

# **Title: Fluid-based proteomics targeted on pathophysiological processes and pathologies in neurodegenerative diseases**

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## **Abstract**

Neurodegenerative dementias constitute a broad group of diseases in which abnormally folded proteins accumulate in specific brain regions and result in tissue reactions that eventually cause neuronal dysfunction and degeneration. Depending on where in the brain this happens, symptoms appear which may be used to classify the disorders on clinical grounds. However, brain changes in neurodegenerative dementias start to accumulate many years prior to symptom onset and there is a poor correlation between the clinical picture and what pathology that is the

most likely to cause it. Thus novel drug candidates having disease-modifying effects, *i.e.*, targeting the underlying pathology and changes the course of the disease needs to be defined using objective biomarker-based measures since the clinical symptoms are often non-specific and overlap between different disorders. Further, the treatment should ideally be initiated as soon as symptoms are evident or when biomarkers confirm an underlying pathology (pre-clinical phase of the disease) to reduce irreversible damage to, *e.g.*, neurons, synapses and axons. Clinical trials in the pre-clinical phase bring a greater importance to biomarkers since by definition the clinical effects are difficult or slow to discern in a population that is not yet clinically affected. Here, we discuss neuropathological changes that may underlie neurodegenerative dementias, including how they can be detected and quantified using currently available bio fluid-based biomarkers and how more of them could be identified using targeted proteomics approaches.

*Abbreviations:*

Alzheimer's disease (AD); amyloid  $\beta$  42 (A $\beta$ 42); amyotrophic lateral sclerosis (ALS); cerebrospinal fluid (CSF); chemokine (C-C motif) ligand 2 (CCL2); chitinase-3-like protein 1 (CHI3L1), a 40 kD chitin-binding protein with a YKL domain YKL-40); corticobasal degeneration (CBD); Creutzfeldt–Jakob disease (CJD); chronic traumatic encephalopathy (CTE); dementia with Lewy bodies (DLB); early endosome antigen 1 (EEA1); enzyme-linked immunosorbent assay (ELISA); fatty acid-binding protein (FABP); frontotemporal dementia (FTD); frontotemporal lobar degeneration (FTLD); growth-associated protein 43 (GAP-43); lysosome-associated membrane protein 1 (LAMP-1); lysosome-associated membrane protein 2 (LAMP-2); light chain 3 (LC3); liquid chromatography (LC); mass spectrometry (MS); mild

cognitive impairment (MCI); neurofilament light (NF-L); neurogranin (Ng); neuron-specific enolase (NSE); parallel reaction monitoring (PRM); Parkinson's disease (PD); Parkinson's disease dementia (PDD); phospho-tau (P-tau); positron emission tomography (PET); posterior cortical atrophy (PCA); progressive supranuclear palsy (PSP); stable isotope labelling kinetics (SILK); synaptosomal-associated protein 25 (SNAP-25); tandem mass tags (TMTs); TAR DNA-binding protein 43 (TDP-43); total tau (T-tau); triggering receptor expressed on myeloid cells 2 (TREM2); vascular dementia (VaD); visinin-like protein 1 (VLP-1).

## **Introduction**

Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Parkinson's disease dementia (PDD), dementia with Lewy bodies (DLB), and frontotemporal dementia (FTD) comprise of a group of brain diseases that are distinguished by a gradual decline in cognitive function and ultimately increased mortality. The most common dementia is AD, which makes up 50% to 70% of prevalent neurodegenerative dementia cases (Winblad *et al.* 2016). AD often starts with short-term memory impairment and continues with a progressive loss of cognitive abilities. PD on the other hand initially affects the motor system and symptoms include slowness of movement, tremor, and rigidity; dementia is a common complication as the disease progresses. In FTD, early symptoms often include changes in social behaviour, personality and language difficulties. These dementia-causing diseases can also have symptoms that overlap, making it difficult to set the correct definitive diagnosis. In addition, autopsy findings have shown that several different pathological changes are often found in the same patients indicating a considerable degree of co-morbidity and suggest that the pathological processes might interact and/or be influenced by similar factors promoting accumulation of aberrant proteins, cognitive decline and other clinical symptoms (Barthelemy *et al.* 2016, Lashley *et al.* 2008). Determining which molecular pathology that is most likely to cause the clinical syndrome (potentially in concert with other pathological changes) will be important to develop effective treatments for neurodegenerative dementias. To that end, well-validated pathology-targeted biomarkers will be important.

Even if several of the neurodegenerative diseases are neuropathologically different, they also share several general processes including protein aggregation, defective organelle trafficking, synaptic dysfunction and neuroinflammation (Tofaris & Buckley 2018). All these biological processes together lead to distinct patterns of regional progression of pathology that often can

be linked to the progression of symptoms. For example, the cause of reductions in synaptic function and neuronal connectivity in AD and other neurodegenerative diseases is unknown, but protein aggregation-related toxicity is potentially a key factor. PD, AD, DLB, Huntington's disease, and amyotrophic lateral sclerosis (ALS) are all examples of so-called conformational diseases or proteopathies. Each conformational disease has a characteristic pathologic signature that includes the accumulation of a particular protein as extracellular deposits and/or intracellular inclusions in specific organs. It is believed that a key pathogenic event in conformational diseases is that a specific predisposed protein assumes an atypical three-dimensional conformation, and starts to aggregate and accumulate, intra- or extracellularly. Hypothetically, proteins in non-native conformation could be more resistant to degradation, and have an enhanced tendency to aggregate. Moreover, misfolded proteins have been shown to act as seeds for aggregation of soluble, monomeric proteins with the same or similar amino acid sequence. Old age is an important risk factor for most conformational diseases, possibly due to a compromised ability to degrade or remove abnormal proteins. Other factors that have been shown experimentally to assist protein aggregation are modifications in amino acid sequence, increased protein concentration, or conditions that promote protein unfolding. Even if it is not yet known whether the protein aggregation is a cause or a consequence of disease, most researchers agree that the abnormal protein aggregation *per se* is central to these disorders. Accordingly, attempts to develop effective therapies for many conformational disorders have been directed toward reducing production, facilitating removal, or inhibiting aggregation of the key proteins.

In the clinic, a dementia diagnosis is based on the pattern of cognitive deficits and the history of the illness. Additional investigations to rule out non-degenerative causes of disease, such as blood tests and structural imaging of the brain are also always performed. The prospect of

disease modification has led to a shift towards the use of biomarkers to diagnose neurodegenerative diseases earlier, also in pre-dementia stages of the disease, and also to identify specific forms of disease with higher specificity (Dubois *et al.* 2014, Jack *et al.* 2018). Since cognitive impairment in older individuals can be caused by multiple, degenerative conditions in the same person, a definite diagnosis of a neurodegenerative dementia presently requires histopathological confirmation at autopsy (Hyman *et al.* 2012).

Generally, hypothesis-driven biomarker discovery include targeting a group of proteins believed to be closely linked to a specific biological process, subset of cells, lesion or tissue. When setting up and evaluating bio fluid-based biomarker assays for brain pathologies, it is important to consider if there are alternative sources of the targeted proteins. These sources can be well documented in the literature, such as red blood cells for  $\alpha$ -synuclein, but also proteins previously considered specific for a certain cell type/tissue can with more thorough investigations be found expressed elsewhere. One example is the post-synaptic protein neurogranin that generally is considered as a neuron-specific protein, but which is expressed in high levels in platelets and moderate levels in B-lymphocytes (Diez-Guerra 2010). However, in some cases individual proteins within a protein family can perform similar functions in different locations and carefully designed bio fluid assays can distinguish between them.

Here, we present some of the neuropathological changes that are common in patients with neurodegenerative dementias and review currently existing as well as tentative bio fluid-based biomarkers and targeted proteomics approaches to identify more of them. The results of the most well studied cerebrospinal fluid (CSF) biomarkers in different neurodegenerative diseases are summarized in Table 1. All biomarkers that are discussed are also depicted in Figure 1.

## **Biomarkers for axonal degeneration**

Communication between populations of neurons is essential for brain function and the unique cytoarchitecture and connectivity of axons and dendrites need to be continually maintained. The maintenance depends on appropriate functionality of, e.g., microtubules, molecular motors, and kinase-based signalling pathways (Brady & Morfini 2017, Gibbs et al. 2015, Morfini et al. 2016, Sayas & Avila 2014). Tauopathies exhibit early degeneration of axons suggesting that alterations in kinase-based signalling pathways and deficits in axonal transport associated with such alterations contribute to the loss of neuronal connectivity triggered by pathogenic forms of tau (Kneynsberg *et al.* 2017). Mutations in specific molecular motor protein subunits are implicated as potential causative factors for various neurodegenerative diseases (Kanaan *et al.* 2013, Morfini *et al.* 2009). Loss of axons is also a hallmark of AD, and has been shown to correlate with the onset of cognitive decline even stronger than A $\beta$  pathology. In some proposed models, onset of neurodegeneration marks the toxic phase of A $\beta$  pathology in the pathogenesis of AD (Jack & Holtzman 2013).

### *Cerebrospinal fluid biomarkers*

The structural axonal protein neurofilament light (NF-L) is a general CSF biomarker for brain axonal degeneration/injury (Zetterberg *et al.* 2016). The CSF NF-L concentration is generally higher in AD patients and high concentration is associated with rapid disease progression (Zetterberg et al. 2016). However, increased CSF NF-L concentration is not specific to AD since several other dementias also display increased concentrations, in particular FTD and vascular dementia (VaD) (de Jong *et al.* 2007, Landqvist Waldo *et al.* 2013, Sjogren *et al.* 2000). These results have been confirmed in a large analysis of retrospective data from the Swedish Dementia Registry (Skillback *et al.* 2014), as well as in atypical parkinsonian disorders

(Hall *et al.* 2012, Magdalinou *et al.* 2015). NF-L concentrations are very high in CSF from patients with Creutzfeldt–Jakob disease (CJD) (Steinacker *et al.* 2016).

Another general axonal injury biomarker is T-tau. Increased concentration of CSF T-tau is a well-established finding in AD (Olsson *et al.* 2016) as well in CJD (Riemenschneider *et al.* 2003) and following stroke (Hesse *et al.* 2001). In AD, patients with high CSF concentrations of T-tau often performed worse on cognitive tests and the cognition often deteriorated faster over time (Wallin *et al.* 2010). This indicates that CSF T-tau concentration reflects the intensity of the neurodegenerative process. Other axonal proteins that have been quantified in CSF are visinin-like protein 1 (VLP-1) and fatty acid-binding protein (FABP). These proteins are enriched in neurons, but their association with AD is weaker than CSF T-tau (Olsson *et al.* 2016). Another candidate biomarker for neuronal loss in AD is neuron-specific enolase (NSE), but its CSF levels have not been able to differentiate between controls and AD patients with any substantial effect sizes (Olsson *et al.* 2016). NSE will therefore most probably be of limited clinical utility. One explanation for this may be that NSE (despite its name) is highly expressed in erythrocytes and the measured NSE concentrations are easily confounded by blood contamination (Ramont *et al.* 2005).

### *Blood biomarkers*

Recently, single molecule array (Simoa) assays for measuring low concentrations of T-tau and NF-L in blood have been developed (Andreasson *et al.* 2016). NF-L concentrations in CSF and serum and plasma correlate well and most of the findings of increased CSF NF-L concentrations in AD, FTD, VaD, and in atypical parkinsonian disorders have been replicated in blood (Zetterberg *et al.* 2016). For plasma tau, the results are promising, but the correlation with the corresponding CSF concentration is either absent (Zetterberg *et al.* 2013) or weak (Mattsson *et al.* 2016). Furthermore, tau concentrations are for unknown reasons higher in plasma than in



serum (unpublished observation in our own research). Plasma T-tau levels are increased in AD, but less compared to CSF and there is no detectable change in the MCI stage of the disease (Zetterberg et al. 2013, Mattsson et al. 2016).

### **Biomarkers for tau pathology**

The major components of neurofibrillary tangles in AD and other so called tauopathies, including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD) and chronic traumatic encephalopathy, is hyper phosphorylated and truncated tau proteins (Grundke-Iqbal *et al.* 1986). Tau is abundant in neurons and highly soluble. Its main function is to bind to and stabilize microtubules in neuronal axons.

Tau exists in six different isoforms, differing by the inclusion of exons 2 and 3 in the N-terminal region and exon 10 in the microtubule-binding region. Tau in brain tissue exists with a large number of post-translational modifications including phosphorylation, glycosylation, and oxidation (Hernandez & Avila 2007). Of a potential 80 serine and threonine phosphorylation sites in the longest isoform, 39 different sites have been verified in the brain (Hanger *et al.* 2007).

### *Cerebrospinal fluid biomarkers*

ELISA assays targeting mid-domain phospho-tau (P-tau) epitopes are currently in use in the clinic. The CSF P-tau concentration is increased in AD patients (Olsson et al. 2016) and correlates weakly with neurofibrillary tangle pathology in AD (Buerger *et al.* 2006, Seppala *et al.* 2012). This finding has recently been replicated in tau positron emission topography (PET) imaging studies of AD patients (Chhatwal *et al.* 2016), although the association is not as strong

as between CSF amyloid  $\beta$  consisting of 42 amino acids (A $\beta$ 42) with amyloid PET (Hansson *et al.* 2018).

A key question is why only AD, and no other tauopathies, such as FTD and related disorders such as PSP, shows increased CSF P-tau concentration. One explanation could be that the tau pathology in these diseases involve disease-specific tau phosphorylation or other modification/processing that the available assays do not recognize. Another conceivable explanation could be that the pathological process that causes the release of P-tau to CSF is more widespread and severe in AD than in other tauopathies. Currently, since no other condition (except for herpes encephalitis and superficial CNS siderosis) features a systematic increase in CSF P-tau it is considered to be the most specific biomarker for AD (Zetterberg 2017).

The relatively low concentration of CSF tau presents a challenge for analysis using liquid chromatography (LC) mass spectrometry (MS). Using targeted enrichment by immunoprecipitation followed by tryptic digestion, CSF tau was identified by LC-MS (Portelius *et al.* 2008). Using this approach, a targeted mass spectrometric method for quantification of several tau tryptic peptides was later demonstrated (McAvoy *et al.* 2014). Results showed a significant difference between AD patients and healthy controls, and the method correlated well with immunoassay results for T-tau. However, the concentrations obtained by mass spectrometry were 17-25 times higher. A possible explanation for this is that the abundance of processed tau forms in CSF, ranging from large protein fragments (Meredith *et al.* 2013) to small, endogenous peptides (Hansson *et al.* 2017). Immunoassays will only recognize some of these fragments depending on whether they contain the epitopes recognized by the antibodies or not. However, many of the fragments will produce the same cleavage peptides upon tryptic digestion and thus contribute to the LC-MS signal.

More recently, an antibody-independent method based on pre-clearing CSF by trichloroacetic acid precipitation and solid-phase extraction of tau, followed by tryptic digestion and targeted LC-MS by parallel reaction monitoring (PRM), was reported that enabled quantification of 18 tryptic peptides covering a large part of the tau amino acid sequence (Barthelemy et al. 2016). While all peptides correlated to total-tau (measured by ELISA), peptides in the central core region were found to be elevated relative to the N- and C-terminal regions, particularly in AD but also in PSP and DLB as well as in controls. In AD, the concentrations of the peptides in the terminal regions were also elevated relative to controls, while this was not observed for the other diseases.

Parallel LC-MS measurement of CSF tau and A $\beta$ 1-42 was recently reported, showing good sensitivity and specificity for separating AD from controls (Pottiez *et al.* 2017). Due to the relatively low abundance of tau in CSF, detection sensitivity presents a major hurdle for the detection of protein modifications of low stoichiometry such as phosphorylation, and it has been difficult to verify the presence of many of the post-translational tau modifications existing in brain. An innovative approach, denoted TMTcalibrator Plus, was used to address this problem by spiking CSF tryptic digests, labeled with tandem mass tags (TMTs), with differently TMT-labeled digests of AD brain tissue extracts (in which phosphorylated tau forms are more abundant), CSF tau peptides below abundances that allowed their identification could be quantified (Russell *et al.* 2017b). Using this method, 47 phosphopeptides covering 31 phosphorylation sites were quantified, albeit in a small number of AD patients and controls, demonstrating the potential of the approach. However, this finding needs to be replicated in larger studies to confirm the results.

In a recent study, a mass spectrometry-based method denoted *stable isotope labelling kinetics* (SILK) (Bateman *et al.* 2007) was employed to measure the kinetics of multiple isoforms of tau in the central nervous system (Sato *et al.* 2018). SILK is based on labelling study participants

(or cells) with a stable isotope-labelled tracer  $^{13}\text{C}_6$ -leucine, and quantifying its incorporation into central nervous system (CNS) proteins over time in CSF, enabling determination of production rate and half-life of tau. The tau production rate in AD patients was found to correlate with amyloidosis, as measured by amyloid PET imaging. This method can shed light on the processing and turnover of specific isoforms and may also be useful in clinical trial to monitor the effect of anti-tau interventions.

Recently, the structures of tau paired helical filaments (PHFs) and straight filaments (SFs) were determined at a high resolution by cryo-electron microscopy (Fitzpatrick *et al.* 2017). Knowledge of the atomic structure of tau filaments may assist in rational design of specific inhibitors of tau aggregation, as well as tracer compounds.

#### *Blood biomarkers*

No reliable assay for measuring blood biomarkers for neurofibrillary tangle pathology is currently available. However, recent studies have reported increased P-tau concentrations in neuron-derived exosomes isolated from serum using antibodies directed against neuron-enriched proteins (Shi *et al.* 2016, Winston *et al.* 2016). These exosomes are isolated from serum using antibodies directed against neuron-enriched proteins. The exosomes are then washed and lysed and their tau content is measured using immunochemical assays.

#### **Biomarkers for synaptic degeneration**

There is mounting evidence that synaptic loss is one of the earliest events in neurodegenerative diseases, particularly AD (Masliah 2001, Selkoe 2002). Furthermore, the synaptic loss is more robustly correlated to cognitive decline in AD than the numbers of plaques or tangles and the rate of decline is directly related to the duration of dementia (Blennow *et al.* 1996, Terry *et al.* 1991). The process of synaptic transmission involves specific synaptic proteins that have

important functions in regulation of the neurotransmitter release including, *e.g.*, vesicle trafficking, docking, and fusion to the synaptic plasma membrane.

### *Cerebrospinal fluid biomarkers*

The significant role of synapses in the disease pathology and progression of both neurodegenerative and neuropsychiatric diseases has prompted a keen interest in detecting synaptic proteins in bio fluids from individual patients. Until recently, most studies on the correlation between cognitive decline in neurodegenerative diseases and synapse loss involved post-mortem tissue. However, during the last five years several research groups have been successful in detecting synaptic proteins in CSF. To date, neurogranin (Ng) is the best-established CSF biomarker for synapse loss or dysfunction associated with AD. Ng is a small neuron-enriched dendritic protein that is involved in long-term potentiation of synapses, particularly in the hippocampus and basal forebrain (Represa *et al.* 1990). Various studies using both ELISAs and mass spectrometry based assays have shown that the levels of Ng in CSF correlates with the severity of brain atrophy, and amyloid pathology, as well as the cognitive decline, and reduction in glucose metabolism (Hellwig *et al.* 2015, Kester *et al.* 2015, Kvartsberg *et al.* 2015a, Kvartsberg *et al.* 2015b, Portelius *et al.* 2015, Tarawneh *et al.* 2016, Thorsell *et al.* 2010). Interestingly, several independent studies have also confirmed that the CSF Ng concentration is only increased in AD, and not in other neurodegenerative disorders (Janelidze *et al.* 2016a, Portelius *et al.* 2018, Remnestal *et al.* 2016, Wellington *et al.* 2016).

Other promising markers of synaptic dysfunction are synaptosomal-associated protein 25 (SNAP-25), synaptotagmin-1, and growth-associated protein 43 (GAP-43) (Brinkmalm *et al.* 2014, Ohrfelt *et al.* 2016b). SNAP-25 and synaptotagmin-1 are both brain-enriched proteins involved in vesicular trafficking. Other members of the SNAP and synaptotagmin families are expressed ubiquitously or enriched in other tissues *e.g.* SNAP-23 in placenta or synaptotagmin-

2 in endocrine glands, but the amino acid sequence differences between the members of the families are sufficient to allow specific detection of e.g. SNAP-25 and synaptotagmin-1 with carefully designed immunoassays or mass spectrometry-based assays. For example, using enrichment with monoclonal antibodies and mass spectrometry-based quantitation with high resolution PRM-MS it has been shown that the CSF concentrations of the SNARE complex protein SNAP-25 are significantly higher in AD, also at the very early stages of the disease (Brinkmalm *et al.* 2014). Using a similar PRM-MS approach it was recently shown that the CSF concentrations of the pre-synaptic vesicle protein synaptotagmin-1 are increased in patients with AD and MCI compared to controls (Ohrfelt *et al.* 2016b). MS-based targeted proteomics has also been used to find increased levels in MCI patients of other proteins involved in synapse formation and stabilization, such as neuronal pentraxin and neuroligins that mediate signalling across the synapse (Duits *et al.* 2018, Brinkmalm *et al.* 2018). This finding was mainly attributable to those patients who at follow-up had progressed to AD dementia which is contrast to AD patients which have slightly decreased CSF neuroligin concentrations compared with control subjects. Using ELISA assays to measure SNAP-25, Ng, and Rab3a in CSF from controls and PD, including a subset of drug-naïve patients it has been shown that the concentrations of SNAP-25 and Ng in PD patients were increased, but no significant disease-driven changes in the concentration of Rab3a was observed (Berezki *et al.* 2017). Another potentially interesting protein is growth-associated protein 43 (GAP-43) which is a marker of neuronal plasticity and critical to the regulation of neuronal morphology and communication (Benowitz & Routtenberg 1997). GAP-43 levels in CSF have been measured with ELISAs and a suspension bead array and elevated levels were found in AD patients (Remnestal *et al.* 2016, Rymo *et al.* 2017, Sjogren *et al.* 2001). However, these studies need to be independently replicated in larger and more diverse cohorts.

### *Blood biomarkers*

For synaptic dysfunction, there are no reliable blood biomarkers yet. Plasma Ng concentrations are unchanged in AD compared to controls even though the concentrations in CSF from the same patients were increased (De Vos *et al.* 2015, Kvartsberg *et al.* 2015b). Moreover, the levels of Ng in CSF and plasma did not correlate which may be due to that largest source of Ng in plasma is produced in the periphery.

Ng and five other synaptic proteins have also been extracted from neuronal-derived exosomes in plasma and quantified by ELISAs (Goetzl *et al.* 2016). In this study, the levels of Ng, synaptophysin, synaptopodin, and synaptotagmin-2 were found to be reduced in patients with FTD and AD compared to controls, but GAP-43 and synapsin-1 levels were lower only in patients with AD.

### **Biomarkers for $\alpha$ -synuclein pathology**

The presynaptic neuronal protein  $\alpha$ -synuclein is the most abundant protein of the intracellular aggregates found in Lewy bodies in PD, DLB and in glial cytoplasmic inclusions of multiple system atrophy (MSA) (review by Dehay *et al.* (Dehay *et al.* 2015)). These diseases are referred to as “synucleinopathies”. Findings of missense mutations in the gene encoding for  $\alpha$ -synuclein and multiplications of the  $\alpha$ -synuclein-encoding gene *SNCA* in families with PD also suggest that the conversion of  $\alpha$ -synuclein from soluble monomers to aggregated, insoluble forms in the brain is an important event in the pathogenesis of synucleinopathies, as reviewed by Fasano *et al.* (Fasano & Lopiano 2008). Interestingly, a truncated form of  $\alpha$ -synuclein (termed non-amyloid component or NAC) is also a major component in the amyloid plaques in patients with AD (Bisaglia *et al.* 2006). Autopsy studies have shown that a sizable part of the AD patients also have Lewy-body pathology (Schneider *et al.* 2009). A complication from a biomarker point of view is that  $\alpha$ -synuclein is widely expressed in healthy individuals. A possible way to

circumvent this problem is to target Lewy-body pathology specific forms of  $\alpha$ -synuclein. Ideally, such  $\alpha$ -synuclein forms are not present or at least present at very low concentrations in healthy controls. One such tentative disease-specific form could be oligomeric  $\alpha$ -synuclein. Another proposed biomarker candidate is  $\alpha$ -synuclein phosphorylated at serine 129 (Landeck *et al.* 2016, Schmid *et al.* 2013). However, Lewy bodies have also been found in neurologically asymptomatic elderly individuals (Parkkinen *et al.* 2005). This is similar to the situation with AD and presence of plaque pathology in healthy controls and has yet not been fully investigated or explained.

#### *Cerebrospinal fluid biomarkers*

CSF  $\alpha$ -synuclein has been measured with various techniques, including different types of immunoassays, and targeted MS-based proteomics (Fairfoul *et al.* 2016, Hong *et al.* 2010, Mollenhauer *et al.* 2017, Parnetti *et al.* 2016, Simonsen *et al.* 2016). Most available assays measure total amount of  $\alpha$ -synuclein but there are also assays targeting oligomeric and phosphorylated  $\alpha$ -synuclein or other modified forms (Fukumoto *et al.* 2010, Hansson *et al.* 2014, Oeckl *et al.* 2016, Schmid *et al.* 2013, Shahnawaz *et al.* 2017, Tokuda *et al.* 2010). Despite considerable variation in assay specificity and performance, the general pattern that has emerged is a decrease in total  $\alpha$ -synuclein and an increase in phosphorylated and oligomeric  $\alpha$ -synuclein in PD (Kang *et al.* 2016, Parnetti *et al.* 2016). Despite the consistency of the overall trend of decreased concentrations of  $\alpha$ -synuclein in CSF, there is a large overlap of individual values, which significantly decreases clinical usefulness (Lewczuk *et al.* 2017). In AD and MCI patients, several studies have found slightly increased levels of CSF  $\alpha$ -synuclein that correlates strongly with CSF T-tau (Mollenhauer *et al.* 2011, Slaets *et al.* 2014, Tateno *et al.* 2012). This strong correlation indicates that  $\alpha$ -synuclein is a non-specific marker of neurodegeneration (Ohrfelt *et al.* 2009). Interestingly, a recent study suggested that the CSF tau/ $\alpha$ -synuclein ratio



could aid in discriminating patients with dementia with Lewy bodies and controls (Llorens *et al.* 2016). Similarly, a longitudinal study of CSF biomarkers in AD and MCI patients found that lower CSF  $\alpha$ -synuclein concentrations than expected compared to tau levels indicated a faster progression of the disease (Wang *et al.* 2018).

### *Blood biomarkers*

Early involvement of the peripheral nervous system points toward PD as a systemic disease (Braak & Del Tredici 2017, Visanji *et al.* 2017). There is therefore a big interest in finding biomarkers of spread of Lewy body pathology in easily accessible bio fluids such as blood. However,  $\alpha$ -synuclein is highly expressed in healthy individuals and >95% of total blood  $\alpha$ -synuclein are derived from red blood cells (Barbour *et al.* 2008). So far conflicting results have been reported for  $\alpha$ -synuclein levels in plasma (El-Agnaf *et al.* 2006, Lee *et al.* 2006, Li *et al.* 2007, Park *et al.* 2011). Similarly, there are conflicting reports of increased or no change in plasma levels of oligomeric  $\alpha$ -synuclein in PD (El-Agnaf *et al.* 2006). Blood contamination of CSF is also a major problem when measuring of  $\alpha$ -synuclein levels in CSF (Mollenhauer *et al.* 2017).

### *Biomarkers in other bio fluids*

Lewy-body pathology has been reported to occur in early stages of PD in the submandibular gland and gut (Beach *et al.* 2016, Uchiyama & Giasson 2016, Uchiyama *et al.* 2016). Studies using fine-needle biopsy or post-mortem tissues from the submandibular salivary gland have been able to differentiate PD from controls with good sensitivity and specificity (Visanji *et al.* 2017). Hence, saliva, which is typically free of blood contamination, could possibly be an ideal bio fluid to study potential biomarkers for PD diagnosis and progression. Recently,  $\alpha$ -synuclein has been shown to be present in saliva, and a study found differences between PD and control

(Devic *et al.* 2011). Another study monitored cheek epithelium, which forms the majority of the cellular component of saliva and is readily accessible clinically, but found no difference between PD and control (Stewart *et al.* 2014).

### **Biomarkers for amyloid $\beta$ pathology**

Extracellular plaques consisting of  $A\beta$  peptides together with neurofibrillary tangles in the medial temporal lobe and cortical areas of the brain are pathological hallmarks of AD and believed to lie at the core of AD (Braak & Braak 1996, Blennow *et al.* 2006). These changes together with inflammatory and oxidative factors ultimately lead to synaptic and neuronal degeneration (Serrano-Pozo *et al.* 2011).

In 1985, it was discovered that plaques consists mainly of a 4-kDa  $A\beta$  peptide (Masters *et al.* 1985), but it is now known that other major species of  $A\beta$  in the plaques include  $A\beta_{4-42}$  and  $A\beta_{1-40}$  (Jarrett *et al.* 1993, Portelius *et al.* 2010).  $A\beta$  is a 36-43 amino acid peptide originating from the amyloid precursor protein (APP) following enzymatic cleavage of  $\beta$ - and  $\gamma$ -secretase where  $A\beta_{1-38}$ ,  $A\beta_{1-40}$  and  $A\beta_{1-42}$  are the most abundant. While  $A\beta_{1-42}$  is secreted mainly from neurons, APP is metabolized by many cell types (Niederwolfsguber *et al.* 1998). Plaques have also been shown to contain deformed neurites as well as being surrounded by microglia and astrocytes (Perl 2010). Rare familial variant of AD (FAD), where mutations in the *APP* as well as *presenilin 1 and 2* (which are the catalytic subunits of the  $\gamma$ -secretase) genes, lead to altered levels of  $A\beta$  (Mullan *et al.* 1992, van Duijn *et al.* 1991, Chartier-Harlin *et al.* 1991, Selkoe 2001). Taken together with the findings of cerebral  $A\beta$  plaque pathology in patients with Down syndrome, who have one extra of the APP containing chromosome 21 (trisomy 21), this led to the hypothesis called the “amyloid cascade hypothesis”, according to which  $A\beta$  is a driving force in the disease process (Hardy & Higgins 1992, Hardy 2009). The hypothesis states that

an increased level of A $\beta$  is the initiating event in the disease that ultimately leads to neuronal degeneration and dementia.

### *Cerebrospinal fluid biomarkers*

Since the discovery of A $\beta$  accumulation in plaques there has been continuous search for biomarkers reflecting the plaque pathology in CSF as CSF is in direct contact with the brain (Masters et al. 1985). In 1992, A $\beta$  was detected in human CSF (Seubert *et al.* 1992) followed by several studies reporting on detection of A $\beta_{1-42}$  as well as the C-terminally truncated A $\beta_{1-40}$  in CSF (Motter *et al.* 1995, Roher *et al.* 2009). The concentrations of CSF A $\beta_{1-40}$  and A $\beta_{1-42}$  in AD patients versus healthy control subjects have been extensively studied, showing that patients with AD have a decreased concentration of CSF A $\beta_{1-42}$  (Olsson et al. 2016). These data supports the hypothesis that A $\beta_{1-42}$  is decreased in CSF of AD patients due to cortical deposition in amyloid plaques (Strozyk *et al.* 2003), which has been verified by several studies using both autopsy and in vivo amyloid PET imaging (Olsson et al. 2016, Blennow *et al.* 2015). Reduced concentrations of CSF A $\beta_{1-42}$  are evident already in patients with mild cognitive impairment (MCI), as well as in pre-clinical stages of AD (Olsson et al. 2016, Bateman *et al.* 2012). A decrease in A $\beta_{1-42}$  concentration in CSF has also been observed in dementia with Lewy bodies (DLB), another neurodegenerative disorder commonly accompanied by cerebral A $\beta$  aggregates (Abdelnour *et al.* 2016).

Measurement of the A $\beta_{1-42}$  concentration in CSF can be performed using antibody-based techniques, such as enzyme-linked immunosorbent assay (ELISA) (Motter et al. 1995, Tamaoka *et al.* 1997, Vanderstichele *et al.* 1998, Sunderland *et al.* 2003) and by antibody-independent techniques, such as mass spectrometry (Leinenbach *et al.* 2014, Korecka *et al.* 2014). ELISAs or immunoassays on other technology platforms are currently used in clinical

routine in some countries and new and fully automated A $\beta$ <sub>1-42</sub> assays are being introduced with significantly better reproducibility and lower lot-to-lot variations compared to manual assays (Bittner *et al.* 2016, Janelidze *et al.* 2016c, Vanderstichele *et al.* 2016, Janelidze *et al.* 2017).

It has been suggested that there is an inter-individual variation of A $\beta$  (including A $\beta$ <sub>1-42</sub>) concentrations in CSF based on the total amount of A $\beta$  peptides produced (as an effect of different efficiency of APP processing). Measurement of several A $\beta$  forms, such as A $\beta$ <sub>1-38</sub>, A $\beta$ <sub>1-40</sub> and A $\beta$ <sub>1-42</sub>, in a single CSF sample can be performed using multiplexed immunoassays (Janelidze *et al.* 2016c, Vanderstichele *et al.* 2016) as well as LC-MS methods (Lame *et al.* 2011, Pannee *et al.* 2013, Korecka *et al.* 2014). By normalizing the A $\beta$ <sub>1-42</sub> concentration to A $\beta$ <sub>1-40</sub>, the most abundant form among the A $\beta$  peptides, the ratios between these different forms have been shown to increase the separation between AD patients and controls using both immunoassays (Wiltfang *et al.* 2007, Hansson *et al.* 2007, Zetterberg *et al.* 2008, Lewczuk *et al.* 2015, Janelidze *et al.* 2016c, Vanderstichele *et al.* 2016) and LC-MS (Pannee *et al.* 2013, Pannee *et al.* 2016, Janelidze *et al.* 2017).

Many different forms of A $\beta$  have been identified and characterized where the concentrations in CSF of several of these, including A $\beta$ <sub>1-16</sub>, A $\beta$ <sub>1-34</sub>, A $\beta$ <sub>5-40</sub> and A $\beta$ <sub>1-42</sub>, have been shown to be influenced by drugs and disease processes (Portelius *et al.* 2011). Recently A $\beta$ <sub>1-17</sub> was identified by MS and quantified using ELISA in both CSF and plasma with possible diagnostic value (Perez-Grijalba *et al.* 2015). It has also been shown that modified forms of A $\beta$  induce A $\beta$  aggregation and plaques formation (e.g., Asp7 isomerization or phosphorylation at Ser8) (Kozin *et al.* 2013, Indeykina *et al.* 2011, Kulikova *et al.* 2014).

### *Blood biomarkers*

A $\beta$  can be measured in plasma, results however show no or low correlation with cerebral  $\beta$ -amyloidosis when using immunoassays (Janelidze *et al.* 2016b, Olsson *et al.* 2016), probably due to the fact that the plasma A $\beta$  concentrations might be influenced by extra-cerebral secretion from platelets, skeletal muscles and vascular walls (Kuo *et al.* 2000, Li *et al.* 1998, Van Nostrand & Melchor 2001) which does not seem to reflect plaque pathology (Zetterberg 2015). However, promising result from three recent studies utilizing quantitative MS showed a clinically significant correlation between plasma A $\beta$  and cerebral  $\beta$ -amyloidosis (Nakamura *et al.* 2018, Kaneko *et al.* 2014, Ovod *et al.* 2017).

In addition to A $\beta$ , some studies have reported on other plasma proteins associated with brain amyloid load, such as interleukin 17, pancreatic polypeptide Y, apolipoprotein A1, IgM, chemokine ligand 13,  $\alpha$ 2-macroglobulin, vascular cell adhesion protein 1, and complement proteins (Voyle *et al.* 2015, Burnham *et al.* 2016, Westwood *et al.* 2016). However, these data have not been replicated or examined in relation to neurodegenerative dementias other than AD and should presently be interpreted with caution. Further, the results have been obtained from multi-marker panels and a biological model of these associations is currently lacking.

### **Biomarkers for TDP-43 pathology**

TDP-43 is a highly conserved and ubiquitously expressed protein, encoded by the *TARDBP* gene. It is involved in gene transcription, exon splicing, regulation of mRNA stability, mRNA biosynthesis, and formation of nuclear bodies (Ou *et al.* 1995, Wang *et al.* 2002). Aggregated forms of TDP-43, truncations and mislocalisation from the nucleus into the cytoplasm, lead to toxic forms of TDP-43 and loss of the physiological function of the protein, which has been suggested to cause neurodegeneration in amyotrophic lateral sclerosis (ALS), frontotemporal lobar degeneration (FTLD) and AD (Arai *et al.* 2006, Hasegawa *et al.* 2008, Josephs *et al.* 2016, Neumann *et al.* 2006, Tremblay *et al.* 2011, Tsuji *et al.* 2012, Yokota *et al.* 2010). TDP-43

pathology has also been described as a consequence of ageing since it has been shown that TDP-43 changes increased with age in control subjects, but was absent in subjects below age of 65 (Geser *et al.* 2010).

Today it is established that aggregated TDP-43 protein is one of the main pathological hallmarks of FTD and ALS. In addition, hyperphosphorylated TDP-43 proteinopathy has also been associated with several other neurodegenerative diseases including AD, in which TDP-43 pathology is found in more than 25% of the cases (Irwin *et al.* 2015, McAleese *et al.* 2017, Wilson *et al.* 2011), and with greater cognitive impairment and dementia independently of AD pathology (Josephs *et al.* 2008). Today, there is no reliable CSF or blood test available for TDP-43 pathology and explorative proteomic studies have so far failed in identifying TDP-43 in blood or CSF. One reason for this could be that TDP-43 has several post-translational modifications including phosphorylation, ubiquitylation and oxidation (Kametani *et al.* 2016). It has also been shown in brain tissue extract that TDP-43 can be truncated generating several C-terminal fragments but the fragments correlation to disease status remain to be elucidated (Lee *et al.* 2011). However, there are some reports on how TDP-43 performs as a biomarker in body fluids.

#### *Cerebrospinal fluid biomarkers*

Using immunoblot it has been shown that a 45-kDa band was significantly increased in ALS and FTLD patients compared to controls, but there was a pronounced overlap between the groups (Steinacker *et al.* 2008). This finding has been replicated in a recent study in which CSF TDP-43 was measured by ELISA and TDP-43 was again shown to be increased in both ALS and FTD compared to controls (Bourbouli *et al.* 2017). Using ELISA to quantify TDP-43 in ALS patients, Guillain-Barre syndrome (GBS) patients and controls the CSF TDP-43

concentrations were shown to be significantly increased in ALS compared to controls and GBS and it was speculated that the concentrations may increase specifically and more rapidly in ALS (Hosokawa *et al.* 2014, Junttila *et al.* 2016, Kasai *et al.* 2009, Noto *et al.* 2011). FTLD includes the pathological spectrum underlying FTD and the two major groups are FTLD-tau (accumulation of tau) and FTLD-TDP (accumulation of TDP-43) (Mackenzie *et al.* 2010). However, a recent study did not find any difference in the TDP-43 levels between FTLD-TDP and FTLD-tau patients while the P-tau/T-tau ratio was significantly different between the groups (Kuiperij *et al.* 2017). Since the protein is expressed throughout the whole body most is probably blood-derived. One study investigated purified exosomes from CSF and quantified two tryptic peptides from TDP-43 using mass spectrometry (Feneberg *et al.* 2014). However, there were no differences between controls, ALS and FTLD patients using this approach.

#### *Blood biomarkers*

In the first study showing that TDP-43 can be detected in plasma from control subjects, AD patients and FTD patients, it was shown that the TDP-43 levels were significantly increased in FTD compared to controls (Foulds *et al.* 2008). In a follow-up study it was shown that the levels of phosphorylated TDP-43 in plasma correlate with the extent of TDP-43 brain pathology in FTLD (Foulds *et al.* 2009). Similarly, it has also been shown that the phosphorylated TDP-43 levels in plasma may be increased in some genetic forms of FTD (*C9orf72* expansion carriers and *GRN* mutation carriers) which previously have been shown to be associated with TDP-43 proteinopathies (Suarez-Calvet *et al.* 2014). One study has shown that the TDP-43 levels in plasma are significantly increased in patients with ALS compared to control and a positive correlation with age in patients and controls was reported (Verstraete *et al.* 2012).

In conclusion, the findings reported so far needs to be replicated and methods able to distinguish between blood versus CNS derived TDP-43 needs to be developed. In addition, if TDP-43 is validated as a biomarker for TDP-43 proteinopathy, its specificity for a certain neurodegenerative disease needs to be carefully elucidated.

### **Biomarkers for dysfunctional proteostasis**

Protein aggregates are characteristics of many neurodegenerative disorders, including AD (plaques and tangles), PD (Lewy bodies), and FTD (TPD-43 pathology). Formation of aggregates may be caused by impaired production or altered turnover, indicating dysfunctional proteostasis. AD exhibits decreased turnover of endocytic and autophagic vesicles as well as accumulation of endo-lysosomal proteins and ubiquitin in the brain. PD has been demonstrated to be connected to mutations associated with lysosomal storage disorders and decreased lysosomal protein expression. Also in FTD, disease causing mutations and genetic profiles suggest altered autophagic function and involvement of the endo-lysosomal system.

#### *Cerebrospinal fluid biomarkers*

There are a limited number of studies conducted to identify biomarkers which are connected with the lysosomal system. In a handful studies monomeric ubiquitin (Oeckl *et al.* 2014, Simonsen *et al.* 2007, Sjödin *et al.* 2017, Constantinescu *et al.* 2010), total ubiquitin (Blennow *et al.* 1994, Heywood *et al.* 2015), and paired helical filament ubiquitin (Wang *et al.* 1991) have been evaluated as potential biomarkers for AD and other neurodegenerative diseases. In all studies where AD was investigated (Sjödin *et al.* 2017, Simonsen *et al.* 2007, Heywood *et al.* 2015, Oeckl *et al.* 2014, Blennow *et al.* 1994, Wang *et al.* 1991) it exhibited increased CSF concentrations compared to controls. In a study comparing MCI-AD to MCI-stable and controls (Simonsen *et al.* 2007) MCI-AD had increased CSF concentrations. Increased CSF



concentrations has also been observed in VaD (Blennow et al. 1994), CJD (Oeckl et al. 2014), and LBD (Heywood et al. 2015) compared to controls, while for ALS, primary progressive aphasia (PPA), behavioral variant of frontotemporal dementia (bvFTD), and FTLD (which included PPA, PSP and bvFTD) (Oeckl et al. 2014) as well as CBD and MSA (Constantinescu et al. 2010) no significant difference was detected in comparison to controls. Measurements on PD and PSP have given contrary results with some studies reporting no difference between PD and controls (Constantinescu et al. 2010, Oeckl et al. 2014) while others have reported higher PD CSF concentrations compared to controls (Sjödín et al. 2017, Heywood et al. 2015). Similarly, ubiquitin levels in PSP have been reported to be similar as to controls (Oeckl et al. 2014) or increased (Constantinescu et al. 2010, Sjödín et al. 2017).

Apart from ubiquitin, a few investigations of lysosomal protein biomarkers have been conducted. CSF LAMP-2 concentrations have been found to be increased in AD (Sjödín *et al.* 2016, Armstrong *et al.* 2014), decreased in PD (Boman *et al.* 2016), and increased in CBD compared to controls (Boman et al. 2016). Recently, LAMP-2 CSF concentrations have also been found to be decreased in female PD patients with LRRK2 mutations (female sporadic-PD patients also exhibited decreased concentrations but not significantly) (Klaver *et al.* 2018). Furthermore, EEA1, RAb3, RAb7, and CatL1 (Armstrong et al. 2014) as well as LAMP-1 (Armstrong et al. 2014, Heywood et al. 2015), and GM2A (Heywood et al. 2015) have been shown to have altered CSF concentrations. For example, CSF concentrations of LAMP-1 have been found to be decreased in PD compared to controls (Boman et al. 2016), CSF concentrations of EEA1 was increased and lysozyme decreased in PSP compared to controls (Boman et al. 2016) and LAMP-1, LC3, and lysozyme were increased in CBD compared to controls (Boman et al. 2016).

## **Biomarkers for glial activation**

Brain glial cells, astrocytes and microglia, are often activated in parallel. Microglia survey the brain, acting as the core of immune defence in the CNS. Astrocytes play a key role in repair mechanisms following CNS injury, but also form part of the blood-brain barrier and provide neurons with nutrients. Glial activation has been linked to deficits in both neuronal function and synaptic plasticity in AD. Recently, a genetic link between AD and variants of the *TREM2* (triggering receptor expressed on myeloid cells 2) gene (Jonsson *et al.* 2013, Guerreiro *et al.* 2013), which is selectively expressed on microglia in the CNS (Takahashi *et al.* 2005, Lue *et al.* 2015), has led to renewed interest in finding biomarkers of glial activation.

### *Cerebrospinal fluid biomarkers*

A disease-specific increase in the CSF concentrations of the secreted ectodomain of TREM2 has recently been demonstrated in AD patients. This increase correlates with increased CSF levels of T-tau and P-tau (Heslegrave *et al.* 2016, Piccio *et al.* 2016, Suarez-Calvet *et al.* 2016). Numerous other studies have also reported increased CSF concentrations of astrocyte-, microglia- and/or macrophage-derived proteins, including chitotriosidase (Watabe-Rudolph *et al.* 2012, Mattsson *et al.* 2011), CD14 (Yin *et al.* 2009) and YKL-40, which actually was discovered in an explorative proteomics study (Craig-Schapiro *et al.* 2010, Olsson *et al.* 2013). Similarly, the C-C chemokine receptor 2, another microglial marker expressed on monocytes, and one of its ligands, C-C chemokine ligand 2 (CCL2), is present at increased concentrations in the CSF of AD patients (Correa *et al.* 2011, Galimberti *et al.* 2006b, Galimberti *et al.* 2006a). So far, most studies found glial protein concentration ranges that overlapped considerably between cases and controls, especially compared to the more prominent changes seen in multiple sclerosis (Ohrfelt *et al.* 2016a), HIV-associated neurocognitive dysfunction (Peluso *et al.* 2017) or other traditional neuroinflammatory conditions. It should also be noted that

microglial and astrocytic activation are difficult to tease apart using CSF-based biomarkers since all of the above-mentioned proteins, except TREM2, can also be released from activated astrocytes. However, since microglial and astrocytic activation are tightly linked, this problem has no practical implications.

Using the TMTcalibrator™ workflow, in which TMT-labelled protein extracts from microglial cell cultures were spiked into TMT-labelled CSF samples, several candidate markers of microglia activation have been differentially quantified in the CSF of patients with AD compared with controls (Russell *et al.* 2017a). However, the sample numbers in this study were small and the detected changes have not yet been replicated. Using selection reaction monitoring mass spectrometry, the secreted ectodomain of TREM2 can be quantified and increased concentrations in AD CSF have been reported (Russell *et al.* 2017a).

### *Blood biomarkers*

Plasma and serum concentrations of biomarkers of microglial activation, such as those mentioned above, have been found to be similar to their CSF concentrations. If they were CNS-derived, the concentration would have been expected to be 100-fold lower (Craig-Schapiro *et al.* 2010). Most likely these high microglial marker concentrations, rather than being due to CNS-related changes, are due to release from monocytes and macrophages in peripheral blood. However, in a few studies, plasma YKL-40 levels have been slightly increased in AD patients (Olsson *et al.* 2016). The difference between AD patients and cognitively normal controls was too small for plasma YKL-40 to be used clinically.

## **Conclusions**

Clinical CSF assays to detect tangle and plaque pathology are currently widely available and tau and A $\beta$  biomarkers are used in many countries to aid in the diagnosis of AD in both prodromal and dementia stages of the disease (Dubois *et al.* 2016). Moreover, several additional biomarkers have been identified that mirror pathological changes common to AD and other neurodegenerative diseases, *e.g.* NF-L and T-tau. For these two biomarkers, ultrasensitive blood tests have been developed. NF-L concentrations in serum and plasma correlate with concentrations in CSF and increased NF-L concentrations in AD, FTD, VaD and in atypical parkinsonian disorders have been replicated in blood. For T-tau further studies are needed to clarify why the correlation with CSF concentration is weak and why the association of plasma T-tau with AD is weaker than for CSF. Furthermore, assays for several novel promising biomarkers to reflect synaptic loss/dysfunction have been developed in the last couple of years. Fluid biomarkers of Lewy body pathology are a highly prioritized matter, but so far reliable and accurate biomarkers for TDP-43 remain to be identified. Our hope is that longitudinal studies of fluid biomarkers will reveal the temporal development of various pathologies during neurodegenerative disease progression and to give clues to how they interact and lead to clinical phenotypes. As multi-morbidity appears to be common in AD and in other neurodegenerative dementias, one imaginable scenario is that, in the future, combinations or panels of biomarkers could be used to sub-classify clinical syndromes according to the pathological signature of individual patients, thus allowing for personalized treatment. Furthermore, such biomarkers could be utilized to stratify clinical trials into well-defined patient groups.

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### Conflicts of interest

None reported.

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### **Figure legend**

A schematic of cell types and pathologies in the central nervous system, including representative biomarkers that can be measured by targeted proteomics techniques.