Master´s thesis

Precious Kwadzo Pomary

Exploring differences in protein metabolites in cerebrospinal fluid from patients with Alzheimer's disease, frontotemporal dementia or amyotrophic lateral sclerosis. A pilot study.

Trondheim, May, 2016

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Thesis, Master of Science in Neuroscience
Trondheim, 2016
Principal supervisor: Professor Linda R. White (PhD)
Co-supervisor: Associate Professor Sigrid Botne Sando (MD, PhD)

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Abstract

Background: FTD is the second commonest presentation of early onset dementia after AD. While the clinical features, pathophysiology and genetics of FTD are heterogeneous, AD is a more homogenous disease. Despite the superficial differences between these disorders, early onset AD and some variants of FTD are sometimes misdiagnosed due to the overlap between their clinical symptoms. Similarly, the genetic and pathological overlaps between FTD and ALS also hinder the distinction between these disorders. The classical hallmark of neurodegeneration is the aggregation of protein molecules in the brain. This is usually associated with inflammation and consequential increase or decrease in concentrations of structural and inflammatory molecules in the CSF. Based on this, CSF metabolites such as T-tau, P-tau and Aβ-42 have been investigated and are well established as the core biomarkers for AD. However, there is currently no biomarker with comparable diagnostic competence for FTD and ALS. Hence, this research sought to explore whether differences could be found in the CSF concentrations of PGRN, YKL-40, NF-L, α-synuclein, Aβ-40, and Aβ-43 in conjunction with the core AD biomarkers in relation to FTD, ALS and AD. Furthermore, it was hoped that differences might prove useful biochemical markers to distinguish these clinical conditions.

Method: CSF samples from 28 patients with AD, 21 patients belonging to the FTD/ALS spectrum of disorders and 27 age-matched healthy controls were analysed with commercial sandwich ELISA kits. The FTD/ALS spectrum was further divided on a clinical basis into FTD, ALS, PSP and FTD+ALS for the purpose of statistical analysis and comparison. The concentrations of PGRN, YKL-40, NF-L, α-synuclein, Aβ-40, Aβ-43 in addition to the core biomarkers for AD were experimentally determined and analysed with IBM-SPSS version 21.

Result: T-tau/Aβ-42 showed 100% sensitivity and specificity in distinguishing patients with AD from healthy controls. In distinguishing patients with FTD from healthy controls, T-tau/Aβ-42 was the best discriminator with a 100% sensitivity and 93% specificity. Patients with ALS were distinguished from healthy controls by the Aβ-42 ratios of YKL-40 and PGRN. While YKL-40/Aβ-42 showed 100% specificity and 80% sensitivity, PGRN/Aβ-42 showed 83% sensitivity and 93% specificity. Also, the CSF concentration of T-tau distinguished patients with FTD from those with ALS. However, neither the metabolites nor their ratios could distinguish between patients with AD from patients with FTD.

Conclusion: In this study with CSF, T-tau showed a significant difference between FTD and ALS. Levels of the inflammatory molecules, PGRN and YKL-40, in addition to the structural molecule NF-L, did not distinguish AD, FTD and ALS in this study. However, their ratios were potentially good discriminators in several comparisons.

Acknowledgment

This research was conducted in the Trønderbrain research group and the thesis was submitted to the department of Neuroscience at the Faculty of Medicine, Norwegian University of Science and Technology.

First and foremost, I am very grateful to my supervisor, Professor Linda White (PhD) for the confidence she had in me by giving me the opportunity to undertake this project. Her guidance, inspiration, suggestions, promptness in responding to questions and above all, her love for precision have been very instrumental in the successful execution of this project. The efforts of my co-supervisor, Associate Professor Sigrid B. Sando (MD, PhD) is equally appreciated. Her experience as a practicing neurologist at the University Hospital in Trondheim has enabled her to diagnose and recruit the best participants for this project. Her willingness to explain her diagnostic approach and the lumber puncture procedure to me is much appreciated. Furthermore, I extend my appreciation to Ina Møller and Lisbeth Botnan for their immense support in the laboratory. I am also grateful to the entire Trønderbrain research group for creating a conducive working environment for me. Last but not the least, I sincerely appreciate the courage exhibited by all the participants (patients and controls) and their respective families for consenting to participate in the Trønderbrain project. Without them, this project would have remained a conceived idea.

May 2016

Precious Kwadzo Pomary

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
APOE	Apolipoprotein E gene
Aβ	Amyloid beta
bvFTD	Behavioural variant frontotemporal dementia
C9orf72	*Chromosome 9 open reading frame 72*
CBD	Corticobasal degeneration
DLB	Dementia with Lewy bodies
DSM	Diagnostic and statistical manual
ELISA	Enzyme linked immunosorbent assay
EOAD	Early Onset AD
FAV	Flail arm variant
FTD	Frontotemporal Dementia
FTLD	Frontotemporal Lobar Degeneration
FUS	Fused in sarcoma
GRN	Granulin gene
HC	Healthy controls
LOAD	Late onset AD
lvPPA	logopenic variant primary progressive aphasia
MMSE	Mini mental state examination

MND	Motor neuron disease
PD	Parkinson’s disease
PGRN	Progranulin
PiD	Pick’s disease
PNFA	Progressive non-fluent aphasia
PPA	Primary progressive aphasia
PSP	Progressive supranuclear palsy
P-tau	Phosphorylated tau
SD	Semantic dementia
SOD	Superoxide dismutase
TDP-43	TAR DNA-binding protein 43
T-tau	Total tau

1 Introduction and Literature Review

Neurodegeneration is a term generally used to describe the progressive loss of structure and function of neurons. This results in a number of disorders which are currently incurable. The most characterised neurodegenerative disorders are known as dementias. Dementia refers to the waning of memory and other cognitive functions relative to the patient's prior level of performance. It also encompasses an array of disease conditions that develop as a consequence of neuronal death or malfunction. The resultant effect of these are changes in the individual's memory, behaviour and thinking abilities (Alzheimer's Association, 2013). The decline in cognitive functions are established based on the individual's history, clinical examinations and neuropsychological tests (McKhann et al., 1984). In the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), dementia has been classified as a subtype of major neurocognitive disorders. According to DSM-V, a disorder can be considered as dementia if an individual experiences cognitive decline in one or more cognitive domains based on information obtained from the individual, a knowledgeable informant or a clinician. In addition, the cognitive impairments should be severe enough to interfere with the individual's activities of daily functioning. Also, there must be a progressive decline in performance on an objective neuropsychological assessment below expected levels for the individual's age (American Psychiatric Association, 2013).

Dementia can manifest in several forms with a variety of causes. Nonetheless, Alzheimer's disease (AD) is by far the leading cause of both early and late onset dementia (described in 1.2.1) (Koedam et al., 2010). Frontotemporal dementia (FTD) is the second most common form of early onset dementia (Neumann, 2013) and often coexists with amyotrophic lateral sclerosis (ALS) which is predominantly a movement disorder (Strong et al., 2009). The co-occurrence of these clinically diverse groups of diseases with partially overlapping pathology led to the idea that both FTD and ALS may be two ends of a continuum.

1.1 Are FTD and ALS two extremes of the same spectrum?

Frontotemporal dementia (FTD) is a progressive neurodegenerative disorder that occurs as a consequence of focal neuronal loss in the frontal and temporal cortices (Mohandas & Rajmohan, 2009; Seelaar et al., 2011), resulting in changes in behaviour, language and personality (Neary, et al., 2005). Memory and visuospatial functions are relatively preserved. FTD is among the leading causes of early onset dementia affecting individuals under the age of 65 (Knopman & Roberts, 2011; Ratnavalli et al., 2002; Rosso et al., 2003) but can also occur in older individuals. The first clinical description of FTD was made by Arnold Pick in 1892. Pick's description of the disease was characterised by protein inclusions known as Pick bodies found at autopsy. This histopathological hallmark of Pick's disease (PiD) was identified by Alois Alzheimer in 1911, though PiD is now considered a subtype of FTD (Kurz & Perneczky, 2009; Rossor, 2001). FTD presents with varied clinical features including extrapyramidal or atypical parkinsonian syndromes. Hence, it is generally regarded as a heterogeneous disorder.

1.1.1 Heterogeneous clinical manifestations of FTD and its overlap with ALS impede diagnosis.

The heterogeneous nature of clinical FTD is clearly evident in the plethora of clinical features it exhibits. The core subtypes of FTD are classified into two broad categories based on the cognitive domains affected in the individuals. These categories are behavioural and language variants. FTD can also manifest in the form of motor dysfunctions or in conjunction with motor neuron disease (MND)/ALS (Irwin et al., 2015).

The behavioural variant of FTD (bvFTD) is primarily characterised by altered personal regulation and social decorum. Such changes have been grouped into positive behaviours and negative behaviours. The positive behaviours are those that are related to disinhibition. These include aggression, irritability, hyperorality, perseveration, excessive jocularity, irresponsibility, unpredictability, inappropriate remarks, restlessness, impulsivity, incontinence and hyper-sexuality. On the other hand, the negative behaviours consist of apathy, rigid thinking, personal neglect, indifference, lack of insight, inattention, distractibility and aspontaneity (Pąchalska et al., 2011). Binge eating, which is one of the manifestations of hyperorality results in weight gain in some patients. Also, due to reduced insight, patients are not usually aware of the changes reported by their knowledgeable informants. Although

visuospatial skills and memory are relatively preserved at the initial stages of bvFTD, the behavioural changes that occur lead to a decline in executive function and the patients find it difficult to plan and generate new ideas (Bang et al, 2015). Some individuals may also develop language problems at later stages of the disease.

The language variants of FTD which is generally referred to as primary progressive aphasia (PPA) usually manifest in the form of difficulty in understanding words, difficulty finding words, and in the worse form of the disorder some individuals eventually become mute. On the basis of the specific aspect of language that has been affected, the language variant has been subdivided into nonfluent/agrammatic variant PPA (nfvPPA), also referred to as progressive non-fluent aphasia (PNFA), semantic dementia (SD) and logopenic variant PPA (lvPPA) (Pan & Chen, 2013).

Progressive non-fluent aphasia usually has an insidious onset and begins with reduced speech fluency. This is accompanied by reduced word output, shortened phrases and a decline in articulation. The decline in articulatory planning makes patients unable to generate correct sentences. There is also a difficulty in understanding grammar and the use of verbs is also impaired. However, the use of nouns is preserved (Gorno-Tempini et al., 2011). Although some patients may develop bvFTD as the disease progresses, most patients have a preserved social decorum. Unlike bvFTD, these patients often recognise their deficits before others do.

Patients with semantic dementia on the other hand, exhibit word finding problems, especially nouns while fluency of speech is preserved. In addition, patients find it difficult to name and recognise words (anomia). As the disease progresses, the patients become unable to recognise the emotions of others. Prosopagnosia (inability to recognise faces) may eventually develop (Seeley et al., 2005).

The third, yet less common variant of PPA, lvPPA is usually associated with the inability to repeat phrases, sentences and to generate spontaneous speech. However, this variant of FTD, unlike other subtypes is not usually associated with behavioural changes except at the terminal stages in some cases (Gorno-Tempini et al., 2011). Its neuropathological features resemble that of AD (Rabinovici et al., 2008).

The majority of FTD patients are diagnosed with bvFTD. Moreover, as the disease progresses, symptoms may overlap and motor dysfunction or parkinsonism may develop in some patients. This is an indication that the different variants do not normally exist as distinct entities in individual patients. They often occur in a mixed form, sometimes overlapping with other disorders such as corticobasal degeneration (CBD), progressive supranuclear palsy (PSP) (Boeve et al.,2003) and amyotrophic lateral sclerosis (ALS) (Murphy et al., 2007). CBD presents in the form of asymmetric parkinsonism with rigidity, limb apraxia, dystonia and sometimes alien limb syndrome (Rebeiz et al., 1968). PSP is also a movement disorder which is often associated with falls, axial rigidity and opthalmoplegia (Steele et al., 1964). Studies have shown that most PPA patients show CBD or PSP pathology at autopsy (Kertesz et al., 2011).

ALS is the commonest presentation of MND. It is a fatal disorder associated with a global loss of motor neurons (upper and lower motor neurons) together with the corticospinal axonal tract which leads to paralysis and subsequent death, usually in less than 5 years (Mitchell & Borasio, 2007). ALS is also heterogeneous in nature. It has three classical clinical presentations namely: bulbar onset ALS, classic limb onset (Charcot) and progressive muscular atrophy (PMA). Other phenotypically distinct subtypes often considered atypical presentations of ALS are referred to as flail leg (FL) and flail arm variants (FAV) of ALS. Patients with these atypical presentations of ALS have a higher survival chance compared to those with the classical presentations so long as the disease is restricted to the legs or arms. (Wijesekera et al., 2009).

Although ALS has generally been considered as a movement disorder, clinical data indicate that approximately 50% of ALS patients show some cognitive deficits similar to FTD and about 30% of FTD patients demonstrate clinical features of ALS (Lomen-Hoerth, Anderson, & Miller, 2002). When FTD occurs with ALS, the patient's limbs become weak and the muscles waste. Death typically occurs as a result of respiratory complications. These intertwined clinical manifestations of the various variants of FTD together with extrapyramidal syndromes and ALS serve as barriers to the clinical distinction of these disorders.

1.1.2 Clinical diagnosis of FTD is a diagnosis of exclusion

The clinical diagnosis of FTD is made by first ruling out other possible explanations for the observed symptoms. In order to successfully diagnose FTD based on the classical clinical features, the actual changes in the patient's interpersonal conduct relative to his or her

premorbid behavioural states is highly relevant. This is because such changes span across a broad spectrum from inactivity and inertia to highly active antisocial and disinhibited sexual, physical and verbal behaviours. Individual patients can exhibit any combination of characters anywhere on this broad spectrum (Neary et al., 1998). Moreover, the deficiency in the sensitivity of the usual dementia tests in identifying the non-cognitive components of FTD makes both the clinical diagnosis of FTD and the ability to distinguish FTD from non-organic psychiatric disorders difficult (Greck, et al., 2000). Also, clinical differences do not always translate into aetiological differences. All these often lead to misdiagnoses of FTD as other forms of neurological disorders, especially AD (Snowden et al., 2002).

Taking into consideration the above arguments regarding the misdiagnosis of FTD due to overlapping clinical features both within subclasses of FTD and between FTD and other disorders, it is obvious that a good knowledge of just the clinical features of FTD alone is not sufficient for a definitive diagnosis of the disease. Nonetheless, a high fidelity diagnosis can be achieved when the clinical features are combined with measurable pathophysiological entities.

1.1.3 Clinical features of some variants of FTD might not predict underlying pathophysiology

The heterogeneity of FTD is not limited to only its clinical manifestations. Frontotemporal lobar degeneration (FTLD), a term used to describe the neuropathological aspects of FTD, also exhibits heterogeneity. FTLD describes the pathological and genetic aspects of a set of disorders predominantly characterised by selective degeneration of the frontal and temporal lobes (Neary et al., 1998). Immunohistochemical analysis of brain tissues from FTD patients has revealed five neuropathological subgroups. These subgroups have been created based on the composition of the abnormal intracranial protein inclusions found at autopsy. At present, FTLD is divided into the following major categories, namely; FTLD-tau (when the inclusion bodies contain microtubule-binding protein tau) and FTLD-TDP (when the inclusion bodies consist of transactive response (TAR) DNA-binding protein 43 (TDP-43)). The third category encompasses FTLD subtypes that are negative for tau and TDP-43. This category is further divided into two ubiquitin positive subgroups; FTLD-FUS (*fused in sarcoma* protein) and FTLD-UPS (ubiquitin proteasome system) in addition to a ubiquitin negative group; FTLD-NI (no inclusion) (Lashley et al., 2015; Mackenzie et al., 2010). This classification scheme, which is

based on the current understanding of the disease pathology, has been summarised in Figure 1.1. A better understanding of FTLD mechanisms in the future will probably lead to the generation of a new classification scheme that will reflect contemporary research findings.

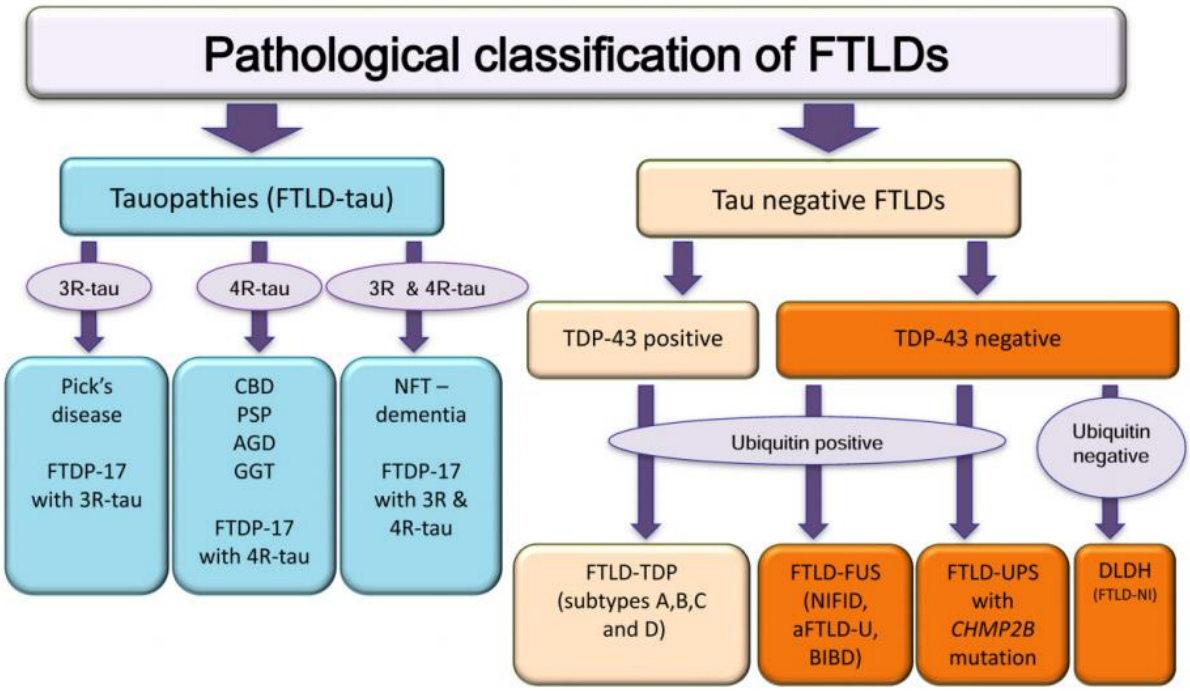


Figure 1.1. A schematic representation of the pathological classification of frontotemporal lobar degeneration. Adapted from (Lashley et al., 2015)

1.1.3.1 FTLD-tau

The tau protein in its unfolded state is a significant component of the microtubule assembly and stabilization machinery. It also plays a vital role in axonal transport. However, the misfolded version of the tau protein has been implicated in the majority of neurodegenerative disorders including FTD (Williams, 2006). Such heterogeneous neurodegenerative disease groups that are characterised by the accumulation of abnormal tau proteins in the brain are commonly referred to as tauopathies.

Pick's disease (PiD) is a form of tauopathy and characterised by Pick bodies as its pathological hallmark. Pick bodies consist of spherical agryophilic inclusions in neurons of the frontal lobes. This inclusion bodies have been shown to contain phosphorylated microtubule associated protein tau MAPT (Murayama et al., 1990). In addition to PiD, PSP, CBD and FTD with parkinsonism linked to chromosome 17 (FTDP-17) also exhibit tauopathies (Mackenzie et al.,

2010). Six different isoforms of tau can be found in the brain. They form two major groups, each consisting of three isoforms. Depending on the number of sequence repeats in the isoform groups, they are either referred to as 3-repeat-tau (3R) or 4-repeat-tau (4R-tau). Tau pathology is also subdivided on the basis of the predominant tau species present in the inclusion. The predominant species in PiD and FTDP-17 inclusion is the 3R-tau group. CBD, PSP and some FTDP-17 inclusions are dominated by 4R-tau while another subset of FTDP-17 together with neurofibrillary tangle (NFT) dementia contain both 3R and 4R-tau in their inclusions. The tau inclusions can be located in both neuronal and non-neuronal cells (Kovacs, 2015).

1.1.3.2 FTLD-TDP shares a common pathology with ALS

The 43 kDa protein TDP-43 is initially located in the nucleus. Its major function is to facilitate protein synthesis through its role in transcription and translation. However, transformations in TDP-43 cause the protein to misfold. The translocation of the misfolded protein to the cytoplasm is believed to result in the pathogenesis of the majority of FTLD-TDP (Hu & Grossman, 2009). FTLD-TDP is further divided according to the location of the inclusions in individual cells, in addition to brain area. On these grounds there are FTLD-TDP types A, B, C and D. Although FTD and ALS affect different parts of the nervous system and exhibit different symptoms, they have been considered to be two extremes of the same disease spectrum with overlaps in their clinical, genetic and neuropathological features. A neuropathological overlap is evident in the identification of TDP-43 neuronal inclusions in the majority of FTD and ALS patients (Davidson et al., 2007; Mackenzie, Rademakers, & Neumann, 2010; Neumann et al., 2006). Figure 1.2 shows the extent of overlap between FTD and ALS pathology. One of the proposed mechanisms for FTD/ALS with TDP-43 inclusions is that mutations in *TARDBP,* granulin *(GRN),* chromosome 9 open reading frame 72 (*C9orf72),* or valocin-containing protien *(VCP)* genes result in the transloction of pathologic TDP-43 into the cytoplasm. This causes RNA dysfunction and abnormal translation. The end result is neurodegeneration and muscle denervation (Irwin et al., 2015). This mechanism is illustrated in Figure 1.3

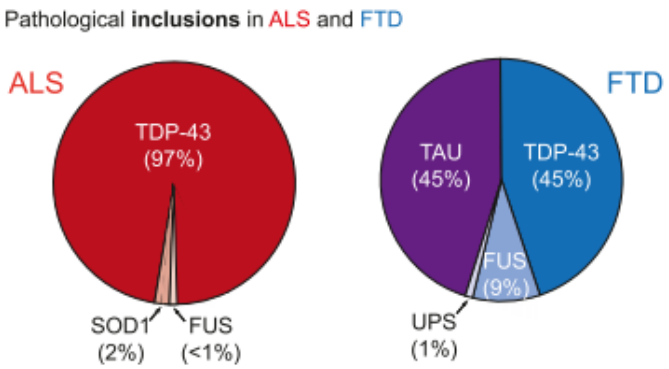


Figure 1.2. An illustration of the extent of pathological overlap between FTD and ALS modified from (Ling et al., 2013)

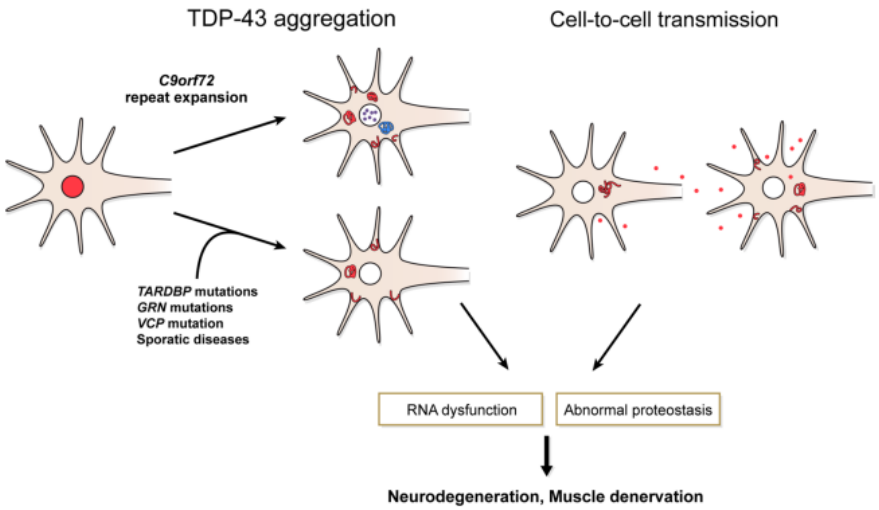


Figure 1.3. Mechanism for TDP-43 mediated FTLD-TDP/ALS. Adapted from (Irwin et al., 2015)

1.1.3.3 Tau and TDP-34 negative subgroups (FTLD-FUS, FTLD-UPS, FTLD-ni)

The majority of the tau negative and TDP-43 negative forms of FTLD are associated with fused in sarcoma (FUS) protein. However, the inclusions found in FTLD-UPS are negative for tau, TDP-43 and also FUS (Urwin et al., 2010). Yet there is another subgroup that has no identifiable inclusion bodies and it is designated FTLD-ni (Lashley et al., 2015). The relationship between the various clinical manifestations of FTD and the relationship with movement disorders and the associated neuropathology are summarised in Figure 1.4.

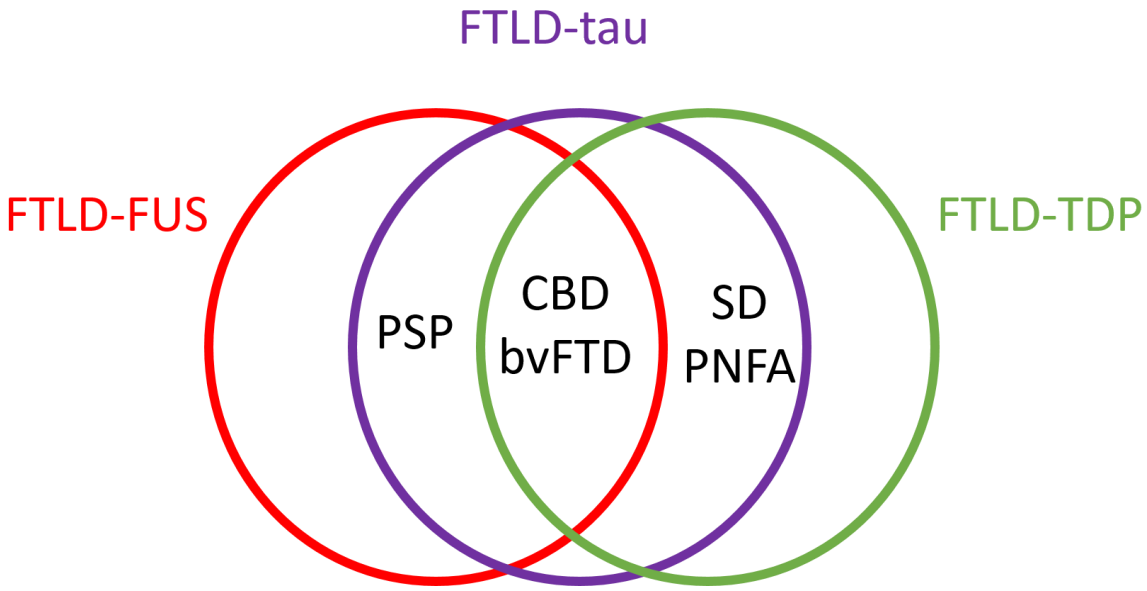


Figure 1.4 Summary of the relationship between clinical manifestations of FTD and the underlying pathology.

From Figure 1.4, it can be observed that tau and TDP-43 pathology can be found in almost all forms of clinical FTD. In most instances, the clinical syndromes of FTD do not necessarily reflect specific histological subtypes. Hence, it is almost impossible to predict neuropathological subtypes on the bases of clinical features alone due to the intertwined relationship between the clinical syndromes and neuropathology. Furthermore, since some forms of FTD tend to run in families, it is reasonable to consider the genetic mutations underlying some of the pathologies. Some of these mutations have been identified and are discussed in the next section.

1.1.4 Genetic mutations associated with FTD and ALS

The genetic study of familial forms of neurodegenerative diseases can throw more light on the underlying pathogenic mechanisms. It has been found that heritable forms of FTD constitute approximately 30 to 50 percent of all cases of frontotemporal dementia (Rohrer et al., 2009). The majority of such cases are usually associated with three main gene mutations, namely; *MAPT*, *GRN* and *C9orf72* (Rohrer et al., 2009; Rohrer et al., 2015). Other genetic causes of FTD are linked to mutations in the genes for charged multivesicular body protein 2B (*CHMP2B*), ubiquilin 2 (*UBQLN2*) and *VCP* (Ferrari et al., 2001; Ling et al., 2013). Between 13-25% of familial cases of FTD are accounted for by *GRN* mutations. These mutations can cause up to

50% reduction in the GRN transcript. Mutations in *C9orf72, TDP* and *FUS* also been identified in ALS cases. In addition, superoxide dismutase (SOD1) mutations are also associated with ALS but not FTD. These mutations together contribute to approximately 50% of familial ALS cases (Ling et al., 2013). Insertion/deletion mutations of TARDBP have also been found in FAV-ALS (Solski et al., 2012). Figure 1.5 shows the genetic overlap between FTD and ALS and also suggests that both disorders are part of a continuous spectrum.

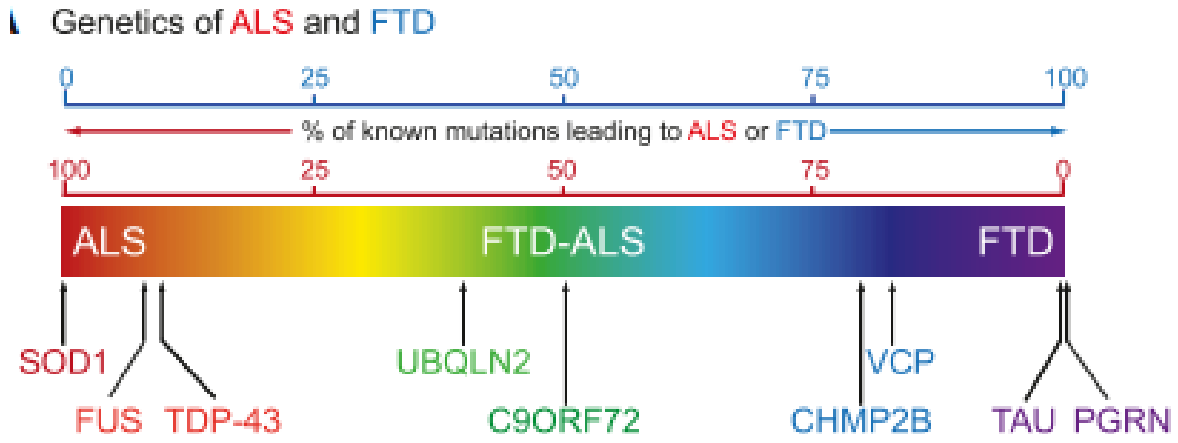


Figure 1.5. The overlap between the genetic aspects of FTD and ALS. Modified from (Ling, et al. 2013)

1.2 Alzheimer's Disease (AD)

Alzheimer's disease is a devastating neurodegenerative disease that affects millions of people globally. It has an insidious onset and progressively causes memory dysfunction and cognitive impairment. At the early stages of the disease, sensory, motor or coordination are relatively intact. AD is the leading cause of dementia and the number of individuals affected is expected to double by 2030 and triple by 2050. This projected increase is attributed to the current increase in life expectancy worldwide (Prince et al., 2013).

1.2.1 Clinical Aspects of AD (Early onset AD vs Late onset AD)

Unlike FTD, AD is a more homogenous disease with respect to its clinical presentation in that the majority of AD cases are amnestic in nature. The prototypical AD described by Alois Alzheimer in 1906 has maintained its distinct clinical characteristics as being an amnestic syndrome of the hippocampal type. The underlying pathology originates from the hippocampus and medial temporal lobe. This makes memory tests important clinical predictors of AD in most patients. Although age is the main risk factor for AD, there are other risk factors. Most of them are considered to be environmental, including traumatic brain injury (Plassman & Grafman, 2015), cardiovascular diseases (Wiesmann et al., 2013), low cognitive reserve (Bauckneht et al., 2015) and level of education (Sando et al., 2008a). Genetics can also play an important role.

While AD is predominantly a disease of the elderly, there is an appreciable number of cases that occur in younger individuals. When AD occurs in an individual below the age of 65, it is considered as early onset AD (EOAD) and those that start above this arbitrary cut-off age are regarded as late onset AD (LOAD) (Harvey et al., 2003; Lobo et al., 2000). This cut-off age for dementias which has no particular biological significance was probably chosen to reflect the social division between the working and the retired population (Rossor et al, 2010). Although both EOAD and LOAD are associated with similar clinical features, it has been observed in a large retrospective cohort study that patients with EOAD tend to exhibit non-memory cognitive deficits five times more than LOAD patients. The non-memory cognitive deficit is mainly in the form of visuospatial dysfunction or apraxia and language impairment in the presence of relatively intact executive function and vision in most of the cases (Koedam et al., 2010). This observation could be a possible explanation for the frequent misdiagnosis of EOAD

and FTD in the clinical setting, since both occur in younger individuals and often exhibit similar clinical features.

1.2.2 Diagnostic criteria for AD

In 1984, diagnostic criteria for AD were developed by the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) workgroup. This was done for the purpose of standardising diagnosis by clinicians and for research. Under these criteria, AD was regarded as a clinical-pathological entity. Hence, a definitive clinical diagnosis of AD could not be made in the absence of post-mortem proof. This shortfall in the criteria restricted the diagnosis of AD in the clinical setting to probable AD. However, this can only be made when a patient reaches the threshold of dementia (McKhann et al., 1984). A possible setback with these diagnostic criteria is that some individuals who are actually suffering from AD might not receive a diagnosis during the prodromal phase. On the other hand, some individuals who might be suffering from non-Alzheimer's dementia might also be wrongly diagnosed with AD. These two scenarios highlight both the insensitive and nonspecific nature of the 1984 diagnostic criteria. These limitations have resulted in misdiagnosed patients being used in some clinical trials, and prompted the development of revised diagnostic criteria which take into account early diagnosis. The revised criteria have yet to be officially accepted.

The revised diagnostic criteria for AD introduce the use of biomarkers, currently only for research purposes. These criteria encompass the entire spectrum of the disease process, ranging from preclinical AD to dementia due to AD in addition to atypical presentations and mixed AD (Dubois et al., 2014). It is now possible to biologically study potential AD development in cognitively normal patients with altered biomarker levels, since these criteria include biomarkers believed to reflect the underlying pathology of AD.

1.2.3 AD Pathophysiology is a reflection of two major lesions

The pathological features of Alzheimer's disease are considered to be the main hallmarks of the disease: the extracellular deposition of neuritic plaques and intracellular neurofibrillary tangles (NFT) (Figure 1.6) (Blennow et al., 2006; Tomlinson et al., 1970). The protein culprits are amyloid beta (Aβ) and hyperphosphorylated tau (Ptau) respectively (Blennow et al., 2006).

AD neuropathology normally affects the medial temporal lobe during the initial stages and progressively engulfs the neocortical-associated areas at later stages (Blennow et al., 2006; de Leon et al., 1993) resulting in atrophy in these regions (Figure 1.7). Neuropathological changes tend to occur several years or decades before recognisable clinical symptoms appear (Hulette et al., 1998; Price & Morris, 1999).

Several hypotheses have been propounded to explain the mechanism behind AD pathogenesis, the first of which is the cholinergic hypothesis. This hypothesis is based on the premise that cholinergic innervations from the basal forebrain to the cerebral cortex are selectively lost in AD brains (Coyle et al., 1983). This hypothesis paved the way for the development of cholinesterase inhibitors which are still used to for symptomatic treatment. Two other hypotheses were later proposed based on the two major pathological hallmarks of AD; the amyloid hypothesis and the tau hypothesis.

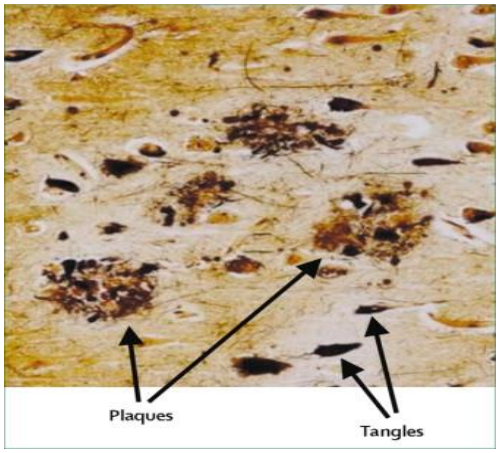


Figure 1.6. Extracellular plaques and intracellular tangles in the cortex forms the pathological hallmarks of AD (Blennow, et al., 2006)

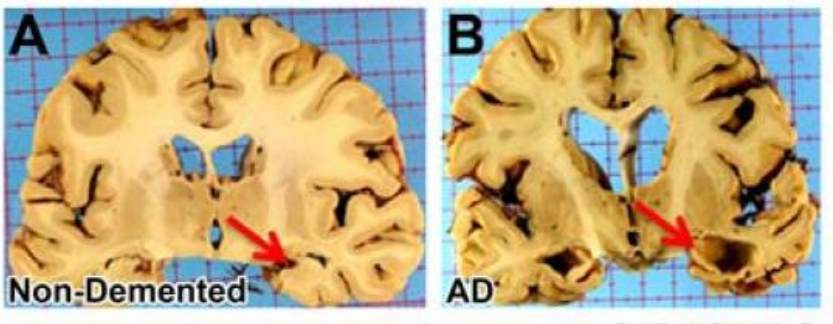


Figure 1.7. Comparison of demented and non-demented brains. Red arrows indicate normal and atrophied hippocampal formation in non-demented (A) and AD (B) brains respectively. Modified from (Niedowicz, et al., 2011)

According to the Aβ hypothesis, the accumulation of Aβ is the main causative agent for AD pathology. Other pathological features such as neurofibrillary tangles, vascular damage, and neuronal loss are secondary to Aβ deposition (Hardy & Higgins, 1992). The fulcrum of the Aβ hypothesis is that the deposition of Aβ in the brain is a vital step that triggers a cascade of pathophysiological events which eventually leads to AD (Karran et al., 2011). Aβ accumulation in the brain is currently attributed to an imbalance between the rate of clearance of the deposited Aβ and the rate of production of Aβ (Tarasoff-Conway et al., 2015). Considering the fact that Aβ pathology occurs very early in the disease process, it is likely that its accumulation serves as a precursor or an actuator for other pathologies such as tau accumulation. Hence, Aβ accumulation may be necessary but not sufficient on its own to cause AD (Musiek & Holtzman, 2015). The failure of this hypothesis to explain how Aβ deposition causes neuronal death led to its modification into the amyloid cascade-inflammatory hypothesis. The reasoning behind this proposition is that AD occurs as a consequence of the inflammation induced by the extracellular deposition of amyloid beta. This microglial-driven inflammation is further enhanced by tau aggregates (McGeer & McGeer, 2013). This idea gains some support from research that shows that non-steroidal anti-inflammatory drugs provide some amount of resilience against the development of AD (Zandi et al., 2002). Besides, a review by Herrup indicates that dementia due to AD persists even after amyloid plaques have been cleared from the brain during vaccine trials which target plaques. This has inspired reconsideration of the amyloid beta hypothesis in tandem with other hypotheses to improve future AD research (Herrup, 2015).

Contrary to Aβ deposition which occurs much earlier and probably starts more than decades before AD symptoms appear, abnormal tau accumulation usually occurs much later and coincides with cognitive decline. This forms the foundation for the tau hypothesis. The amount of NFT accumulated in the brain tends to correlate with the severity of dementia due to AD (Ghoshal et al., 2002). Also, the use of drugs targeted at tau have also been demonstrated to relieve cognitive decline in AD patients (Duff, Kuret, & Congdon, 2010). In this regard, the tau hypothesis seems to be superior to the amyloid hypothesis.

1.2.4 Genetic aspects of AD

1.2.4.1 APOE ε4 polymorphism is the greatest genetic risk factor for AD

APOE is the genetic blueprint for the chaperone protein apolipoprotein E. Three major alleles of this gene exist, namely ε2, ε3 and ε4. The ε3 allele is the most common and is found in more than half of the general population (Zannis & Breslow, 1981). The epsilon 4 allele of the apolipoprotein E gene (*APOE ε4*) is the major genetic risk factor for AD. Its effect is said to be dose dependent. This implies that the risk of AD increases with the number of copies inherited (Corder et al., 1993). *APOE ε4* has been demonstrated to increase the risk of AD by lowering the age of onset (Sando et al., 2008b; Saunders et al., 1993). Although it is the most important risk factor, the inheritance of this allele alone is not sufficient to cause AD since not all AD patients possess *APOE ε4* and not all individuals who have inherited *APOE ε4* develop AD (Myers et al., 1996). The reason for the increase in AD prevalence among carriers of *APOE ε4* is still debated but it is possibly related to the chaperone ability to clear deposited Aβ. The *APOE ε4* allele has been shown to have less ability in this respect, while the *APOE ε2* allele is most efficient. Carriers of the *APOE ε2* allele have some limited protection against AD (Berge et al., 2014).

1.2.4.2 Mutations are responsible for familial AD

The most numerous mutations associated with familial AD are found in the presenilin-1 gene. Presenilin is one of the subunits of γ-secretase, a catalytic component of the amyloid precursor protein (APP) cleavage machinery. The activity of γ-secretase results in the production of varied lengths of Aβ peptide (De Strooper et al., 2012). When the presenilin gene is mutated, it is believed to increase the activity of β-secretase. The cleavage of APP by β-secretase, followed by γ-secretase results in the production of Aβ peptides. Some of these peptides may aggregate and eventually form plaques. The mechanism for cleavage of APP to produce Aβ peptides and its subsequent accumulation has been illustrated in Figure 1.8. Mutations in the presenilin-2 and APP genes can also lead to familial AD (Ryan & Rossor, 2010). However, these are even less common.

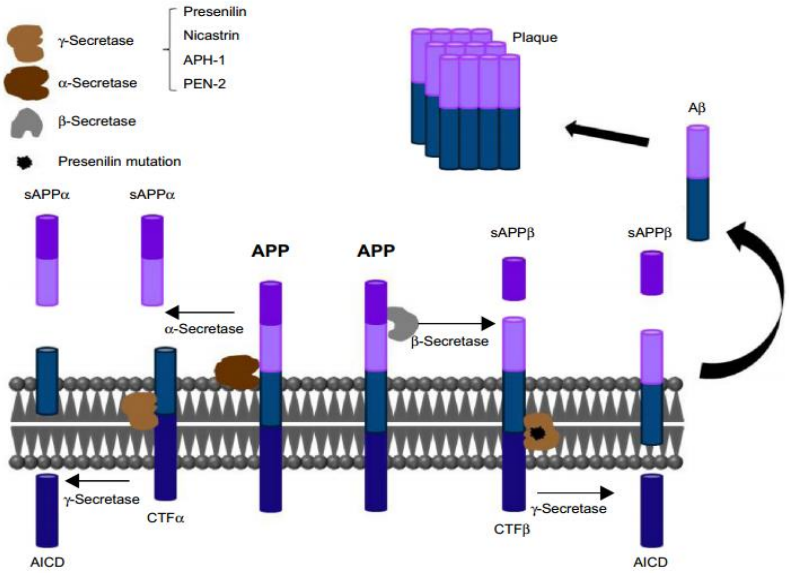


Figure 1.8. The accumulation of Aβ as a result of a presenilin mutation. Adapted from (Cai, An et al. 2015)

1.3 Biomarkers are useful for the diagnosis of neurodegenerative diseases

A biomarker is a measureable entity that reflects a normal physiological state, a disease state or the efficacy of a disease modifying drug. Biomarkers define pathology during life in a minimally invasive fashion. They are usually useful for prognosis, understanding the disease, identifying candidates for disease-modifying treatments and also defining endpoints in treatment trials. Different methods have been applied in identification and quantification of biomarkers that are indicative of neurodegeneration. These include neuroimaging, protein quantification in body fluids, and in some instances genetic analysis.

1.3.1 Neuroimaging-based biomarkers are indicators of brain metabolic rate and anatomical localisation of disease pathology.

Neuroimaging techniques are used for determining the anatomical localisation and quantification of pathological lesions in the brain. Some are also used to determine the rate of metabolic activities in a particular brain region. Structural and volumetric magnetic resonance imaging (MRI) are used to quantify brain atrophy by measuring the size of specific brain areas. This serves as a marker for neurodegeneration. In the case of FTD, MRI enables the detection of frontal and temporal lobe atrophy. In AD, medial temporal lobe atrophy is a major indicator of the disease. These measurements can distinguish AD from FTD patients (Fukui & Kertesz, 2000).

Brain metabolism and perfusion can be identified by using 18-fluorodeoxyglucose positron emission tomography (FDG PET) or single photon emission tomography (SPECT) scans respectively. For instance AD brains show hypometabolism in the temporo-parietal and cingulate cortices while FTD brains show reduced metabolic activity in the temporal and frontal regions on PET scans (Davison & O'Brien, 2014). The deposition of Aβ in AD brains can also be identified with amyloid-PET imaging. Amyloid PET involves the injection of a radioactively labelled tracer drug that selectively binds to amyloid plaques. It is useful for distinguishing AD from FTLD since it measures Aβ accumulation, which is found in AD brains but absent in FTLD brains. Nonetheless, the observation of Aβ accumulation in the brain is not specific for AD since similar pathological levels have been observed in some cognitively normal individuals (Pike et al., 2007).

While the changes observed during neuroimaging for AD reflect to some extent the underlying pathophysiology, imaging for FTD does not specify the underlying FTLD pathologies (Moodley et al., 2015).

1.3.2 CSF-based biomarkers can predict neurodegeneration.

Body fluid-based biomarkers are usually proteins whose measurable values either increase or decrease in response to physiological or pathological activities. They are often obtained from fluids such as blood (serum or plasma) and cerebrospinal fluid (CSF). CSF is a clear transparent fluid produced in the choroid plexus by ependymal cells. It provides buoyancy and also bathes the brain. Its proximity with the brain means that it is more likely to have a biochemical signature of the brain's metabolic activities. Although CSF is not as easily obtainable as serum and plasma, it remains the body fluid of choice for prospecting potential disease specific biomarkers due to its close association with brain tissues. Several studies have consistently shown that changes in the concentrations of certain substances in CSF correlate with neurodegenerative disorders.

1.3.2.1 Core CSF biomarkers distinguish AD from controls and other neurodegenerative disorders.

In order to serve as a CSF biomarker for a neurodegenerative disorder, the measureable substance should reflect the central pathologic processes that are associated with the disorder. Generally, changes in the CSF concentrations of such substances usually signify synaptic degradation, neuronal death or neuroinflammation. In the case of AD, the pathological processes include; synaptic loss and axonal degradation in addition to the accumulation of amyloid beta (Aβ), and hyperphosphorylated/ubiquitinated forms of tau proteins in the brain (Blennow, 2004). It is however not surprising that total tau (T-tau), phosphorylated tau (P-tau) and Aβ-42 are the most studied biomarkers for AD. Their concentrations and ratios in CSF have usually shown consistency in differentiating patients from controls and other neurodegenerative disorders. For instance, patients with AD exhibit low CSF concentrations of Aβ-42 and high P-tau and T-tau concentrations compared to cognitively normal individuals (Blennow, 2004). Also, the ratio of Aβ-42 to Aβ40 has been demonstrated to decrease in patients with mild cognitive impairment (MCI) who later developed AD as compared to stable MCI individuals. This makes the Aβ-42/Aβ-40 ratio a

better predictive value than the raw concentration of Aβ-42 or Aβ-40 (Hansson et al., 2007; Sauvee et al., 2014). Other ratios such as T-tau/Aβ-42, T-tau/Aβ-40 and P-tau/Aβ-42 have also been shown to increase in AD patients compared to controls and patients with other dementia syndromes (Seeburger et al., 2015). Moreover, Seeburger and colleagues further suggested that it is possible to have single baseline cut-off concentrations for CSF Aβ and tau for well characterised and homogeneous populations which can permit the diagnosis of AD with high specificity and sensitivity (Seeburger et al., 2015). In reality, this may not be applicable in the clinical setting where physicians are presented with heterogeneous patient populations. A universal cut-off concentration for these biomarkers is therefore desirable.

1.3.2.2 Other CSF metabolites also have the potential to distinguish neurodegenerative disorders and controls.

In addition to the core biomarkers of AD, other CSF-based metabolites have also been studied in relation to neurodegeneration. These substances include but are not limited to, other Aβ species like Aβ-43, neurofilament light, progranulin, YKL-40 and alpha synuclein.

CSF Aβ-43 has been identified to be of potential diagnostic importance. However, the CSF levels of Aβ-43 and its combination with both total and phosphorylated tau have been found to exhibit diagnostic patterns similar to Aβ-42 (Bruggink et al., 2013). Nonetheless, Aβ-43 and its tau T-tau ratio have been shown to be better at distinguishing progressive MCI from stable MCI patients compared to Aβ-42 (Lauridsen et al., 2016).

Neurofilament light is an important neuronal component that has been studied in relation to neurodegeneration. It is a structural constituent of the neurofilaments of the neuronal cytoskeleton. Filaments contain several subunits, the main ones being termed; neurofilament light (NF-L), neurofilament medium (NF-M) and neurofilament heavy (NF-H) (Lee et al., 1993). Studies have shown that an increase in the concentration of CSF NFL is associated with certain neurodegenerative disorders including AD, FTD and ALS. For instance, at the onset of AD, CSF NFL levels tend to increase, correlating with changes in brain structure and cognitive decline, and reflecting the degradation of large myelinated axons implicated in AD neurodegeneration (Zetterberg et al., 2015). Scherling and colleagues also demonstrated that increased CSF concentrations of NFL also correlate with disease severity in FTD (Scherling et al., 2014).

Structural damage to the nervous system associated with ALS is also reflected in an increased level of NFL in CSF (Weydt et al., 2016).

Another protein of interest associated with neurodegeneration is progranulin (PGRN). PGRN is a protein that regulates the outgrowth and survival of neurites and is expressed in both neurons and microglia (Van Damme et al., 2008). Mutations in the *granulin* gene (*GRN*) have been ascribed to FTLD-TDP and ALS. *GRN* mutations result in altered concentrations of PGRN in the body fluids of mutation carriers. Therefore, its measured values may be capable of differentiating between asymptomatic and symptomatic carriers of the mutated forms of *GRN* (Finch et al., 2009). However, this capability may not be applicable to dementias that lack *GRN* mutations (Morenas-Rodriguez et al., 2015). PGRN is also believed to influence inflammatory proteins such as YKL-40 due to its involvement in inflammatory processes (Alcolea et al., 2015).

YKL-40 (also known as chitinase-3 like-1, cartilage glycoprotein), is a 40 kDa glycoprotein that is expressed in a variety of cells. It has an amino acid sequence which is homologous to fungal and bacterial chitinase. Nevertheless, it lacks chitinase activity. Although the complete biological function of YKL-40 is not well understood, its pattern of expression has been implicated in pathological inflammatory processes associated with a variety of diseases (Kazakova & Sarafian, 2009). Interestingly, elevated levels of YKL-40 have been reported to be an indicator of neuroinflammation associated with AD. It has also been observed that YKL-40 increases in the CSF of FTD patients. Furthermore, when combined with Aβ-42, YKL-40 may serve as a prognostic biomarker for preclinical AD. For instance, the risk of developing mild cognitive impairment might be predicted by the YKL-40/ Aβ-42 ratio (Craig-Schapiro et al., 2010). However, the diagnostic accuracy of YKL-40 has been found to be below that of the CSF core biomarkers for AD (Janelidze et al., 2016).

Alpha synuclein is generally associated with Parkinson's disease and dementia with Lewy bodies. In such disorders, it is deposited in the brain as Lewy body inclusions. However, it is also regarded as a marker for healthy aging since its plasma concentration tends to decrease between the third and fifth decades of life (Koehler et al., 2015). It was recently demonstrated that a combination of CSF measurements of alpha synuclein and ubiquitin carboxyl-terminal

hydrolase L1 (UCH-L1) may aid differential diagnosis of parkinsonian syndromes (Mondello et al., 2014).

1.4 Diagnostic bottlenecks associated with FTD, ALS and AD

Just like other forms of dementia, AD and FTD are not usually definitively diagnosed in living patients but only at autopsy. The diagnosis in a living individual is usually regarded as probable. The difficulties associated with such diagnosis could be within the context of the same disorder such as distinguishing various variants of FTD from each other. In the case of AD, the asymptomatic stages are difficult to detect. Conversely, it could also be between different disorders such as distinguishing FTD from AD or FTD+ALS. This situation is exemplified in a review by Irwin and colleagues, which indicated that some studies in which the participants were clinically confirmed FTD cohorts at the initial stages later produced 30% AD diagnoses at autopsy (Irwin et al., 2013). This diagnostic uncertainty is partially due to the absence of standard laboratory tests for the diagnosis of most dementias and the subjective nature of neurological tests used in the clinical environment. Also, there are levels of overlap between the symptoms and pathology associated with these disorders. An example of pathological overlap is the disease spectrum that spans from FTD through FTD+ALS to ALS. The overlaps also extend to the genetic causes of these disorders. A typical example is the fact that presenilin mutations which are regarded as one of the most common causes of familial AD have also been observed as an underlying cause of some clinical phenotypes of familial FTD (Bernardi et al., 2009) . Furthermore, the overlap between the levels of major biomarkers for AD (CSF tau and Aβ) and other forms of dementia sometimes become a limiting factor hindering the use of such biomarker values for differential diagnosis (Bibl et al., 2011).

While levels of core CSF biomarkers for AD such Aβ, total tau, phosphorylated tau, and their corresponding ratios can be useful in the diagnosis of AD, none of them has so far been proven to aid early diagnosis and prognosis. In contrast, other forms of dementia such as FTD, lack such specific biomarkers comparable to the core biomarkers of AD (Bibl et al., 2011). In addition, most drugs used in clinical trials have failed to have any effect on the progression of AD and other neurodegenerative disorders. This has been attributed to late diagnosis and in some instances, misdiagnosis of these disorders. The culminating effect is the inability of the patients to benefit from potential disease-modifying drugs. This phenomenon underscores

the need for well-validated biomarkers for accurate and early diagnosis of AD (Blennow et al., 2015).

2 Aims, Hypothesis and Objectives

2.1 Aims

There is a need to research the potential of CSF biomarkers which will assist early diagnosis, prognosis and differentiation between AD, FTD and ALS. It is therefore prudent to re-examine the core biomarkers vis-à-vis novel molecules for these diseases as we strive to obtain sensitive and disease-specific diagnostic tools. In this regard, this research is aimed at determining whether the CSF concentrations of the core biomarkers together with Aβ-40, Aβ43, NF-L, α-synuclein, PGRN and YKL40 show differences between the clinical syndromes of FTD, ALS or AD.

2.2 Hypotheses:

- There may be an association between the concentrations of Aβ-40, Aβ43, NF-L, α-synuclein, PGRN, YKL40 and the core AD biomarkers in the CSF of patients with the clinical syndromes of FTD, ALS or AD.
- The concentrations of Aβ-40, Aβ43, NF-L, α-synuclein, PGRN, YKL40 and the core AD biomarkers in the CSF of patients with FTD, ALS or AD may assist differential diagnosis.

2.3 Objectives:

Based on the aims of this study, the main objectives were to:

- analyse CSF samples with sandwich ELISA to obtain the concentrations of the core biomarkers for AD in addition to Aβ-40, Aβ43, NF-L, α-synuclein, PGRN and YKL40 in healthy controls and patient groups.
- statistically compare the concentrations of the biomarkers in order to evaluate their suitability for differentiating healthy controls, AD and patients with FTD or ALS.

3. Materials and Methods

3.1. Study Subjects (Patients and Controls)

All materials relating to the study subjects were obtained by clinicians. The study subjects and controls were selected from the participants of the Trønderbrain project. The Trønderbrain project which was started in 2003 seeks to explore the link between genetic inheritance and risk of developing neurodegenerative disorders especially dementia, the mechanisms of such disorders and to find new tools for early and accurate diagnosis. All the participants are ethnic Norwegians from central Norway. The ages for the controls ranged from 47 to 64 years. Also, the age at onset for all patients ranged from 45 to 64 years. However, since not all patients were included in the study at the age of onset, the age of inclusion started from 46 to 69 years. The common ethnic background made the participants a genetically comparatively homogeneous group. This homogeneity in addition to the narrow age range provided a common ground for comparison between the different disease groups and the controls. The patients were diagnosed and classified by an experienced neurologist at the Department of Neurology at the University Hospital in Trondheim, Norway. All diagnoses were made based on the individuals' medical history, neurological examination, laboratory tests, cerebral MRI and neuropsychological tests. The later included mini mental state examination (MMSE) to distinguish patients with AD from non-AD dementia patients and controls.

The mini mental state examination which was introduced by Folstein et al., (1975), is a 30 point questionnaire used in the clinical setting for the purpose of screening for cognitive impairment and dementia. It is also used in the estimation of the severity of cognitive impairment in individuals over time. This makes it a useful tool in determining the progress of cognitive impairment and also the response of an individual to a particular treatment. The MMSE has also been useful in the clinical setting for distinguishing between different types of dementias based on the patient's performance with respect to the various sections of the test.

During the MMSE test the patients in this study were asked to perform tasks covering a number of areas such as the time and place of the test, arithmetic (serial seven), language usage and comprehension, repetition of list of words, and basic motor skills. The time difference between the instruction and the performance of each task spanned the range of 10 seconds to 1 minute. The score for each correctly performed task started from a minimum

of 1 to a maximum of 5. Total scores greater than or equal to 27 points were considered to be indicators of normal cognition. Below this threshold, scores were categorised as indicators of severe, moderate or mild cognitive impairment. The scores were also corrected for the patients' level of education and age since such factors have been known to influence MMSE scores. On the other hand, very low scores mostly correlated with the presence of dementia. These decisions were however taken after other mental disorders in addition to physical deficits such as hearing impairment and motor defects which could also influence a patient's performance on the MMSE test were all ruled out. AD patients usually obtained significantly lower scores on memory, orientation to time and place compared to FTD and ALS groups. The questions used for diagnosing the patients in this study can be found in appendix A.

Furthermore, CSF samples from healthy individuals, comparable in terms of gender and age and lacking first-degree relatives with dementia were used as controls. The healthy controls were also taken through the same neuropsychological tests just like the patients in order to exclude any form of neurodegenerative disorder. These samples were obtained from the Neurological Research Biobank. All the clinical diagnoses used in this study have been supported by neuroimaging (MRI) results. The study consisted of 76 individuals in total: 27 controls, 28 AD, and 21 FTLD/ALS group. Table 3.1 contains a breakdown of the distribution of the patients and controls.

Table 3.1. Gender distribution of the various patient groups and controls.

Diagnosis	Males	Females	Total
Controls	13	14	27
AD	10	18	28
bvFTD	2	4	6
PNFA	1	1	2
SD	0	1	1
PSP	2	1	3
ALS+FTD	1	0	1
Pure ALS	3	2	5
F- ALS	1	0	1
FAV-ALS	1	1	2
Total	**36**	**40**	**76**

3.2 Ethical Considerations

It was ensured that all the participants in this research provided written informed consent. This was done by either the patients or their parents/ legal guardians in situations where the level of cognitive impairment did not permit the patients to do it themselves. The protection of the privacy of the data obtained from all the participants has also been taken into consideration. Moreover, the project has approval from the Regional Committee for Medical Research Ethics (REK Midt 2010/226 and 2014/487). It has also been approved and supported by the Research Council of Norway. Samples from the Neurological Research Biobank are available for research projects with ethical approval (REK Midt 2013/467).

3.3 Collection of CSF samples

CSF samples were collected from all participants through a process called spinal tap or lumbar puncture. During the lumbar puncture, each patient was asked to either lie down sideways or sit down and bend forward in order to space out the vertebrae. The appropriate location for the puncture was then identified by the physician and marked. The skin surrounding the puncture area was sterilised and the needle carefully inserted through the skin and the meninges to L4/L5 or L5/S1 level of the vertebral column. The CSF samples were collected into polypropylene cryovials. If necessary, samples were centrifuged to remove blood cells and other debris. They were then aliquoted and stored at -80^{o}C until the day of analysis. The use of aliquots frozen only once prevented multiple freeze thaw cycles which could alter the protein integrity of the samples. On the day of analysis, the samples were gradually thawed in ice-water prior to the actual analysis.

3.4 Analysis of CSF samples

The various proteins under study (T-tau, P-tau, Aβ-40, Aβ-42, Aβ-43, progranulin, YKL- 40, α-synuclein, and nurofilament light) were analysed by enzyme linked immunosorbent assay (ELISA). ELISA is a biochemical procedure for detecting and quantifying particular proteins of interest in a sample. There are different approaches for performing ELISA. However, the method used in this study is referred to as sandwich ELISA. Although the protocols for the individual commercial kits used in this research differ from each other, the underlying principles for all of them remained the same.

Sandwich ELISA is a special form of ELISA in which the protein of interest (antigen) is bound between two antibodies; namely, the capture antibody and the detection antibody. The capture antibodies were usually monoclonal antibodies specific for the protein of interest while the detection antibodies were mostly polyclonal antibodies also specific for the protein of interest and linked to an enzyme (streptavidin-horse radish peroxidase (STREP-HRP)). This first stage (which was done by the manufacturers of the commercial kits) involved the coating of the ELISA pates with the capture antibodies to ensure that the capture antibodies were the only substances that were bound to the walls of the ELISA plate. The excess capture antibodies were washed off and the exposed surfaces were blocked with bovine serum albumin (BSA) or

detergent. This step reduced the background signals, and hence increased the sensitivity of the sandwich ELISA.

When the samples, standards and controls were later added to the wells in duplicates, the proteins of interest were bound specifically to the immobilised capture antibodies in the pre-coated wells. Other proteins which did not bind were later washed away. This was then followed by the addition of the detection antibodies which were coupled to enzymes. Unbound enzyme-substrate conjugates were washed off. A substrate solution or chromogen (3, 3', 5, 5'-tetramethylbenzidine (TMB)) was then added, which reacted with the enzymes. The enzyme-substrate reaction produced a colour change, usually from blue to yellow (Figure 3.2). At the end of the reaction, a stop solution was added to the wells to terminate the reactions. All steps that preceded the enzyme substrate reactions were accompanied with an appropriate duration of incubation and washing as directed by the kit manufacturers. Figure 3.1 is a summary of the entire procedure described above. The plates were then read in a plate reader at specific wavelengths designated by the kit manufactures. Standard curves were generated by plotting the optical densities (OD) against the concentrations of the standards. These curves were used to estimate the concentrations of the samples.

All samples, controls, standards and reagents were added to the wells in the same order that they appeared on the plate charts (appendix B) to ensure equal reaction times. The samples for the various patient groups and the controls were randomised on the 96-well plates. This ensured that, in the event of any edge effect, no particular category of samples was severely affect at the expense of the others. All the instructions that accompanied the various kits were strictly followed as directed by the respective kit manufacturers. Table 3.2 contains a brief summary of the individual kits and the dilution factors applied to the samples during analysis. The protocols for the kits can be found in appendix C.

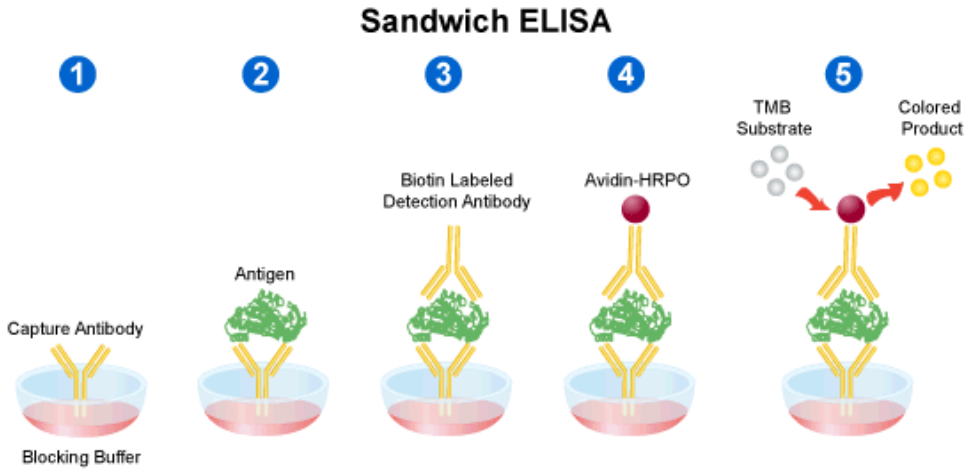


Figure 3.1. An illustration of the sandwich ELISA method. Modified from http://www.leinco.com/sandwich_elisa

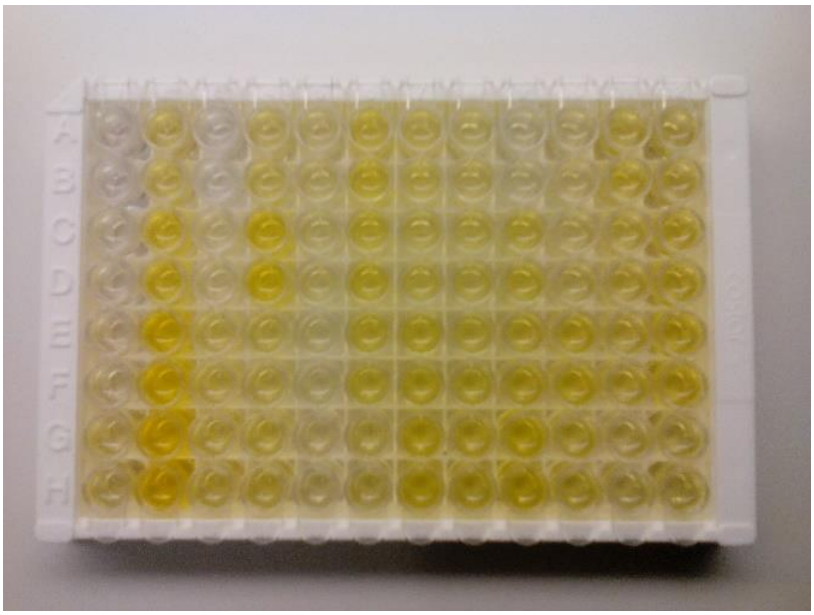


Figure 3.2. An image of the 96-well ELISA plate indicating the final colour change from blue to yellow. The standards for creating the standard curve are in the first two columns.

Table 3.2. An overview of the commercial kits, their lot numbers, expiry dates, target proteins and the corresponding dilution factors for the CSF samples.

Kit Manufacturers	Lot	Expiry date	Protein of interest	CSF dilution factor
FUJIREBIO INNOTEST hTau Ag	401796	31-12-2016	T-tau	Na
FUJIREBIO INNOTEST PHOSPHOS-TAU$_{(181P)}$	401938	31-10-2016	P-tau	Na
IBL Human Amyloid β$_{(1-40)}$	230472	31-03-2017	Aβ-40	1:200
FUJIREBIO INNOTEST β-AMYLOID$_{(1-42)}$	401989	31-10-2017	Aβ-42	na
IBL Amyloid - beta $_{(1-43)}$	ID-501	10-03-2016	Aβ-43	na
Uman Diagnostics NF-LIght®	70320	31-07-2016	NF-L	1:2
Invitrogen™	1683926A	01-2017	α-synuclein	Na
AdipoGen®	K1451508	08-2016	PGRN	1:15
R & D Systems™ Quantikine®	33027	04-10-2016	YKL-40	1:400

Abbreviations: na – not applicable

3.5 Statistical analysis

IBM® SPSS® version 21 was the statistical package used for analysing the data obtained. In order to select an appropriate statistical test for the analysis, graphical displays such as Q-Q plots and box plots were used to first and foremost visualise the distribution of the data. This was then followed by the conduction of a normality test. The p-values of the Shapiro-Wilk and Kolmogorov-Smirnov tests were used to decide whether the data were significantly different from the normal distribution. The normality tests indicated that most of the data deviated from the normal distribution which was contrary to one of the assumptions for the use of a parametric test. Some of the box plots also showed some extreme outliers which would have made the use of a parametric tests inappropriate due to the violation of the normal distribution assumption. Based on the small sample size, results from the normality tests and graphical displays, non-parametric tests such as Kruskal-Wallis and Mann-Whitney test were used to analyse the data. These non-parametric tests required no assumptions regarding the distribution of the data.

Kruskal-Wallis test was used to compare more than two groups while the Mann-Whitney test was used for pairwise comparison of the diagnostic groups. In the absence of pathological and genetic data, the heterogeneous FTD/ALS (n=21) group consisting of FTD, ALS, PSP and FTD+ALS (See Table 4.10 for the distribution), was first of all split on a clinical basis into two groups, FTD (n=9) and ALS (n=8). PSP and FTD+ALS were not included in the split groups because of their clinical distinctness and their smaller numbers (n=3 and 1 respectively) which could not permit statistical analysis. The Kruskal-Wallis test was used to compare the split groups to the controls and AD. In this analysis, a p-value of less than or equal to 0.05 was considered to be statistically significant. The Mann-Whitney U test was later used to for pairwise comparison of metabolites with statistically significant p-values from the Kruskal-Wallis test. In the case of the Mann-Whitney U test, p-values less or equal to 0.01 were considered statistically significant to allow for multiple comparisons and reduce the risk of type 1 errors. Spearman correlation coefficients were also calculated in order to determine the existence of any associations between the metabolites. In addition, receiver-operator characteristic (ROC) curves were generated for the metabolites to ascertain their diagnostic suitability in terms of sensitivity and specificity. The measures of central tendency and spread that were reported for the descriptive statistics were median and range. Boxplots were also used for the visual display of the distribution of data obtained for the metabolites and how they vary between healthy controls and the disease

groups. A boxplot (box and whisker plot) consists of a rectangular box with two vertical lines that extend from the box. The line in the middle of the box represents the median value (50th percentile). The top and bottom ends of the box represents the upper quartile (75th percentile) and lower quartile (25th percentile) respectively while the upper and lower whiskers represent the maximum and minimum values respectively. Empty circles outside the whiskers are referred to as outliers while the asterisks are known as extreme outliers (Figure 3.3).

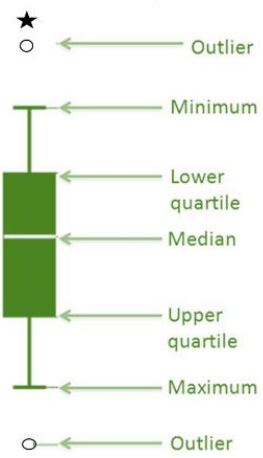


Figure 3.3. Diagrammatic representation of box and whisker plot.

4 Results

4.1 Demographic data

Table 4.1 contains a summary of the demographic data for the controls, AD, FTD, ALS, FTD+ALS and PSP patients. From the statistical analysis of the demographic data, results with p values less than 0.05 and 0.01 were considered statistically significant for the Kruskal-Wallis and Mann Whitney U tests respectively. The male to female ratios were found to be equally distributed among the disease groups and controls as shown by the Chi-Square test which exhibited no significant difference among the groups. The Kruskal-Wallis results indicated a strong significant difference among the groups in terms of their MMSE scores ($p < 0.001$), though the Mann-Whitney U test for pairwise comparison showed no significant difference between the MMSE scores for AD and FTD patients. The significant difference was found between the healthy controls and the AD and FTD patients; $p < 0.001$ and $p = 0.001$ respectively. There were no MMSE scores available for the pure ALS group since the patients did not exhibit signs of cognitive impairment, hence there had been no need to conduct the MMSE test on them. The age at onset for the AD, FTD and ALS patients in addition to the age at inclusion for all participants were not significantly different among the groups. However, there was a significant difference in the duration of disease according to the Kruskal-Wallis test, $p = 0.001$. Since the samples sizes for the PSP and FTD+ALS patients were small, their demographic data were not included in the statistical analyse but presented as raw values in Table 4.1. Also, due to the heterogeneous nature of the FTLD/ALS disease group, the distribution of the concentrations of the metabolites and their ratios for the various diseases that make up this group are presented in Table 4.10.

Table 4.1 Summary of demographic data

	HC	AD	FTD	ALS	PSP	FTD+ALS	p-value
Total number of CSF samples	27	28	9	8	3	1	
Gender (M:F)	13:14	10:18	3:6	5:3	2:1	1:0	0.479
Age at inclusion, median (range)	**58** (47-64)	**61** (52-69)	**61** (46-67)	**59.5** (47-65)	**62** (56-66)	**59**	0.172
Age at onset, median (range)	na	**57** (47-64)	**59** (45-64)	**58.5** (46-64)	**58** (51-60)	**57**	0.847
Disease duration, median (range)	na	**3** (1-11)	**2** (1-10)	**1** (1-2)	**5** (4-6)	**2**	0.001*****
MMSE score, median (range)	**30** (28-30)	**24** (11-30)	**24** (10-28)	na	**27** (19-28)	**22**	< 0.001*****

*****significant at alpha level of 0.01. PSP and FTD+ALS values were not included in the statistical analysis. Age at inclusion, Age at onset, disease duration and MMSE scores for the FTD+ALS patient are single values. Abbreviation; na: not applicable, HC: healthy controls.

4.2 CSF metabolite levels

4.2.1 Kruskal-Wallis test results for metabolite concentrations

Regarding the Kruskal-Wallis test for the concentrations of the metabolite between the various groups (HC, AD, FTD and ALS), p values less than 0.05 were considered to be statistically significant. There was a statistically significant difference among the core biomarkers for the controls, AD, FTD and ALS patients ($p < 0.001$). Aβ-40/Aβ-42 and T-tau/Aβ-42 ratios also showed significant differences ($p < 0.001$) between the controls and the patient groups. Also, Aβ-40/Aβ-43 showed a significant difference for the groups being compared ($p = 0.004$). In addition, there was a significant difference among the groups for T-tau/Aβ-42, $p < 0.001$. Aβ-40 was not significantly different among the groups.

Among the other metabolites, only NF-L ($p < 0.001$) and YKL-40 ($p = 0.001$) exhibited a statistically significant difference between the control and patient groups when the Kruskal-Wallis test was conducted. In contrast, neither α-synuclein nor PGRN showed any significant difference between the disease groups and the controls. The ratios for the metabolites; YKL-

40/Aβ-42, PGRN/Aβ-42, YKL-40/Aβ-43, PGRN/Aβ-43 and NFL/Aβ-43 had p-values less than 0.001 while NFL/Aβ-42 had a p-value of 0.006, which were all statistically significant. Table 4.2 contains a summary of the Kruskal-Wallis result as well as the descriptive statistics.

Table 4.2 Descriptive statistics

Metabolite & ratios	HC, n=27 median (range)	AD, n=28 median (range)	FTD, n=9 median (range)	ALS, n=8 median (range)	Kruskal-Wallis test p-value
T-tau_(pg/ml)	**222.8** (134.1-533.1)	**997.7** (260.6-1410.5)	**610.6** (260.6-1410.5)	**226.3** (182.9-435.5)	<0.001
P-tau_(pg/ml)	**37.2** (21-84.9)	**77.7** (30.7-134.2)	**64.4** (28.6-271.7)	**28.7** (18.5-52.2)	<0.001
Aβ-40 (pg/ml)	**11376** (7002-33373)	**13609.2** (7706.4-18038.8)	**13306** (6459.6-18725)	**9810** (4478.2-16088.2)	ns
Aβ-42_(pg/ml)	**830.5** (473.3-1579.5)	**409.6** (243.4-890.2)	**538** (227.6-938.2)	**557.5** (315.6-926.2)	<0.001
Aβ-43_(pg/ml)	**26.9** (6.8-62.2)	**12.7** (5.4-52.5)	**22** (6-39.4)	**26.7** (13.5-41.2)	<0.001
Aβ-40/Aβ-42	**15** (12.2-21.1)	**30.4** (18-43.8)	**17.7** (14.3-59.5)	**16.9** (12.8-18.4)	<0.001
T-tau/Aβ-42	**0.28** (0.16-0.59)	**2.52** (0.73-3.04)	**1.03** (0.43-15.12)	**0.42** (0.34-0.58)	<0.001
NF-L_(ng/ml)	**2729.1** (557-11289.4)	**7167.4** (4335.5-18175.6)	**7156** (28-16998.1)	**5253.7** (2439.6-17055.6)	<0.001
α-synuclein_(pg/ml)	**272** (171-2358)	**267** (182-1468)	**894** (198-4782)	**257** (213-2804)	ns
PGRN_(pg/ml)	**4995** (2280-7920)	**4065** (2625-7035)	**5317.5** (3795-7140)	**5467.5** (4575-13665)	ns
YKL-40_(ng/ml)	**134.1** (65.4-301.7)	**165.7** (136-319)	**212.5** (123.7-418.3)	**212.1** (165.2-317.8)	0.001
YKL-40/ Aβ-42	**0.16** (0.09-0.40)	**0.41** (0.36-0.64)	**0.37** (0.14-1.84)	**0.30** (0.23-0.84)	<0.001
PGRN/ Aβ-42	**6.4** (2.9-8.9)	**10.7** (7.5-18.2)	**8.3** (5.9-31.4)	**11.52** (6.7-14.8)	<0.001
NFL/ Aβ-42	**4.1** (0.6-11.1)	**17** (11-24.5)	**12.4** (0.04-74.7)	**7.4** (3.4-54)	0.006
Aβ-40/Aβ-43	**405** (321.9-1666.3)	**1034.3** (305.9-1676.8)	**555.6** (364.3-2274)	**381.6** (255.5-600.4)	0.004
T-tau/ Aβ-43	**8** (5.6-21.6)	**90.4** (12.6-109.6)	**24.6** (11.7-578.4)	**10.4** (6.9-13.9)	<0.001
YKL-40/Aβ-43	**4.3** (2.2-14.8)	**13.3** (6.1-25.1)	**12** (3.1-70.3)	**8.9** (5.8-19.6)	<0.001
PGRN/Aβ-43	**168.8** (74.5-845.9)	**384.8** (126.5-676.4)	**798.8** (126.6-1199.4)	**280.7** (171-415.6)	<0.001
NFL/Aβ-43	**106.3** (20.5-409.9)	**520.3** (346-906.8)	**443** (1-2855.4)	**219.9** (85.8-1264.2)	<0.001

Abbreviation: ns = not significant.

4.2.2 Pairwise comparison of metabolite concentrations with the Mann-Whitney U test

Only the metabolites and their ratios that were significantly different among the controls and disease groups from the Kruskal-Wallis test were further analysed with the Mann-Whitney U test. In this case, p values less than 0.01 were considered to be statistically significant. It was generally observed that all the metabolites and their ratios analysed at this stage showed a significant difference between healthy controls and AD patients except YKL-40 which had a significant level exactly at the adjusted level of significance ($p = 0.01$). On the contrary, neither the metabolites nor their ratios showed a significant difference between AD and FTD patients. Table 4.3 shows a summary of the Mann-Whitney U test results for the pairwise comparison of the metabolites between the healthy controls, AD, FTD and ALS patients. Also, a "special cases" section below has been dedicated to the variations of the metabolites among the PSP and FTD+ALS patients relative to the healthy controls, AD, FTD and ALS patients. This separation was done since the number of samples for PSP and FTD+ALS, ($n = 3$ and 1 respectively) were considered too small to warrant statistical analysis. Also, there was no pairwise comparison between AD and ALS since both diseases are clinically distinct.

Table 4.3 Significance levels for the pairwise comparison of the metabolites and their ratios

Metabolite & ratio	p-value Mann-Whitney U test				
	HC vs AD	HC vs FTD	HC vs ALS	AD vs FTD	FTD vs ALS
T-tau_(pg/ml)	<0.001**	<0.001**	ns	ns	0.003*
P-tau_(pg/ml)	<0.001**	(0.015)	ns	ns	0.007*
Aβ-42_(pg/ml)	<0.001**	ns	ns	ns	ns
Aβ-43_(pg/ml)	<0.001**	ns	ns	(0.031)	ns
Aβ-40/Aβ-42	<0.001**	(0.028)	ns	ns	ns
T-tau/Aβ-42	<0.001**	<0.001**	(0.020)	ns	(0.018)
NF-L_(ng/ml)	0.003*	0.003*	0.004*	ns	ns
YKL-40_(ng/ml)	(0.010)	0.003*	0.002*	ns	ns
YKL-40/ Aβ-42	<0.001**	0.002*	0.004*	ns	ns
PGRN/ Aβ-42	<0.001**	ns	0.002*	(0.042)	ns
NFL/ Aβ-42	0.001*	0.009*	ns	ns	ns
Aβ-40/Aβ-43	0.002*	ns	ns	ns	ns
T-tau/ Aβ-43	<0.001**	<0.001**	ns	ns	(0.013)
YKL-40/Aβ-43	<0.001**	0.002*	0.001*	ns	ns
PGRN/Aβ-43	<0.001*	ns	0.005*	ns	ns
NFL/Aβ-43	<0.001*	0.005*	0.003*	ns	ns

*significance < 0.01.

**significance < 0.001.

Trends (p < 0.05 but ≥ 0.01)

Abbreviation: ns = not significant

4.2.3 Variations in CSF core biomarker levels in the control and disease groups

T-tau showed a statistically significant increase in patients with AD and FTD compared to controls (p < 0.001). It was also significantly higher in FTD compared to ALS (p=0.003). There was no significant difference between the controls vs ALS and the AD vs FTD. Sample #63 was an extreme outlier in the FTD group while #73 was an outlier in the ALS group, yet it was within the range for the controls, AD and FTD groups as shown in Figure 4.1

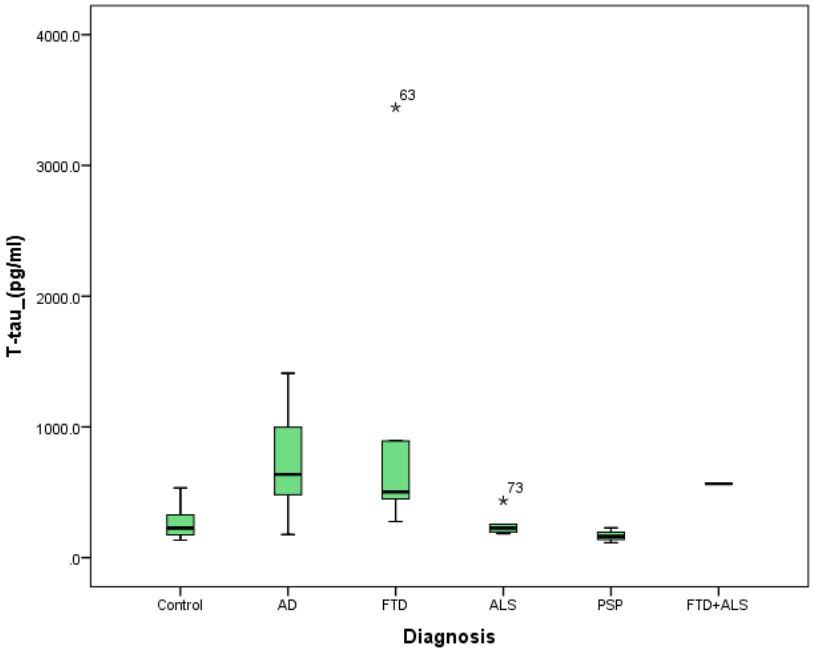


Figure 4.1. Boxplots for T-tau levels in the CSF of healthy controls and the patients.

There was a significant increase in P-tau for the AD patients compared to controls ($p < 0.001$) and a significant increase in FTD patients compared to the ALS patients ($p = 0.007$). However, there was no significant difference between controls vs FTD, controls vs ALS and AD vs FTD. Samples #26, #63 and #73 were outliers for controls, FTD and ALS patients respectively as shown in Figure. 4.2

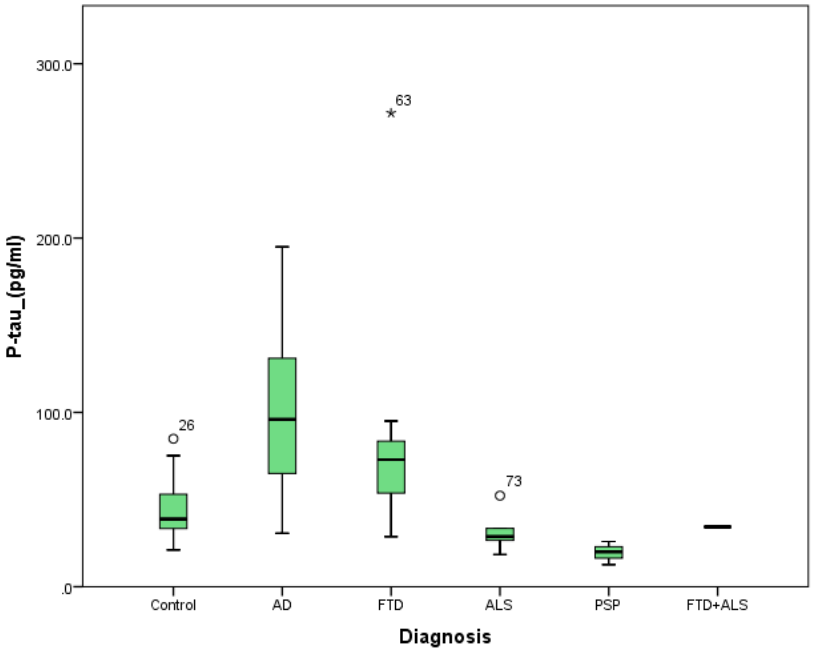


Figure 4.2. Boxplots for P-tau levels in the CSF of healthy controls and the patients.

Aβ-42 was decreased only in the AD group compared to the controls ($p < 0.001$). There was no significant difference in the Aβ-42 levels for the other pairs that were compared. Figure 4.3 shows that sample #26 was an outlier in the control group while #29 and #31 were outliers in the AD group. The outlier in the control group was outside the range for any of the disease categories and the controls.

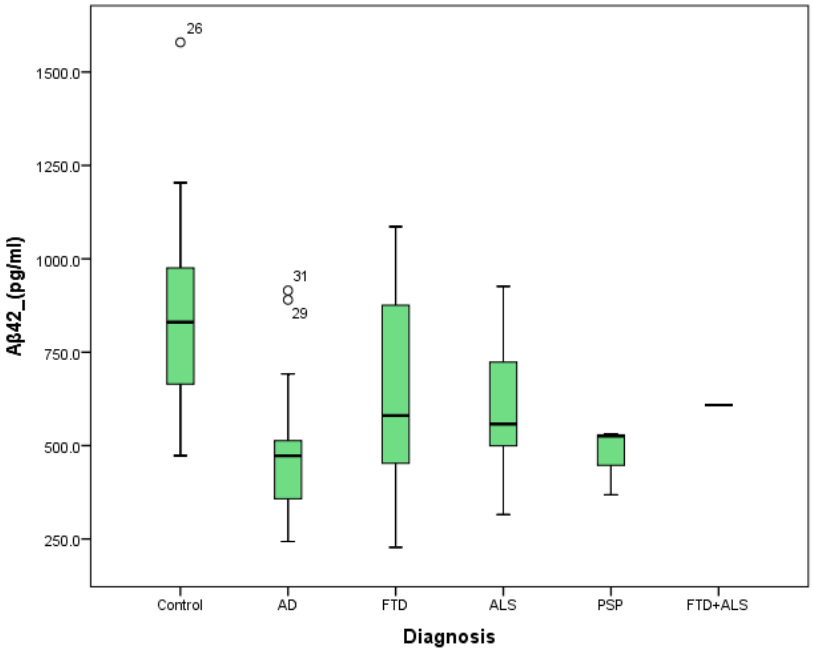


Figure 4.3. Boxplots for Aβ-42 levels in the CSF of healthy controls and the patients.

No pairwise comparison was performed on the raw data for Aβ-40 data since there was no significant difference in the levels of Aβ-40 among the controls, AD, FTD and ALS groups according to the Kruskal-Wallis test. One control, #26 was an outlier with values outside the range for all the metabolites measured (Figure 4.4).

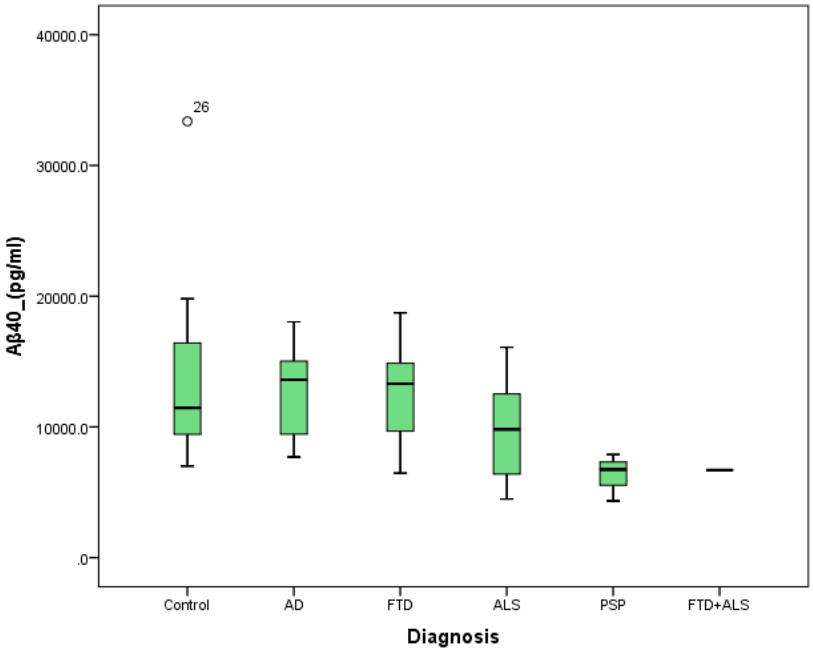


Figure 4.4. Boxplots for Aβ-40 levels in the CSF of healthy controls and the patients.

With regards to the core biomarker ratios, Aβ-40/Aβ-42 was significantly higher in the AD group compared to the controls, ($p < 0.001$). There was no significant difference among the other pairs compared. Sample #26 ratio was an outlier in the control group while sample #63 was an outlier in the FTD group, Figure 4.5.

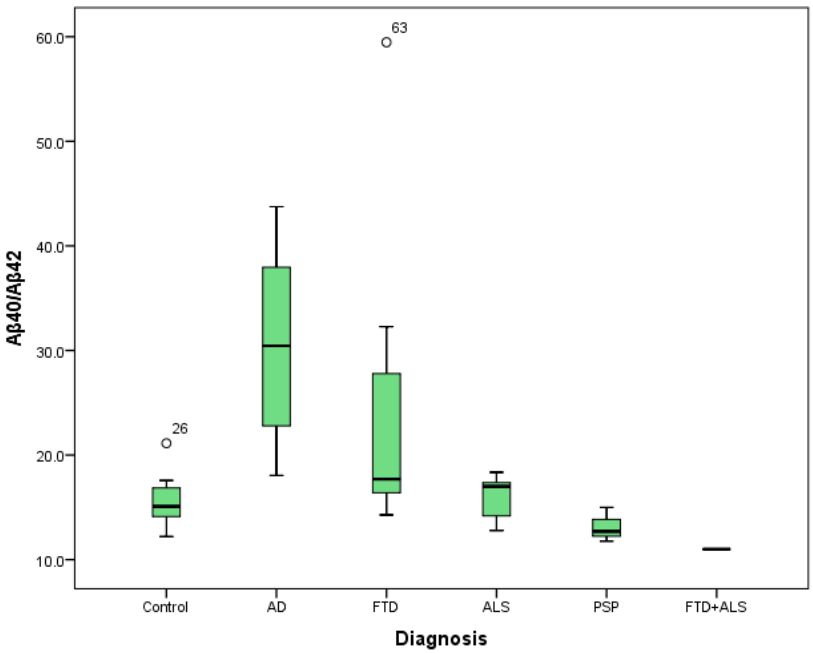


Figure 4.5 Boxplots for the Aβ-40/Aβ-42 ratios in the CSF of healthy controls and the patients.

The T-tau/Aβ-42 ratios was significantly higher for AD and FTD compared to controls ($p < 0.001$). The other pairs showed no significant difference. Sample # 63 in the FTD group was an extreme outlier with a ratio far outside the range for all the other disease groups and the controls, Figure 4.6.

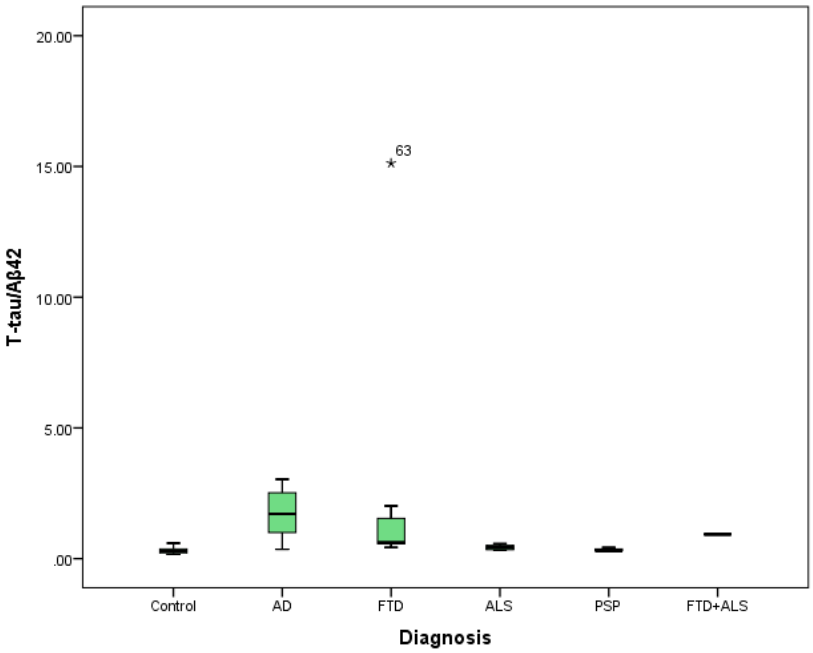


Figure 4.6. Boxplots for the T-tau/Aβ-42 ratio in the CSF of healthy controls and the patients.

4.2.4 Variations in the CSF levels of other metabolites in the controls and disease groups

The Aβ-43 isoform of amyloid-β peptide was significantly decreased in the AD group compared to the controls ($p < 0.001$). There was no significant difference between the other pairs. The control group had two outliers. One of them, sample #23 was lower than the median value for the AD patients while the other, #26 was the highest Aβ-43 levels in this study. The AD groups also had two outliers, # 29 and #31 which were higher than the median values of the controls, Figure 4.7.

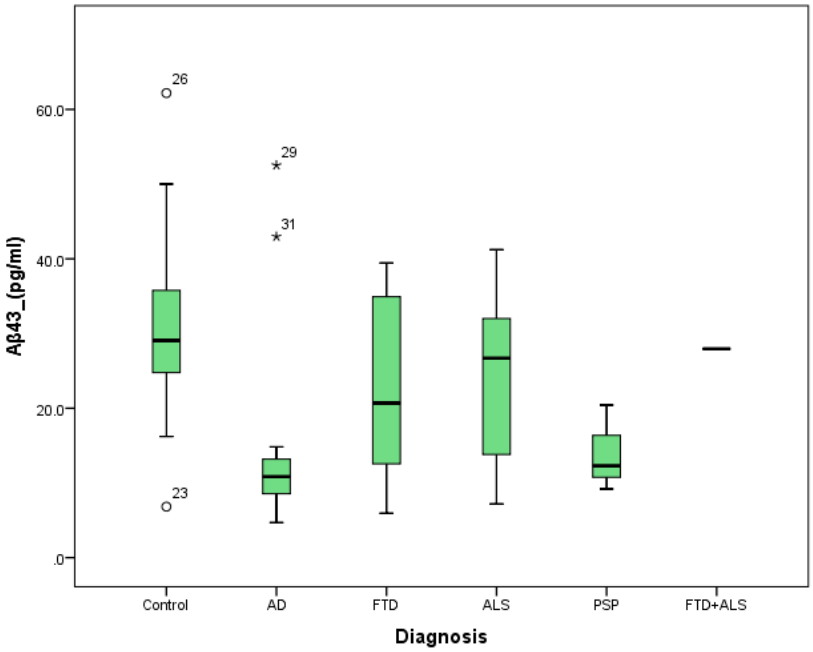


Figure 4.7. Boxplots for Aβ-43 levels in the CSF of healthy controls and patients.

The CSF values for α-synuclein showed no significant difference for any of the comparisons. However, samples #3, #26, and #57 were outliers for the controls, AD and FTD respectively, Figure 4.8.

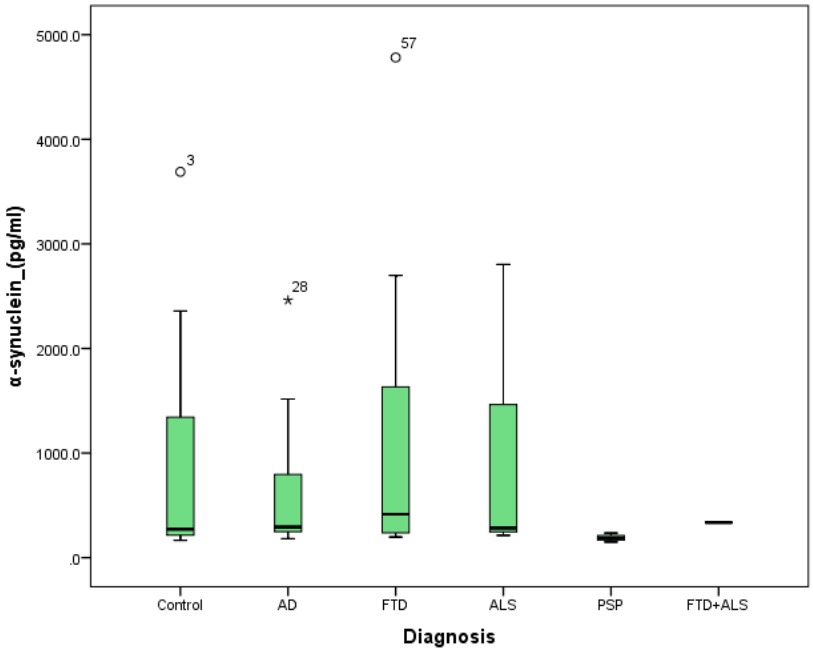


Figure 4.8. Boxplots indicating the levels of α-synuclein in the CSF of healthy controls and patients.

Patients with AD, FTD and ALS showed a significant increase in CSF NF-L concentration compared to the controls, p = 0.003, 0.003 and 0.003 respectively, with the median values progressively increasing from AD to FTD and ALS. However, there was no significant difference among the disease group pairs. Sample #13 was an outlier in the control group, while #29 and 34 were outliers in the AD group (Figure 4.9).

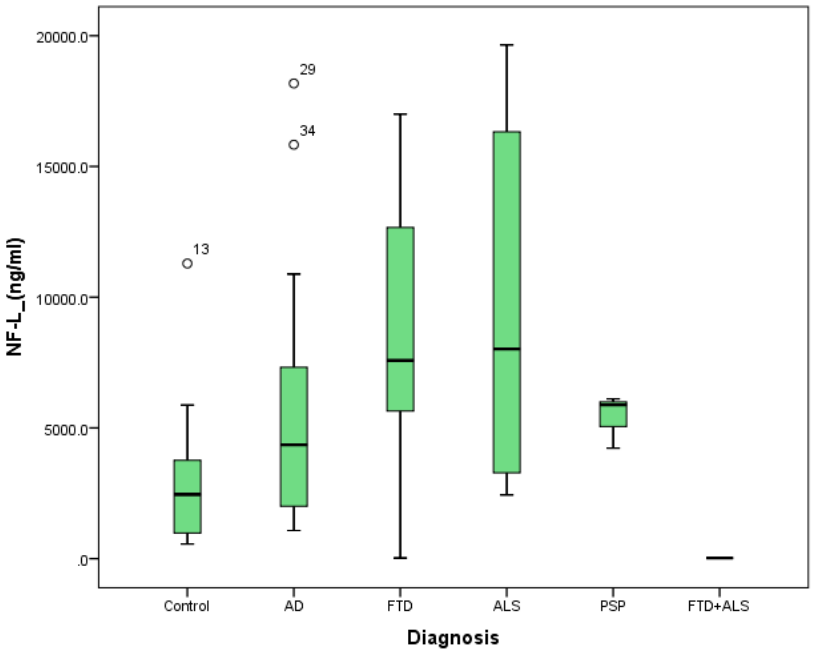


Figure 4.9. Boxplots for NF-L indicating the levels of NF-L in the CSF of healthy controls and patients.

Patients with FTD and ALS had a significantly higher level of YKL-40 compared to the healthy controls. However, the level in AD patients was on the border of the adjusted level of significance (p = 0.01). No significant differences were observed for the comparison between the disease groups. Samples #11 and #27 were in outliers in the control group, 34 in the AD group, and 61 and 63 in the FTD group. Sample #75 was an outlier in the ALS group (Figure4.10).

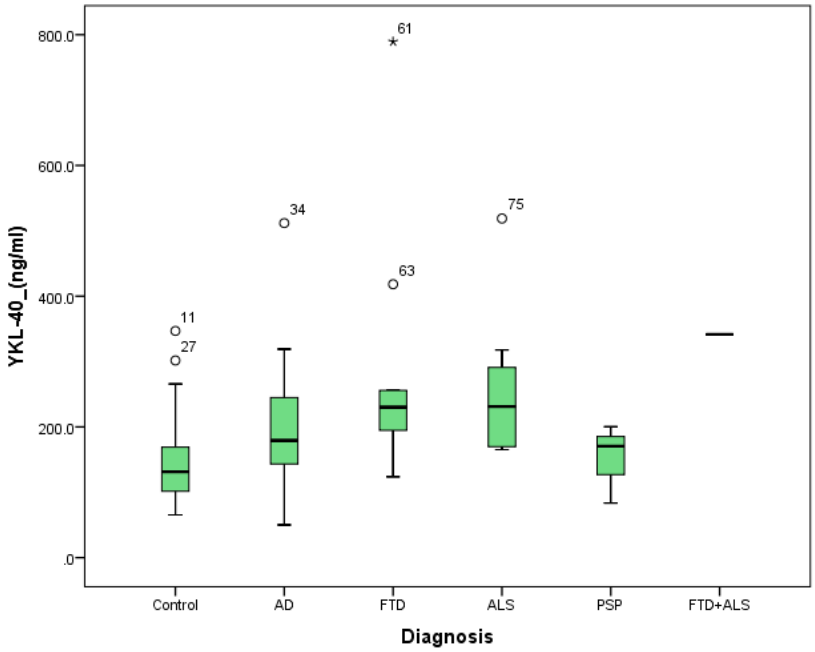


Figure 4.10. Boxplots for YKL-40 indicating the levels in the CSF of healthy controls and patients.

Neither the patient groups nor the controls showed any significant differences in the CSF levels of PGRN though there were a number of outliers (Figure 4.11).

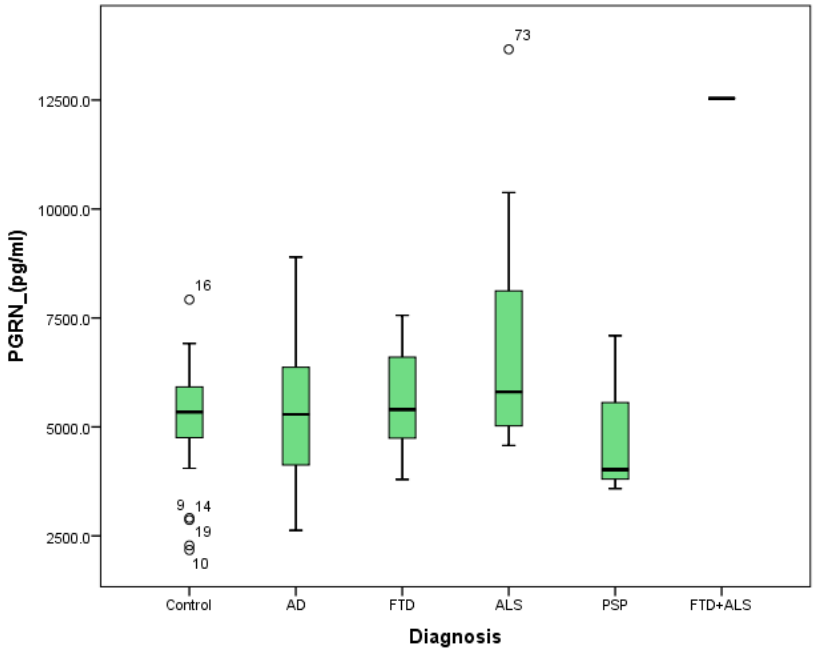


Figure 4.11. Boxplots for PGRN indicating the levels in the CSF of healthy controls and patients.

Patients with AD, FTD and ALS had a significantly higher YKL-40/Aβ-42 ratio compared to the healthy controls, $p < 0.001$, $p = 0.002$ and 0.004 respectively. On the other hand, the ratios were not significantly different within the disease groups. Samples #25, 34 and 64 were outliers for the controls, AD and FTD respectively (Figure 4.12).

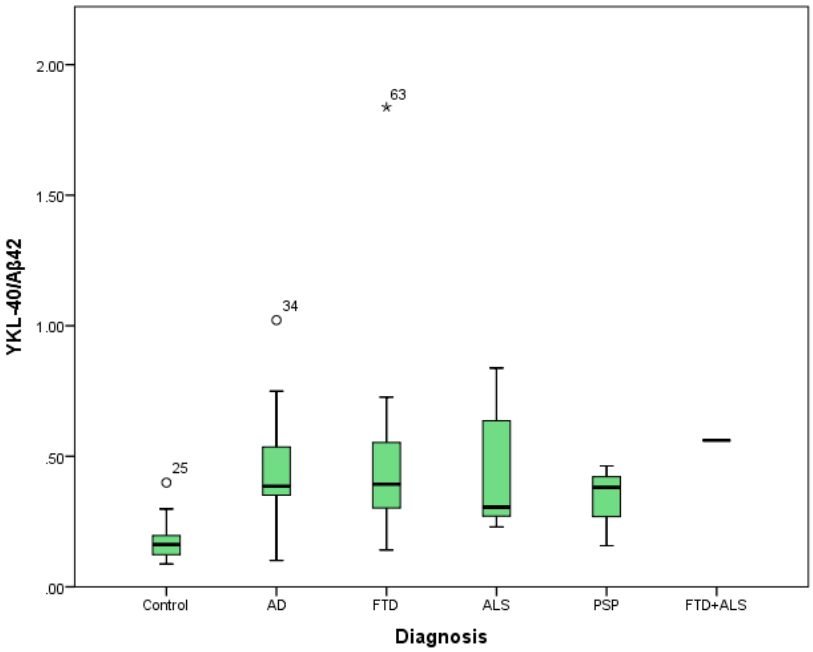


Figure 4.12 Boxplots for the YKL-40/Aβ-42 ratio in the CSF of healthy controls and the patients.

PGRN/Aβ-42 ratio was significantly higher in AD and ALS than the controls, $p < 0.001$ and $p = 0.002$ respectively. No significant difference existed between FTD and controls and also between the disease groups. Sample #63 is an outlier for FTD (Figure 4.13).

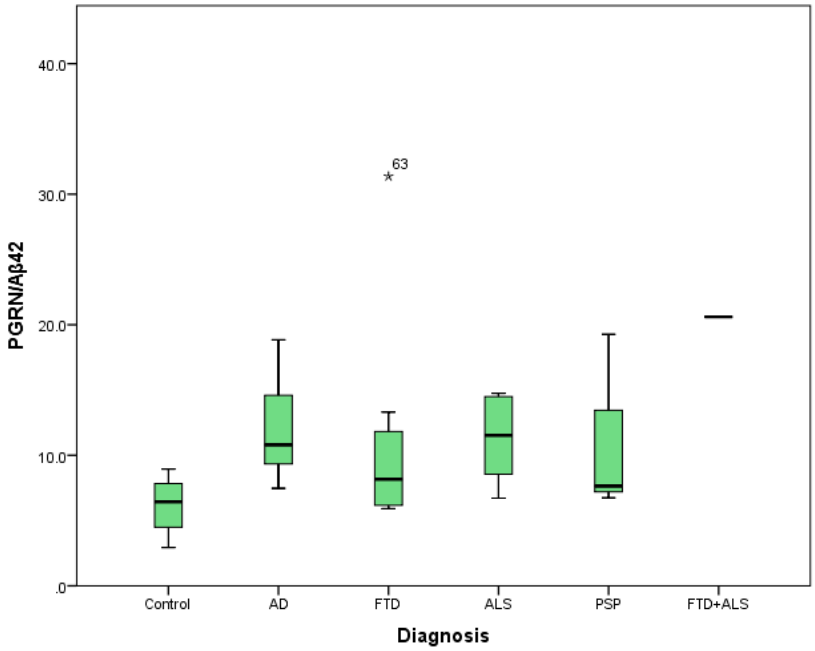


Figure 4.13. Boxplots for the PGRN/Aβ-42 ratio in the CSF of healthy controls and the patients.

The NF-L/Aβ-42 ratios for AD and FTD were significantly higher than the controls, p = 0.001 and 0.009 respectively. None of the other pairs compared showed a significant difference from each other. Samples #13 and #63 were outliers for the controls and FTD respectively (Figure 4.14).

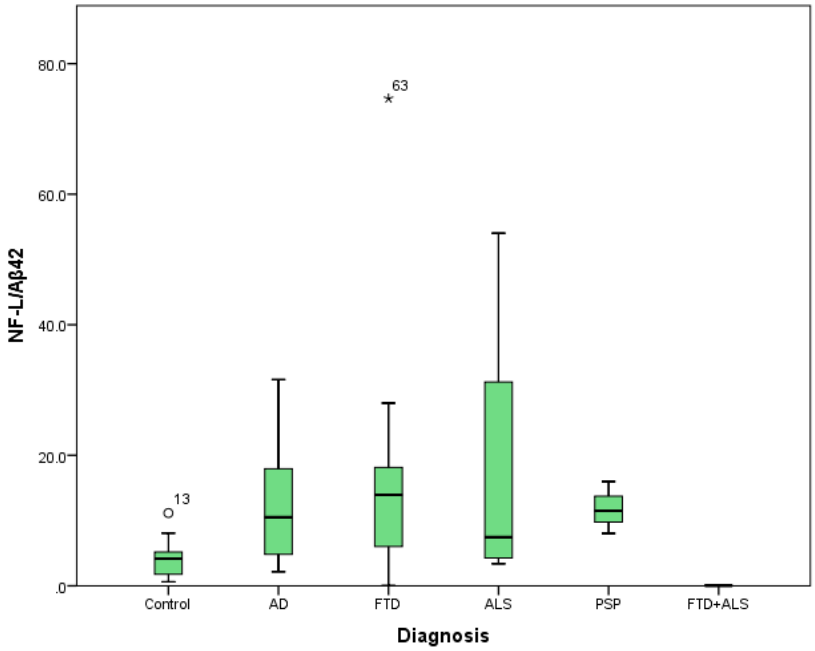


Figure 4.14. Boxplots for the NF-L/Aβ-42 ratio in the CSF of healthy controls and the patients.

3.2.5. Variations in the levels of Aβ-43 and metabolite ratios for the controls and disease groups

In general, the Aβ-40/Aβ-43, T-tau/Aβ-43, YKL-40/Aβ-43, PGRN/Aβ-43 and NF-L/Aβ-43 ratios exhibited a similar pattern to their corresponding Aβ-42 ratios among the controls, AD, FTD and ALS patients. The only exception was the observation of a significantly higher NF-L/Aβ-43 ratio in ALS patients compared to the controls while the NF-L/Aβ-42 ratio was not significantly different between the controls and the ALS patients. The corresponding p values are stated in Table 4.3 and boxplots in Appendix D. Table 4.4 contains a summary of the changes in the levels of the metabolites and their ratios among the disease groups relative to each other and the controls.

Table 4.4. Summary of the metabolite comparisons for AD, FTD and ALS patients relative to healthy controls and FTD relative to AD and ALS patients.

	Changes in the levels of metabolites and ratios				
Metabolite & ratio	HC vs AD	HC vs FTD	HC vs ALS	AD vs FTD	ALS vs FTD
T-tau	↑	↑	-	-	↑
P-tau	↑	-	-	-	↑
Aβ-42	↓	-	-	-	-
Aβ-43	↓	-	-	-	-
Aβ-40/Aβ-42	↑	-	-	-	-
T-tau/Aβ-42	↑	↑	-	-	-
NF-L	↑	↑	↑	-	-
YKL-40	* -*	↑	↑	-	-
YKL-40/ Aβ-42	↑	↑	↑	-	-
PGRN/ Aβ-42	↑	-	↑	-	-
NFL/ Aβ-42	↑	↑	-	-	-
Aβ-40/Aβ-43	↑	-	-	-	-
T-tau/ Aβ-43	↑	↑	-	-	-
YKL-40/Aβ-43	↑	↑	↑	-	-
PGRN/Aβ-43	↑	-	↑	-	-
NFL/Aβ-43	↑	↑	↑	-	-

Red arrows (↑) indicated a significant increase while green arrows (↓) indicate a significant decrease. Dashed lines (-) indicate no significant difference. *-* exactly on the border of significance.

Correlation analysis

Correlation between Aβ-40, Aβ-42, Aβ-43, and YKL-40 for the healthy controls and AD patients

YKL-40 tended to be positively correlated with Aβ-40, Aβ-42 and Aβ-43 among the controls (r_s = 0.626, 0.575 and 0.441 respectively) and the AD patients (r_s = 0.700, 0.488 and 0.401 respectively). All the correlations were statistically significant at an alpha level of 0.05 except the correlation between YKL-40 and Aβ-40 among the heathy controls which was significant at an alpha level of 0.01.

Correlation between Aβ-43, T-tau and NF-L for the healthy AD and FTD patients

NFL levels were significantly negatively correlated with Aβ-43 in AD (r_s = -0.683, p = 0.042) and FTD (r_s = -0.683, p = 0.042). NF-L also correlated positively with T-tau in AD patients (r_s = -0.683, p = 0.042) but not FTD.

3.2.6. Special cases

The special cases include 3 patients with PSP and 1 patient with FTD+ALS. Hence, they were not included in the statistical analysis.

PSP:

The values from these three patients showed that both the T-tau and P-Tau medians were low compared to the other patient groups (Figure 4.1 and 4.2). For Aβ-42, levels lay within the distribution for the AD, FTD and ALS patients (Figure 4.3), but Aβ-40 values were among the lowest recorded (Figure 4.4). The Aβ-40/Aβ-42 ratio was therefore also low (Figure 4.5) as was the T-tau/Aβ-42 ratio (Figure 4.6).

Among the other metabolites, PSP patients had Aβ-43 values within the distribution range for the other patient groups, but was low compared to the control group (Figure 4.7). All three patients had particularly low α-synuclein concentrations (Figure 4.8). The NF-L, YKL-40 and PGRN levels were all within the measured ranges for the other groups (Figures 4.9, 4.10, 4.11). Ratios between these metabolites and Aβ-42 were also within the limits of the other patient groups (Figures 4.12, 4.13. 4.14). The pattern of variability observed for the Aβ-43 ratios was similar to the Aβ-42 ratios as shown in Appendix D.

FTD+ALS:

Since there was only one patient with both FTD and ALS, only the most important results are highlighted here. The FTD+ALS patient had a particularly low level of NF-L (Figure 4.9), but a comparatively high level of YKL-40 compared to the range for the patient groups and controls (Figure 4.10). Interestingly, the PGRN level was also well above the ranges for the disease groups and controls, though similar to outlier #73 for the ALS group (Figure 4.11).

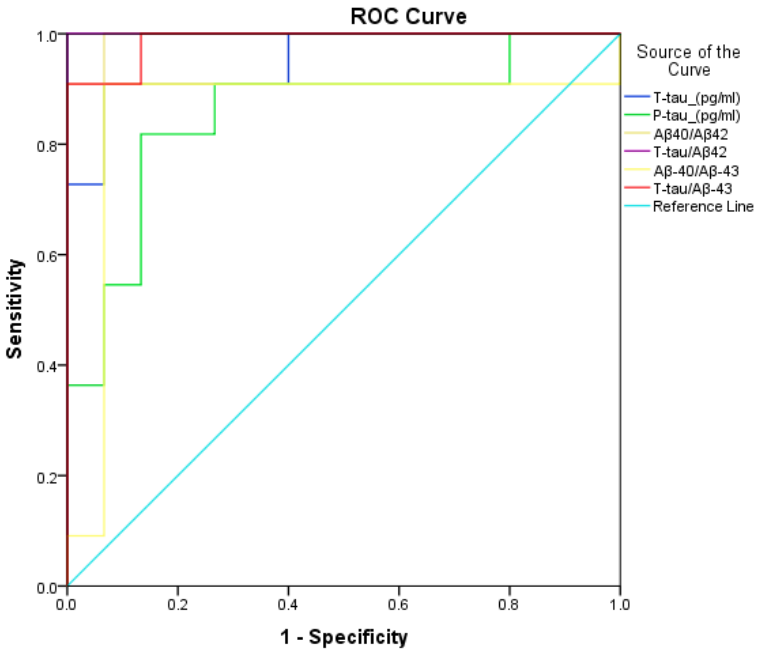
4.3 Differential Diagnostic performance of the metabolites and their ratios

The diagnostic performance of the metabolites that were significantly different between the disease groups and controls according to the Mann Whitney U test were evaluated with ROC curves. The corresponding cut-off values, area under the curve (AUC), sensitivity and specificity were recorded in tables.

4.3.1 Differentiating AD patients from controls with core biomarkers and their ratios with Aβ-42 and Aβ-43.

Among the core biomarkers, T-tau/Aβ-42 was a perfect discriminator between healthy controls and AD patients with 100% sensitivity and specificity at a cut-off value of 0.65. This was followed by T-tau/Aβ-43 with sensitivity and specificity of 91% and 100% respectively at a cut-off value of 22.2. However, at a cut-off value of 11.9, the sensitivity and specificity were 100% and 87% respectively. The rest of the core biomarkers were all good at discriminating between healthy controls and AD patients. However, between related biomarkers and their ratios, T-tau was a better discriminator than P-tau, Aβ-43 was better than Aβ-42 and Aβ-40/Aβ-42 was better than Aβ-40/Aβ-43 for distinguishing between healthy controls and AD patients. Table 4.5 shows the diagnostic performance of the Aβ peptides, tau proteins, and ratios in differentiating between healthy controls and AD patients while Figure 4.15 contains the ROC curves.

(a)



(b)

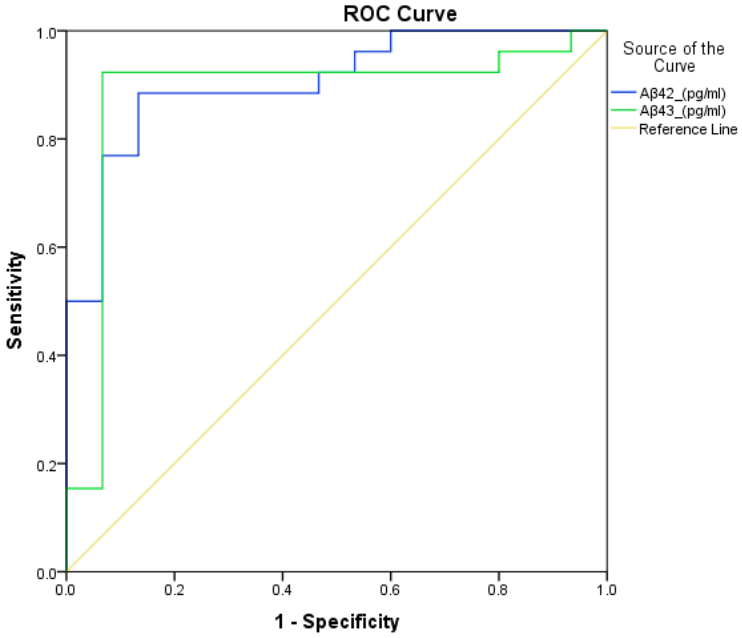


Figure 4.15 a and b. ROC curves for the core biomarkers and related metabolites as well as their ratios, indicating their ability to distinguish between AD patients and healthy controls

Table 4.5 Area under the curve (AUC), cut-offs, sensitivity and specificity for core biomarkers in distinguishing AD patients from healthy controls

	AUC	p-value	Cut – off	Sensitivity	Specificity
T-tau	0.952	<0.001	440	91	93
P-tau	0.855	0.002	47.8	91	73
			60.7	82	87
Aβ-42	0.945	<0.001	628	88	87
Aβ-43	0.861	0.002	15	92	93
Aβ-40/Aβ-42	0.994	<0.001	17.8	100	93
			21.3	91	100
Aβ-40/Aβ-43	0.855	0.002	653	91	93
T-tau/Aβ-42	1.000	<0.001	0.65	100	100
T-tau/Aβ-43	0.988	<0.001	11.9	100	87
			22.2	91	100

4.3.2 Differentiating AD patients from controls with the other metabolites and their ratios

The best discriminator between AD and healthy controls among the other metabolites was the PGRN/Aβ-42 ratio with a sensitivity and specificity of 92% and 93% respectively at a cut-off value of 8.5. The other ratios, YKL-40/Aβ-42, PGRN/Aβ-42, YKL-40/Aβ-43, PGRN/Aβ-43 and NF-L/Aβ-43 were also good at distinguishing AD patients from controls. On the other hand, NFL, YKL-40 and NFL/Aβ-42 ratio poorly distinguished AD patients from controls. The corresponding sensitivities and specificities for the metabolites are in Table 4.6 and the ROC curves are in Figure 4.16.

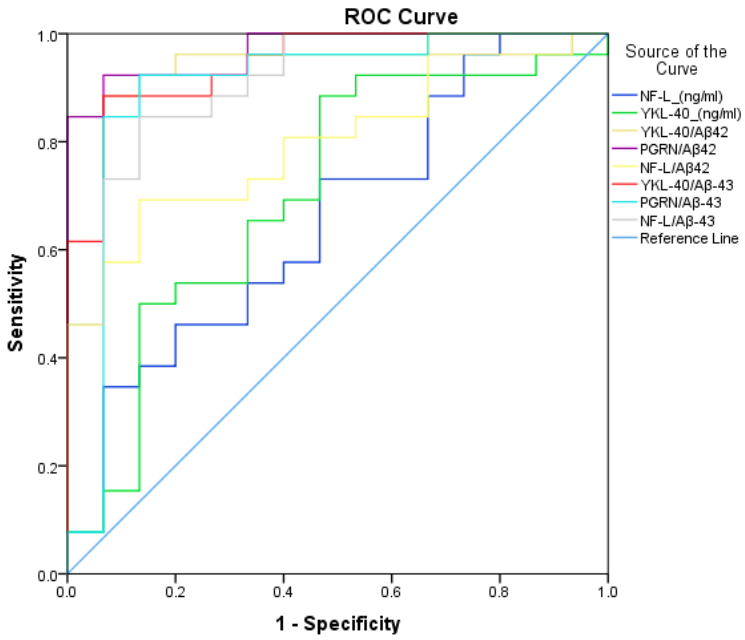


Figure 4.16. ROC curves for the other metabolites and their ratios, indicating their ability to distinguish between AD patients and healthy controls

Table 4.6 Area under the curve (AUC) cut-offs, sensitivity and specificity for other metabolites in distinguishing AD patients from healthy controls

	AUC	p-value	Cut – off	Sensitivity	Specificity
NF-L	0.659	0.093	2761	73	53
YKL-40	0.705	0.030	135	89	53
YKL-40/Aβ-42	0.923	<0.001	0.21	96	80
			0.27	92	87
PGRN/Aβ-42	0.969	<0.001	8.5	92	93
NF-L/Aβ-42	0.810	0.001	4.85	73	67
YKL-40/Aβ-43	0.944	<0.001	12	89	93
PGRN/Aβ-43	0.900	<0.001	241	92	87
			299	85	93
NF-L/Aβ-43	0.913	<0.001	202	84	86

4.3.3 Differentiating FTD patients from controls with core biomarkers, other metabolites and their ratios with Aβ-42 and Aβ-43

Tau and its ratios with Aβ-42 and Aβ-43 excellently distinguished FTD patients from healthy controls. The best distinguisher between FTD patients and healthy controls was T-tau/Aβ-42, with 100% sensitivity and 93% specificity at a cut-off value of 0.43. On the other hand, NF-L, YKL-40, YKL-40/Aβ-42, NF-L/Aβ-42, and YKL-40/Aβ-43 showed a good ability to distinguish FTD patients from controls. Patients with FTD were fairly distinguished from healthy controls by NF-L/Aβ-43 as shown in (Figure 4.17) and (Table 4.7).

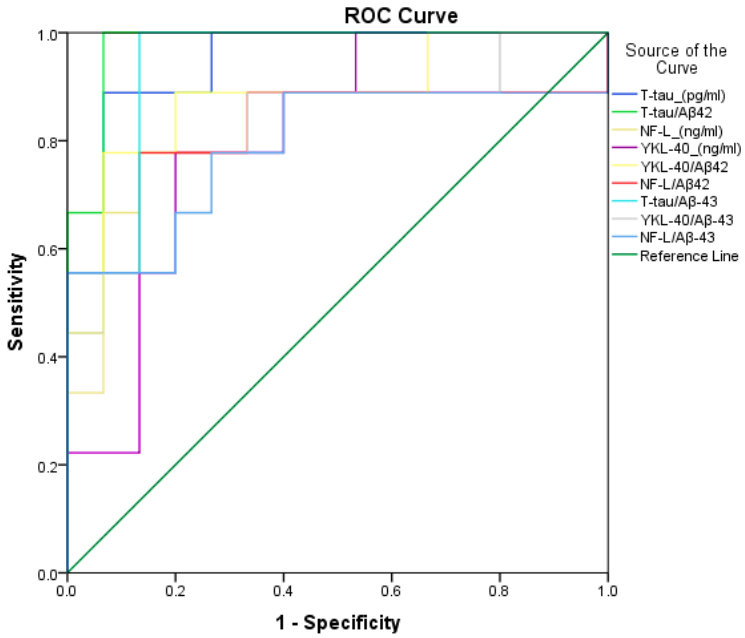


Figure 4.17. ROC curves for the core biomarkers, other metabolites and their ratios, indicating their ability to distinguish between patients with FTD and healthy controls

Table 4.7 Area under curve (AUC) cut- off, sensitivity and specificity for core and other metabolites in distinguishing patients with FTD from healthy controls

	AUC	p-value	Cut – off	Sensitivity	Specificity
T-tau	0.941	<0.001	404	89	93
T-tau/Aβ-42	0.978	<0.001	0.43	100	93
NF-L	0.815	0.011	3817	89	67
YKL-40	0.807	0.013	183	78	80
YKL-40/Aβ-42	0.881	0.002	0.20	89	80
NF-L/Aβ-42	0.822	0.009	5.9	78	87
T-tau/Aβ-43	0.941	<0.001	11.4	100	86
YKL-40/Aβ-43	0.815	0.011	5.6	89	67
			6.3	78	73
NF-L/Aβ-43	0.793	0.019	145	78	73

4.3.4 Differentiating ALS patients from controls with the other metabolites and their ratios

Table 4.8 shows that YKL-40/Aβ-42 and PGRN/Aβ-42 showed an excellent ability to distinguish ALS patients from healthy controls. However, at a 0.21 cut off level for YKL-40/Aβ-42, the sensitivity was higher compared PGRN/Aβ-42 (100 and 82 respectively), while the specificity of PGRN/Aβ-42, (93%) was also higher than YKL-40/Aβ-42, (87%) at a cut-off value of 26 for YKL-40/Aβ-42. The rest of the metabolites and their ratios performed fairly in the differentiation. Figure 4.18 shows the corresponding ROC curve.

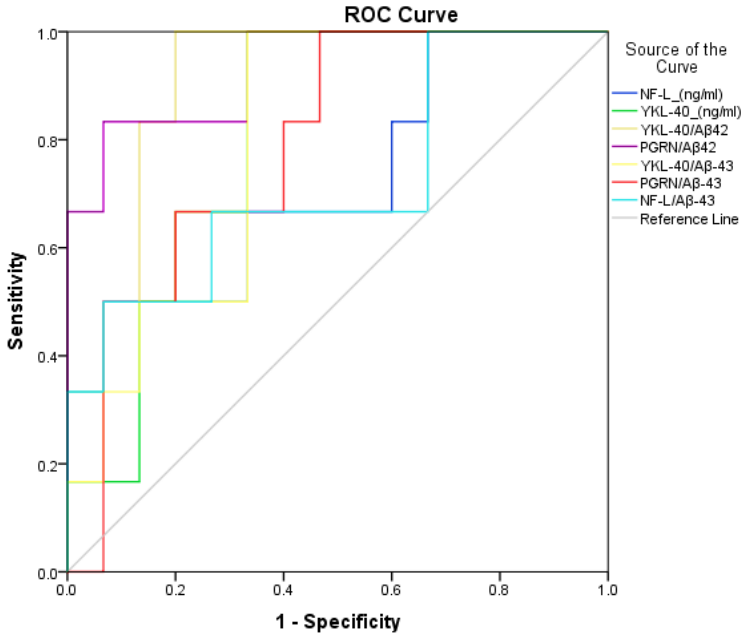


Figure 4.18. ROC curves for the other metabolites and their ratios, indicating their ability to distinguish between patients with ALS and healthy controls

Table 4.8 Area under the curve (AUC), cut-offs, sensitivity and specificity for the other metabolites in distinguishing patients with ALS from healthy controls

	AUC	p-value	Cut – off	Sensitivity	Specificity
NF-L	0.722	0.119	3860	67	67
YKL-40	0.811	0.029	162	100	67
YKL-40/Aβ-42	0.911	0.004	0.21	100	80
			0.26	83	87
PGRN/Aβ-42	0.933	0.002	8.4	83	93
YKL-40/Aβ-43	0.800	0.036	5.7	100	67
PGRN/Aβ-43	0.789	0.043	172	83	60
NF-L/Aβ-43	0.722	0.119	143	67	73

4.3.5 Differentiating FTD from ALS with T-tau and P-tau

Only T-tau and P-tau were able to distinguish between FTD from ALS as shown in Figure 4.19. Both biomarkers exhibited the same level of specificity (83%). However, the sensitivity was higher for T-tau than P-tau, making T-tau the best distinguisher (Table 4.9).

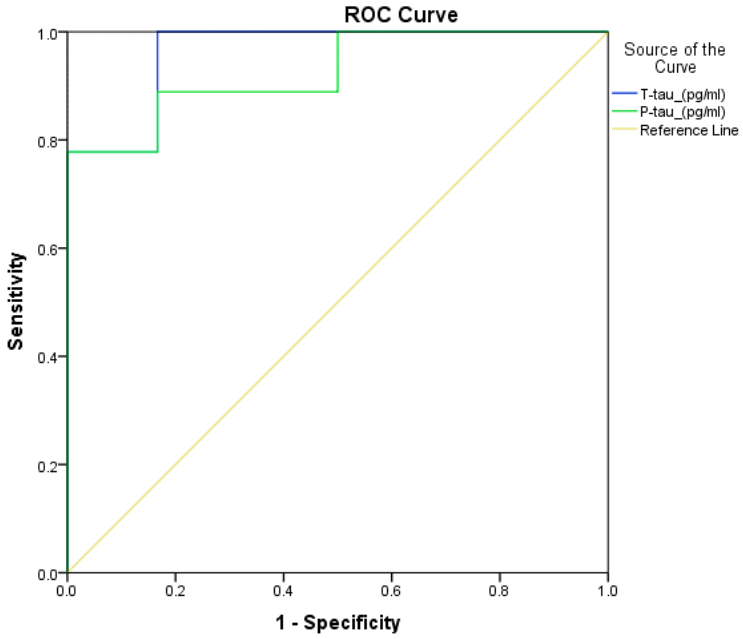


Figure 4.19. ROC curves for T-tau and P-tau, indicating their ability to distinguish between patients with FTD or ALS.

Table 4.9 Area under the curve (AUC), cut-off, sensitivity and specificity for T-tau and P-tau in distinguishing patients with AD from healthy controls

	AUC	p-value	Cut – off	Sensitivity	Specificity
T-tau	0.963	0.003	266	100	83
P-tau	0.926	0.007	41	89	83

Table 4.10 Disease duration and CSF concentrations of biomarkers for the heterogeneous FTLD/ALS group

DB-ID	Diagnosis	Duration	T-tau	P-tau	Aβ-40	Aβ-42	Aβ-43	NF-L	α-synuclein	PGRN	YKL-40	Aβ-40/Aβ-42	T-tau/Aβ-42
56	bvFTD	1	502.8	56	15058.2	875.8	39.4	6730.7	1373	5400	123.7	17.2	0.57
57	bvFTD	10	892.2	83.5	10322	442.8	9.9	7581.4	4782	5235	153.5	23.3	2.01
58	bvFTD	1	449.7	53.7	13074.6	761.8	35.9	3883.4	415	4545	230	17.2	0.59
59	bvFTD	2	276.7	28.6	6459.6	452.4	17	12667.1	198	3795	250.1	14.3	0.61
60	bvFTD	3	407.3	49.5	14669.4	938.2	34.9	5642.4	234	5550	195	15.6	0.43
61	bvFTD	4	490	95	-	1086	20.7	15125.6	336	7560	789.4	-	0.45
62	PNFA	4	718.6	72.9	9022	495.8	12.6	8987	237	6600	194.8	18.2	1.45
63	PNFA	1	3443	271.7	13537.4	227.6	6	16998.1	2699	7140	418.3	59.5	15.12
64	SD	2	894	75.8	18725	580.3	27.1	28	1632	4740	255.9	32.3	1.54
65	Pure ALS	1	255.2	26.6	6385.4	499.4	25	15595.2	2804	5745	317.8	12.8	0.51
66	Pure ALS	1	205.6	29.5	11147	607.3	29.9	2591.7	251	5190	173.2	18.4	0.34
67	Pure ALS	1	195.9	28	8473	507.8	14.1	3971.2	213	5865	165.2	16.7	0.39
68	Pure ALS	1	-	-	-	-	7.2	19649.1	1707	5865	211.1	-	-
69	Pure ALS	1	435.6	52.2	16088.2	926.3	41.2	6536.2	1222	13665	251.1	17.4	0.47
70	f-ALS	1	182.9	18.5	4478.2	315.6	13.5	17055.6	241	4575	264.3	14.2	0.58
71	FAV-ALS	1	-	-	-	-	34.1	9500.9	304	10380	518.8	-	-
72	FAV-ALS	2	247	33.4	12526	723.5	28.4	2439.6	263	4860	166.2	17.3	0.34
73	PSP	4	162.9	20	6749.8	531.5	12.3	6111.4	150	3585	83.6	12.7	0.31
74	PSP	5	227.5	25.9	7885.4	525.9	20.4	4223.9	236	4020	200.3	15	0.43
75	PSP	6	114.1	12.6	4335.4	368.3	9.2	5880.7	187	7095	170.5	11.8	0.31
76	ALS+FTD	2	565.5	34.3	6696.8	608.6	28	26	336	12540	341.6	11	0.93

DB-ID: Database identification (De-identified patient ID). Protein levels are given as pg/ml except for YKL-40 and NF-L which are given as ng/ml

5 Discussion, Conclusion and Future Perspectives

The increase or decrease in the levels of some metabolites in CSF usually correlate with physiological or pathologic changes in the brain. These changes sometimes reflect the fundamental mechanisms for neurodegenerative disorders including AD, FTD and ALS. Since CSF serves as the window to the brain, it is logical that changes in the CSF levels of such metabolites have been the bedrock of biochemical biomarker research aimed at early and differential diagnosis of most neurodegenerative disorders. On this premise, this research also sought to explore other metabolites in addition to the core biomarkers in order to access their potential for differential diagnosis of AD, FTD and ALS.

CSF T-tau, P-tau, and Aβ-42 have been designated core biomarkers for AD due to their consistency in distinguishing AD patients from healthy controls (Blennow, 2004). The results for the core biomarkers from this exploratory research have also produced trends similar to those that have been established over the years for the core biomarkers in terms of their potential to distinguish AD patients from healthy controls. That is, both T-tau and P-tau increased while Aβ-42 decreased significantly in AD patients compared to controls. Nevertheless, the 100% discriminatory ability exhibited by the T-tau/Aβ-42 ratio is certainly due to the limited number of samples used in this study. However, the observation of these trends buttresses the reliability of the results obtained from the use of other assays for the analysis of the other metabolites.

Another species of amyloid beta that was introduced in this study is Aβ-43. It is not currently considered a core biomarker for AD. However, its diagnostic performance mimics Aβ-42 in distinguishing AD patients from healthy controls, a pattern which was earlier reported by (Bruggink et al., 2013). Also, Aβ-43 was better at distinguishing AD patients from healthy controls than Aβ-42 as shown by the observation of higher sensitivity and specificity for Aβ-43 compared to Aβ-42. This capability of Aβ-43 to perform better than Aβ-42 was recently demonstrated by Lauridsen et al. (2016) to distinguish MCI patients that eventually developed AD from those who remained stable. Aβ-43 therefore has the potential to complement Aβ-42 for diagnostic purposes.

The aggregation of amyloid beta into plaques which eventually leads to the decrease in the CSF levels of Aβ is known to be more prominent in Alzheimer's disease but not FTD or ALS. Because of this, none of the Aβ species distinguished FTD and ALS patients from healthy controls.

Moreover, Aβ-42 has been reported to accumulate in motor neurons of the spinal cord in ALS patients (Calingasan, Chen, Kiaei, & Beal, 2005) hence reducing its CSF level. This probably accounted for the inability of Aβ-42 to distinguish AD from ALS.

Neither T-tau, P-tau nor their corresponding ratios were able to distinguish between ALS patients and the healthy controls. However, both were able to distinguish FTD from ALS patients. This could be as a result of the anatomical extent of neuronal degradation associated with these disorders. FTD and AD tend to affect a larger portion of the brain (frontal, temporal and parietal lobes) and are therefore associated with massive neurodegeneration while ALS mainly affects upper and lower motor neurons. Thus, the amount of T-tau released from the neurons into the CSF in ALS patients might not result in any significant increase from the levels found in healthy controls. Also, AD and FTD are tauopathies with the formation of NFTs as a major event in AD and the formation of tau inclusion bodies in the majority of FTD brains. Since the FTD group in this study was composed of patients with the clinical diagnosis of bvFTD, SD and PNFA which mostly share a common neuropathology (FTLD-tau), tau levels in CSF would probably not be expected to distinguish AD from FTD, especially since both NFT in AD and tau inclusions in FTD brains are composed of hyperphosphorylated forms of tau. It is also reasonable that although T-tau is known to be a biomarker for neurodegeneration in general, it could not distinguish between ALS patients and healthy controls as ALS is not a tauopathy.

There is currently no consensus on the differential diagnostic capacity of P-tau in terms of distinguishing controls from ALS patients since some studies have recorded a decrease in the levels of P-tau (Grossman et al., 2014) while others showed no change (Wilke et al., 2015) in ALS patients. T-tau and P-tau, which are core biomarkers for AD, also have the potential to be used in the differential diagnosis of FTD and ALS provided the underlying pathology for FTD is a tauopathy (FTLD-tau).

One patient in the FTD group whose clinical diagnosis was PNFA, had an extremely high level of T-tau and P-tau, far beyond levels for the AD group. This patient also had a very low level of Aβ-42 and Aβ-43 which accounted for the corresponding high ratios obtained when they were combined with the tau species and the other metabolites. This patient was probably a mixed dementia patient with massive neuronal degradation and AD pathology. Moreover, some variants of PPA are also known to exhibit AD pathology (Rabinovici et al., 2008). The severity of the neuronal damage also corresponded with the observation of the lowest MMSE score in this patient compared to the rest of the patients.

Neurofilament light

The breakdown of neurons results in an increase in the amount of structural metabolites in CSF. As a structural constituent of neurons, neurofilament light was expected to increase in the CSF of all the patient groups. In consonance with this, the CSF levels of NF-L were high in AD, FTD and ALS patients compared to the healthy controls. However, the inability of NF-L to distinguish between all the disorders under study reinforced the fact that neuronal breakdown occurs in all these diseases, resulting in the increased level of structural molecules in CSF. In contrast to the inability of T-tau and P-tau to distinguish between ALS patients and healthy controls which was partly attributed to the anatomical extent of the disease, NFL distinguished between ALS patients and healthy controls. A possible anatomical explanation for this disparity is that NFL is highly expressed in axons. Therefore, the destruction of long axonal projections of the motor neurons probably increased the CSF levels of NFL regardless of the small portion of the brain affected in ALS compared to AD and FTD. Neuronal degradation is therefore a common factor for FTD, AD and ALS (Scherling 2014, Zetterberg 2015, Weydt 2016). Hence, structural molecules such as NFT may be useful in distinguishing patients with neurodegenerative disorders from healthy controls but not between different neurodegenerative disorders.

α-synuclein

Another pathological mechanism shared by neurodegenerative disorders is the loss of synaptic function. The level of the synaptic molecule α-synuclein is known to decrease with advancing age (Koehler et al., 2015) and also in neurodegenerative disorders such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Results from this study showed no differences in the CSF levels of α-synuclein between patients and healthy controls. Taking into account the fact that all the participants in this study were age matched, the observed equivalent levels of α-synuclein among the patient groups and the controls was not different from what was expected. This observation reduces the possibility of the inclusion of mixed dementia patients with Lewy body pathology in this study. It was also an indication that patients with the clinical diagnosis of PSP had an accurate diagnosis and were not misdiagnosed PD patients. Although there were no PD patients among the participants in this study for comparison, this observation will be useful in the research setting to ensure that patients with extrapyramidal syndromes such as PSP and CBD are not misdiagnosed with atypical Parkinson's disease which could result in erroneous conclusions. Therefore, α-synuclein might not serve as a good candidate

biomarker for the differential diagnosis of AD, FTD and ALS but its levels in CSF should be measured in studies involving extrapyramidal syndromes. It can also be useful in determining mixed dementia patients with either PD or DLB pathology.

Inflammation in AD, FTD and ALS

Neurodegeneration resulting from neuroinflammation is normally implicated in several neurodegenerative disorders such as AD, FTD and ALS. High expression of cytokines (IL-1 and the tumour necrosis factor (TNF) family of cytokines) has been suggested to contribute to neuroinflammation and neurodegenerative diseases (Singhal et al., 2014). Epidemiological data also suggest that the use of non-steroidal anti-inflammatory drugs probably lowers the risk of occurrence of AD among users of such drugs provided the use of the drugs has preceded dementia onset (Anthony et al., 2000; Zandi et al., 2002).

In this study, PGRN and YKL-40, both of which are associated with neuroinflammation were analysed. PGRN did not distinguish between any of the disease groups controls. However, one PSP and the single FTD+ALS patient had higher PGRN levels compared to all the disease groups. The reason for such exceptional levels in individual patients is not known, but could be genetic. The participation of PGRN in inflammation had been observed in studies that demonstrated that PGRN interacts with pro-inflammatory cytokines. In one such study, the loss of function mutation in *GRN* among FTLD patients was associated with an increased serum level of IL-6 among patients compared to controls and non-mutated patients. However, this trend was not found among asymptomatic mutation carriers (Bossu et al., 2011). This interaction between PGRN and pro-inflammatory molecules could accelerate the degradation of neurons and microglia. For this reason, PGRN levels could be a potential biomarker for distinguishing FTD and ALS patients with *GRN* mutations from other neurodegenerative disorders and healthy controls among the Norwegian population.

Another molecule implicated in neuroinflammation is chitinase-3 like-1 (YKL-40). As an inflammation-related glycoprotein, YKL-40 levels were higher in the disease groups than the healthy controls but were not able to distinguish between the disease groups. This is probably due to the involvement of an inflammatory process in all these neurodegenerative diseases. Strikingly, it was only the YKL-40/Aβ-42 and PGRN/Aβ-42 ratios that showed the highest capability of distinguishing ALS patients from healthy controls. This therefore signifies that inflammation plays a major role in ALS pathology.

The relevance of metabolite ratios in differential diagnosis.

Biomarker ratios have consistently been proven to be of diagnostic significance with regards to neurodegenerative disorders. The core biomarker ratios, T-tau/Aβ-42 and Aβ-40/Aβ-42 have been used in several studies to distinguish between AD and controls. In this current study, the best distinguishers between AD, FTD and ALS and controls were all ratios. The only exception was the distinction between FTD and ALS. The most intriguing observation was the high distinction capability of PGRN/Aβ-42 in separating AD patients from controls and also the ability of both YKL-40/Aβ-42 and PGRN/Aβ-42 to distinguish ALS patients from healthy controls, though the raw values for YKL-40 and PGRN performed poorly in this regard. Putting it all together, it is obvious that biomarker ratios in general have the potential to distinguish between AD, FTD, ALS and healthy controls.

Ratios consisting of Aβ-43 were also used in this study for the purpose of comparison to the Aβ-42 ratios. The results indicated that the Aβ-42 ratios exhibited a better diagnostic performance compared to the Aβ-43 ratios. This could be due to the ability of the ratios to serve as buffers by nullifying individual differences which might result in extremely low or high values for some metabolites in some individuals. The strength of the extent of the buffering depends on the strength of correlation that exists between the metabolites that contributed to the ratios. This could be a possible explanation for the high diagnostic performance of Aβ-42 ratios compared to Aβ-43 ratios since the correlation between Aβ-42 and YKL-40 was greater than between YKL-40 and Aβ-43. This property of biomarker ratios may be useful in determining the progression of a particular disease since they are more likely to be resistant to individual differences.

Conclusion

On the basis of current data obtained in this exploratory study, most of the metabolites and their ratios with Aβ-42 and Aβ-43 had the ability to distinguish between AD patients and healthy controls except the CSF concentrations of PGRN, α-synuclein and Aβ-40. However, none of the metabolites performed better than the Tau/Aβ-42 ratio. In contrast, none of the metabolites or their ratios were able to distinguish between AD and FTD. However, T-tau showed a significant difference between FTD and ALS patients. Additionally, the general increase in the levels of YKL-40 in AD, FTD and ALS patients compared to healthy controls is an affirmation of the significant role of inflammation in neurodegeneration. Since inflammation is common to all neurodegenerative disorders, the use of inflammatory molecules as biomarkers may distinguish between patients with neurodegenerative disorders and controls, although this may not necessarily translate into distinguishing neurodegenerative disorders from each order. Furthermore, since AD pathology overlaps with FTD, and FTD pathology overlaps with ALS, a panel of biomarkers may be needed in order to successfully distinguish them from each other based on any individual patient's profile, though a single biomarker would be much desirable.

Limitations of this study and future perspectives

It took 15 years to collect enough clinical samples of CSF to begin this study. This particular project was only the beginning, and 'n' is small in all groups. However, the project has produced promising results and will continue, so n will increase over time. On the other hand, rare cases like FTD+ALS will always belong to a very small group.

Considering the small sample sizes used in this study, it is possible that results that were considered to be statistically significant might prove otherwise in larger studies and vice versa for non-significant results. Also, errors that might have occurred within assays are likely to greatly impact the results. In order to deal with the limitations of this study, future studies with larger sample sizes that might nullify potential errors within individual assays and also permit the use of parametric statistical analysis with higher statistical power may corroborate current results and provide a better understanding of how these biomarkers perform in differential diagnosis of the clinical syndromes of FTD, ALS, and AD. In addition, longitudinal studies that will assess how these biomarkers change over time with respect to these disorders will also be of interest to the field but would require international cooperation to be large enough. Furthermore, the role of inflammation in neurodegeneration requires further investigation. It is obvious that pro-inflammatory molecules which may be common to most inflammatory pathways may obscure their potential as differential diagnostic molecules. Hence, inflammation-oriented research regarding neurodegenerative disorders should aim at identifying disease specific inflammatory molecules.

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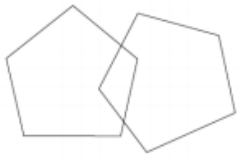
Appendices

Appendix A

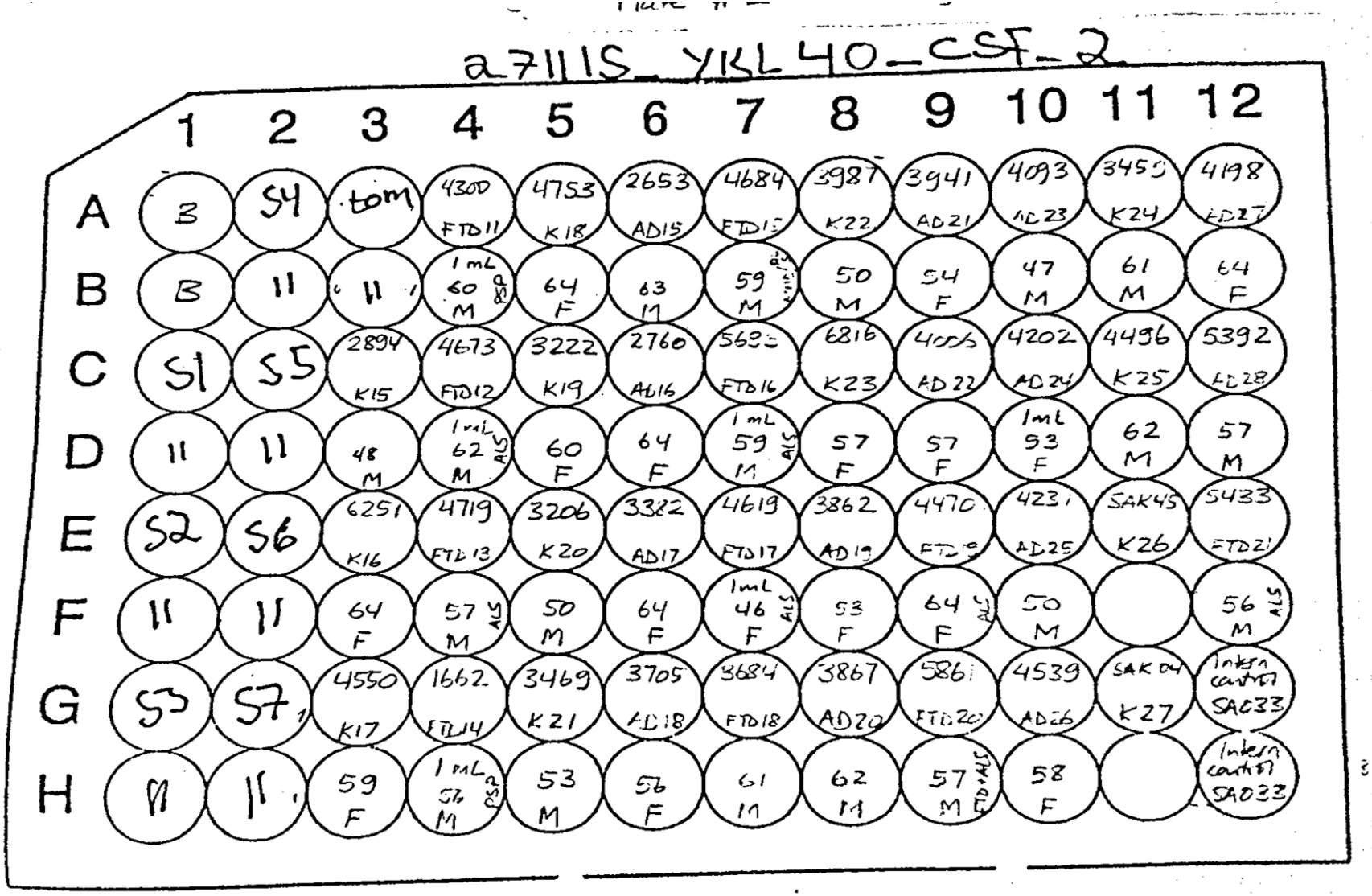
Mini Mental State Examination (MMSE)

STANDARDIZED MINI-MENTAL STATE EXAMINATION (SMMSE)

	QUESTION	TIME ALLOWED	SCORE
1	a. ***What year is this?***	10 seconds	/1
	b. ***Which season is this?***	10 seconds	/1
	c. ***What month is this?***	10 seconds	/1
	d. ***What is today's date?***	10 seconds	/1
	e. ***What day of the week is this?***	10 seconds	/1
2	a. ***What country are we in?***	10 seconds	/1
	b. ***What province are we in?***	10 seconds	/1
	c. ***What city/town are we in?***	10 seconds	/1
	d. ***IN HOME – What is the street address of this house?*** ***IN FACILITY – What is the name of this building?***	10 seconds	/1
	e. ***IN HOME – What room are we in? IN FACILITY – What floor are we on?***	10 seconds	/1
3	**SAY:** ***I am going to name three objects. When I am finished, I want you to repeat them. Remember what they are because I am going to ask you to name them again in a few minutes.*** Say the following words slowly at 1-second intervals - **ball/ car/ man**	20 seconds	/3
4	***Spell the word WORLD. Now spell it backwards.***	30 seconds	/5
5	***Now what were the three objects I asked you to remember?***	10 seconds	/3
6	**SHOW** wristwatch. **ASK:** ***What is this called?***	10 seconds	/1
7	**SHOW** pencil. **ASK:** ***What is this called?***	10 seconds	/1
8	**SAY:** ***I would like you to repeat this phrase after me: No ifs, ands or buts.***	10 seconds	/1
9	**SAY:** ***Read the words on the page and then do what it says.*** Then hand the person the sheet with CLOSE YOUR EYES on it. If the subject reads and does not close their eyes, repeat up to three times. Score only if subject closes eyes	10 seconds	/1
10	**HAND** the person a pencil and paper. **SAY:** ***Write any complete sentence on that piece of paper.*** (Note: The sentence must make sense. Ignore spelling errors)	30 seconds	/1
11	**PLACE** design, eraser and pencil in front of the person. **SAY:** ***Copy this design please.***  Allow multiple tries. Wait until person is finished and hands it back. Score only for correctly copied diagram with a 4-sided figure between two 5-sided figures.	1 minute	/1
12	**ASK** the person if he is right or left-handed. Take a piece of paper and hold it up in front of the person. **SAY:** ***Take this paper in your right/left hand*** (whichever is non-dominant), ***fold the paper in half once with both hands and put the paper down on the floor*** . Score 1 point for each instruction executed correctly. Takes paper correctly in hand Folds it in half Puts it on the floor	30 seconds	/1 /1 /1
	TOTAL TEST SCORE		**/30**

Appendix B

ELISA Plate chart



Appendix C: ELISA Protocols

Appendix C1: T-tau Protocol

INNOTEST hTAU Ag

IVD CE

1. Take the strip-holder with the required number of strips. Place any unused strip in the plastic minigrip bag with the silicagel desiccant.
2. Prepare **Conjugate working solution 1** according to the preparations for use.
3. Add **75 µL** of **Conjugate working solution 1** to each well of the antibody-coated plate.
4. Add **25 µL of each CAL, RVC, and the CSF samples** to duplicate wells of the antibody-coated plate.
5. Make sure that the calibrators, Run Validation Controls, and CSF samples are adequately mixed by carefully tapping the stripholder or by shaking 1 minute at 1000 rpm. **Cover** the strips with an adhesive sealer. **Incubate** overnight (14-18 hrs) in an incubator at 25 ± 2°C.
6. Prepare **Conjugate working solution 2** -aq prefing to the preparations for use-just before the end of step 5.
7. **Wash** each well **5 times** (see 'Directions for washing').
8. Add **100 µL** of **Conjugate working solution 2** to each well. **Cover** the strips with a new adhesive sealer and **incubate** for 30 ± 3 minutes in an incubator at 25 ± 2°C.
9. Prepare **Substrate working solution** just before the end of step 8.
10. **Wash** each well **5 times** (see 'Directions for washing').
11. Add **100 µL** of **Substrate working solution** to each well. **Incubate** for 30 ± 3 minutes at 25 ± 2°C in the dark.
12. To stop the reaction, add **50 µL** of **Stop Solution** to each well in the same sequence and at the same time intervals as the Substrate Solution. Tap the stripholder carefully to ensure optimal mixing.
13. **Read** (within 15 minutes after step 12) the absorbance at 450 nm (single wavelength). For dual wavelength analysis, 620 nm or 690nm can be used as the reference wavelength.

Appendix C2: P-tau Protocol

INNOTEST PHOSPHO-TAU(181P)
IVD CE

1. Take the strip-holder with the required number of strips. Place any unused strip in the plastic minigrip bag with the silicagel desiccant.
2. Prepare **Conjugate working solution 1** according to the preparations for use.
3. Add **25 µL** of **Conjugate working solution 1** to each well of the antibody-coated plate.
4. Add **75 µL of each CAL, RVC, and the CSF samples** to duplicate wells of the antibody-coated plate.
5. Make sure that the calibrators, Run Validation Controls, and CSF samples are adequately mixed by carefully tapping the stripholder or by shaking 1 minute at 1000 rpm. **Cover** the strips with an adhesive sealer. **Incubate** overnight (14-18hrs) in the fridge at 2-8°C.
6. Prepare **Conjugate working solution 2** -according to the preparations for use- just before the end of step 5.
7. **Wash** each well **5 times** (see 'Directions for washing').
8. Add **100 µL** of **Conjugate working solution 2** to each well. **Cover** the strips with a new adhesive sealer and **incubate** for 60 ± 3 minutes in an incubator at 25 ± 2°C.
9. Prepare **Substrate working solution** just before the end of step 8.
10. **Wash** each well **5 times** (see 'Directions for washing').
11. Add **100 µL** of **Substrate working solution** to each well. **Incubate** for 30 ± 3 minutes at 25 ± 2°C in the dark.
12. To stop the reaction, add **50 µL** of **Stop Solution** to each well in the same sequence and at the same time intervals as the Substrate solution. Tap the stripholder carefully to ensure optimal mixing.
13. **Read** (within 15 minutes after step 12) the absorbance at 450 nm (single wavelength). For dual wavelength analysis, 690 nm or 620 nm can be used as the reference wavelength.

Appendix C3: Aβ-40 Protocol

1) Determine wells for reagent blank. Put 100 µL each of "4, EIA buffer" into the wells.
2) Determine wells for test sample blank, test sample and diluted standard.
   Then, put 100 µL each of test sample blank (tube-8), test sample and dilutions of standard (tube-1-7) into the appropriate wells.
3) Incubate the precoated plate overnight at 4°C after covering it with plate lid.
4) Wash each well of the precoated plate 5 times with wash buffer using a washing bottle or a plate washer in following way.
   After shaking off (or aspiration of) the solution in wells, fill each well with wash buffer and shake off the wash buffer completely from the precoated plate. This procedure must be repeated 5 times. Then, drain the precoated plate completely on paper towel.
   In case of using a plate washer, we recommend manually washing in the manner mentioned above at the last one time.
   Please refer to 5) and 6) in SPECIAL ATTENION below, and be careful not to miss a well.
5) Pipette 100 µL of labeled antibody solution into the wells of test samples, diluted standard and test sample blank.
6) Incubate the precoated plate for 30 minutes at 4°C after covering it with plate lid.
7) Wash the precoated plate 7 times in the same manner as 4).
   In case of using a plate washer, we recommend manually washing in the manner mentioned above at the last two times.
8) Take the required quantity of "6, Chromogen" and put it into a disposable test tube. Then, pipette 100 µL from the test tube into every well. Please do not return the rest of used chromogen in the test tube into "6, Chromogen" bottle in order to avoid contamination.
9) Incubate the precoated plate for 30 minutes at room temperature in the dark. The solution of Chromogen will turn blue.
10) Add 100 µL of "7, Stop solution" to all wells. Mix the solution by tapping the side of precoated plate. The solution will turn yellow by addition of "7, Stop solution".
11) Remove any dirt or drop of water on the bottom of the precoated plate and confirm there is no bubble on the surface of the solution. Then, run the plate reader and conduct measurement at 450 nm against a reagent blank.
    The measurement shall be done within 30 minutes after addition of "7, Stop solution".

Appendix C4: Aβ-42 Protocol

INNOTEST β-AMYLOID(1-42)

IVD CE

1. Take the strip-holder with the required number of strips. Place any unused strip in the plastic minigrip bag with the silicag[illegible]siccant.
2. Prepare **Conjugate working solution 1** according to the preparations for use.
3. Add **75 µL Conjugate working solution 1** to each well of the antibody-coated plate.
4. a) *In case a limited number of samples needs to be tested:* add 25 µL of each sample/CAL/RVC to duplicate wells of the antibody-coated plate.
   b) *In case a larger number of samples needs to be tested (more than 6 strips):* transfer 25 µL from each well of the polypropylene plate to duplicate wells on the antibody-coated plate, using a multichannel pipette.
5. Make sure that the calibrators, Run Validation Controls, and CSF samples are adequately mixed by carefully tapping the stripholder or by shaking 1 minute at 1000 rpm. **Cover** the strips with an adhesive sealer. **Incubate** 60 ± 3 minutes in an incubator at 25 ± 2°C.
6. Prepare **Conjugate working solution 2** – according to the preparation for use - just before the end of step 5.
7. **Wash** each well **5 times** (see 'Directions for washing').
8. Add **100 µL** of **Conjugate working solution 2** to each well. **Cover** the strips with a new adhesive sealer and **incubate** for 30 ± 3 minutes in an incubator at 25 ± 2°C.
9. Prepare **Substrate working solution** just before the end of step 8.
10. **Wash** each well **5 times** (see 'Directions for washing').
11. Add **100 µL** of **Substrate working solution** to each well. **Incubate** for 30 ± 3 minutes at 25 ± 2°C in the dark.
12. To stop the reaction, add **50 µL** of **Stop Solution** to each well in the same sequence and at the same time intervals as the Substrate solution. Tap the stripholder carefully to ensure optimal mixing.
13. **Read** (within 15 minutes after step 12) the absorbance at 450 nm (single wavelength). For dual wavelength analysis 620 nm or 690nm can be used as the reference wavelength.

Appendix C5: Aβ-43 Protocol

Amyloid-beta (1-43) High Sensitive ELISA (RE59711)

1) Determine wells for reagent blank. Put 100 µL each of **ASSAYBUF** into the wells.
2) Determine wells for test sample blank, test sample and diluted standard.
   Then, put 100 µL each of test sample blank (tube-8), test sample and dilutions of standard (tube-1-7) into the appropriate wells.
3) Incubate the microtiter plate overnight at 4℃ after covering it with plate lid.
4) Wash each well of the microtiter plate vigorously with Wash Buffer using the washing bottle. Then, fill each well with Wash Buffer and leave the microtiter plate laid for 15-30 seconds. Remove Wash Buffer completely from the microtiter plate by snapping. This procedure must be repeated more than 7 times. Then, remove the remaining liquid from all wells completely by snapping the microtiter plate onto paper towel.
   In case of using a plate washer, after 4 times washing with plate washer, washing with above washing bottle must be repeated 3 times.
5) Pipette 100 µL of Enzyme Conjugate into the wells of test samples, diluted standard and test sample blank.
6) Incubate the microtiter plate for 60 minutes at 4℃ after covering it with plate lid.
7) Wash the microtiter plate 9 times in the same manner as 4).
8) Take the required quantity of **TMB SUBS** into a disposable test tube. Then, pipette 100 µL from the test tube into the wells. Please do not return the rest of the test tube to **TMB SUBS** bottle to avoid contamination.
9) Incubate the microtiter plate for 30 minutes at room temperature in the dark. The liquid will turn blue by addition of TMB Substrate Solution.
10) Pipette 100 µL of **TMB STOP** into the wells. Mix the liquid by tapping the side of microtiter plate. The liquid will turn yellow by addition of TMB Stop Solution
11) Remove any dirt or drop of water on the bottom of the microtiter plate and confirm there is no bubble on the surface of the liquid. Then, run the plate reader and conduct measurement at 450 nm against a reagent blank.
    The measurement shall be done within 30 minutes after addition of TMB Stop Solution.

Appendix C6: α-synuclein Protocol

invitrogen

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Add 50 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
3. Add 50 µL of standards, samples or controls to the appropriate microtiter wells. Samples prepared in Cell Extraction Buffer must be diluted 1:5 or greater in *Standard Diluent Buffer* (for example, 10 µL sample into 40 µL buffer). While a 1:5 sample dilution has been found to be satisfactory, higher dilutions such as 1:10 or 1:25 may be optimal. The dilution chosen should be optimized for each experimental system. .
4. Pipette 50 µL of *Hu α-Synuclein Detection Antibody* solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
5. Cover wells with *plate cover* and incubate for **3 hours at room temperature.** Tap gently on side of plate to thoroughly mix. (See **REAGENT PREPARATION AND STORAGE**, Section B.)
6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING.**
7. Add 100 µL *Anti-Rabbit IgG HRP* Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
8. Cover wells with the *plate cover* and incubate for **30 minutes at room temperature.**
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING.**
10. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
11. Incubate for **30 minutes at room temperature and in the dark.** ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar.** The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
12. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
13. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 2 hours after adding the *Stop Solution*.

Appendix C7: YKL-40 Protocol

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of CHI3L1 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

1. Prepare all reagents, samples, and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of Assay Diluent RD1-34 to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper toweling.
6. Add 200 µL of CHI3L1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or if the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

Appendix C8: PGRN Protocol

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with progranulin antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody **(DET) (see 8.1. Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution **(TMB)**.
☐	12. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 10 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution **(STOP)**. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

Appendix C9: NF-L Protocol

UmanDiagnostics
Rapid detection of brain disease

NF-Light® (Neurofilament light ELISA)

Assay instructions

All assay reagents should be brought to room temperature prior to use.
After each washing cycle the plate should be tapped dry against absorbent paper.
Agitation of the plate at 800 rpm is of HIGH IMPORTANCE.

1. Dilute the CSF samples with equal amount (1+1) of sample diluent to a total minimum volume of 210 µL. The standards reconstituted and diluted according to the standard dilution table are ready to use (i.e. no further dilution should be made).
2. Wash the wells to be used with wash buffer (3x300 µL). The wash buffer added could be either aspirated or removed by knocking the plate against absorbing material immediately before next washing cycle.
3. Add 100 µL of each standard and sample in duplicate. Incubate 1 hour at room temperature (20-25°C) with agitation (800 rpm).
4. Wash the wells with wash buffer (3x300 µL), see point 2.
5. Add 100 µL of freshly diluted tracer (biotin anti-NF-L) antibody to each well. Incubate 45 minutes at room temperature (20-25°C) with agitation (800 rpm).
6. Wash the wells with wash buffer (3x300 µL), see point 2.
7. Add 100 µL of newly diluted conjugate (streptavidin-HRP) to each well. Incubate 30 minutes at room temperature (20-25°C) with agitation (800 rpm).
8. Wash the wells with wash buffer (3x300 µL), see point 2.
9. Add 100 µL of TMB to each well. Incubate 15 minutes at room temperature (20-25°C) with agitation (800 rpm).
10. Add 50 µL of stop reagent to each well and read the absorbance at 450 nm (reference wavelength 620-650 nm).

Appendix D
The Aβ-43 ratios for Aβ-40, T-tau, YKL-40, PGRN and NFL