefoil 2	Tref_Fac2_QES	791.7243	377.1338	QESDQCVMEVSDR	No	not tested
	Tref_Fac2_QES	791.7243	782.7479			
Trefoil 2	TREF_FAC2_NCG	848.8381	332.1084	NCGYPGISPEECASR	No	not tested

List of peptides and transitions included in Multiplex targeted proteomics assay (courtesy of Dr Wendy Heywood, who designed the assay); *Denotes biomarkers identified using 2D-LC-MSe profiling of neurodegenerative CSF; Red colour denotes peptides that could not reliably be detected in CSF and were not included in the final panel.

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