Proteomic studies of cerebrospinal fluid biomarkers for Alzheimer's disease: an update

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Alzheimer's disease (AD) is a neurodegenerative disease affecting the brain. Today there are three cerebrospinal fluid (CSF) biomarkers, amyloid-β consisting of 42 amino acids (Aβ42), total-tau (t-tau) and phosphorylated-tau (p-tau), which combined have sensitivity and specificity figures around 80%. In the current paper, we screened PubMed for articles published the last five years (2012-2017) on proteomic studies on CSF with the criteria that AD had to be included as one of the diagnostic groups. Based on inclusion criteria, XX papers were included. Both mass spectrometry and multi-panel immunoassays were considered as proteomic studies. A large number of pilot data have been reported but so far there is a lack of replicated findings and to date no CSF biomarker discovered in proteomic studies has reached the clinic to aid in the diagnostic work-up of patients with cognitive impairment.

Keywords: Mass spectrometry; cerebrospinal fluid; Alzheimer's disease; proteomics; immunoassays; biomarker

Introduction

Alzheimer's disease (AD) is a disorder affecting the structure and function of the human brain, leading to episodic memory impairment and general cognitive symptoms including difficulties in decision-making and orientation. The disease was first described by Alois Alzheimer in 1906 and is today the most prevalent form of dementia accounting for 50-60% of all cases [1,2]. Alzheimer described brain changes, including extracellular "miliary bodies" (plaques), "dense bundles of fibrils" (tangles) in the interior if nerve cells and degeneration of cortical nerve cells, in a patient with early onset dementia. These changes are now considered the neuropathological hallmarks of AD [2]. Nearly 80 years later, Colin Masters and colleagues isolated plaques from AD and Down's syndrome patients and were able to show that one of the major components of the plaques was cerebral amyloid protein which today is called amyloid β (Aβ) [3]. Almost at the same time it was shown that paired helical filaments, isolated from brains of AD patients, consist of the microtubule-associated protein tau [4,5]. These two breakthroughs can be considered as the start of the "modern" AD research which also forms the basis for the amyloid cascade hypothesis. According to this hypothesis, AD is caused by abnormal accumulation of Aβ in senile plaques in the brain. This process is thought to induce, by unknown mechanisms, hyperphosphorylation of the axonal protein tau, formation of intra-neuronal tau inclusions (neurofibrillary tangles), neurodegeneration and, ultimately, dementia [6].

The core biomarkers of Alzheimer's disease

In 1992, it was shown that Aβ is present in cerebrospinal fluid (CSF) [7] and a few years later it was demonstrated that AD patients have a lower concentration of a 42 amino acid long Aβ peptide (Aβ42) compared to healthy controls [8] which is believed to reflect the plaque pathology [9]. The first publication demonstrating a marked increase in CSF total-tau (t-tau)

and phosphorylated tau (p-tau) in AD was published 1995 [10]. Today, several studies have shown that Aβ42 in combination with t-tau p-tau can be used to diagnose AD with sensitivity and specificity around 85-95% [11]. They are referred to as the core biomarkers because they all are at the core of the disease pathology (plaque and tangles), and they are since 2007 included in the diagnostic research criteria for AD [12] and in the International Working Group (IWG-2) research diagnostic criteria [13]. Lately they have also more frequently been used as inclusion criteria to clinical trials where drugs that may have disease-modifying effects are tested.

Other pathologies associated with Alzheimer's disease

With all the pathological studies published on AD up to date, it is safe to conclude that comorbidity (the presence of more than one disease process) is present in more than half of the AD patients [14]. Below follows some examples of such pathologies.

In 2006, the TAR DNA-binding protein 43 (TDP-43) was identified to be a major constituent of protein aggregates in the brains of patients diagnosed with fronto-temporal lobar degeneration (FTLD) [15]. In addition, TDP-43 has been suggested to cause neurodegeneration. Recent pathological studies have shown that TDP-43 seems to be a secondary feature of several neurodegenerative diseases, including AD and Parkinson disease (PD). Indeed, TDP-43 pathology has been found to be associated with amyloid plaques and tangles and studies have shown that TDP-43 pathology is found in over 50% of AD cases. One of the forms of cerebral amyloid angiopathy (CAA), a disease characterized by accumulation of amyloidogenic proteins in cerebral arteries, arterioles and capillaries, displays aggregated Aβ and previous studies have shown that 25% of the AD patients also have CAA. α-Synuclein, a protein which strongly associated with PD and dementia with

Lewy bodies (LBD), has been shown to aggregate into so called Lewy bodies which develop inside nerve cells. Several pathological studies have demonstrated that α-synuclein pathology can be found in multiple brain regions in almost 50% of the AD cases. Mounting evidence has indicated that synaptic dysfunction is an event that occurs early in the disease progression, earlier than the plaque and tau pathology. The synaptic pathology including synaptic degeneration and loss is widespread in the AD effected brain. Several studies have shown that activation of inflammatory pathways is a common feature in AD and local inflammations in the brain are common pathological findings as comorbidity in the AD affected brain. In conclusion, there are numerous comorbidities associated with AD and several, such as LBD, vascular brain injury, hippocampal sclerosis and TDP-43 inclusions, have been shown to contribute to cognitive impairment.

Why do we need additional biomarkers for AD?

A disease biomarker should reflect the activity of a disease process and ideally be used for diagnosis and prognosis in clinical practice, selection of patients into clinical trials and as a pharmacodynamic marker. It is clear that the three core CSF biomarkers Aβ42 in combination with t-tau and p-tau is not enough to identify "pure AD". In order to identify these patients, we are in great need of additional biomarkers reflecting other pathologies besides the plaque and tau pathology. This will most likely be of outmost importance the day we have a therapy for AD with disease modifying effects, *e.g.*, vaccination and secretase inhibitors. In the present paper, we have examined the literature on what have been published the last five years covering proteomic studies on CSF biomarkers for AD. Articles published in PubMed between 2012 and March 2017 were screened with the following search string; cerebrospinal fluid; Alzheimer's disease, proteomics; mass spectrometry;

xxxxxxxxxxxxxx. The published paper had to include at least one biomarker which could be analyzed either using mass spectrometry or immunoassay in a panel format.

Approaches to biomarker discovery and development

Explorative proteomic studies

In the emerging field of clinical proteomics, the basic premise is to compare, on a global level, protein abundances in clinical cohorts to detect proteins that differ between study groups, for example between disease groups or treatment groups. A particularly attractive aspect of the unbiased proteomic approach is that it may lead to the discovery of biomarkers that are not based on existing hypotheses, thereby stimulating the formulation of new hypotheses on disease mechanisms.

Identifying biomarkers in CSF is, however, a challenging task for several reasons. The uneven distribution of protein concentrations, with the protein content being heavily dominated by a small number of highly abundant proteins, hampers the detection of low-abundant proteins. Furthermore, heterogeneous disease groups and individual proteome variations necessitates the analysis of large cohorts in order to discern disease-associated aberrancies. Thus, a particular challenge of CSF proteomics is to achieve sufficient depth of analysis, demanding sample prefractionation and long analysis times per sample, while also acquiring quantitative information from a sufficient number of study participants. Different analytical strategies are currently being explored to address this challenge.

Label-free quantification

The majority of explorative proteomic studies in CSF published in the last five years use the label-free (LF) approach. The simplest form of LF quantification is spectral counting, in which protein abundances are measured as the count of acquired fragment ion spectra in liquid chromatography-mass spectrometry (LC-MS) data sets, which were matched by

database searching to tryptic peptides of the given protein (ref.). Alternatively, LF quantification can be based on the peak area of the precursor ion in the preceding MS scan or, more sophisticated, by mass spectrometric feature detection of peptide precursors over their high pressure liquid chromatography (HPLC) elution profile (ref. OpenMS, Non-linear dynamics). Attractive feature of LF quantification is that it involves relatively little sample preparation, and that peptides are detected in their native state, thus facilitating the establishment of selected reaction monitoring (SRM) assays.

Perrin et al. evaluated the performance of the LF approach by analysis of pooled and individual CSF samples [14]. While they reported low variability for a subset of 87 proteins they also pointed out some methodological limitations. One such limitation is the fluctuations of the signal response of the LC-MS system over time, which, in the absence of an internal standard, may affect quantification. Since sample multiplexing is not possible with the LF approach, this problem is likely to hamper larger clinical studies, as they require consistent LC-MS performance over several days or even weeks. This is likely one reason why CSF biomarker studies using the LF approach are typically quite small (10-20 patients), and the identified proteins are used primarily to select candidate markers for targeted follow-up studies. For example, in a discovery study of LBD in which LF quantification to analyze a cohort comprised of 10 patients and 15 controls a panel of 26 candidate biomarkers was identified, for which targeted SRM assays were established [16]. This panel was then assayed in a second cohort of similar size, consisting of AD, Parkinson's disease (PD) and DLB patients and controls, identifying four novel protein markers; ENPP2, transthyretin, Pro-orexin and LAMP1, that were elevated in AD compared to the other groups, and several proteins that were elevated in all disease groups. They also found several markers for LBD, and validated the data in LBD, AD, PD and controls [16].

A similar approach was used in a discovery study to identify AD biomarkers in a cohort consisting of 10 AD patients and 10 non-demented controls [17]. Two candidate markers; neurosecretory protein VGF and NPTXR, were selected among the identified proteins and assayed in a second cohort consisting of longitudinal samples.

Also using LF proteomics, the CSF proteome of 14 persons who will develop AD due to mutations in the *PSEN1* and *APP* genes was compared, with five related non-mutation carriers, identifying 56 proteins that differed between the groups [18]. Similarly, the proteomes of 12 frontotemporal dementia (FTD) patients with TDP43 pathology (n=12), eight FTD patients with tau pathology and 10 individuals with subjective memory complaints were recently described [19]. Among 56 proteins that differed between the patient groups, five were analyzed in a second cohort by enzyme-linked immunosorbent assay (ELISA), resulting in the confirmation of one; YKL-40 as a promising marker to distinguish the pathologies. YKL-40 is actually one of very few biomarkers that have shown consisting results across different studies and it was first identified as a biomarker for AD in 2010 by using two-dimensional difference gel electrophoresis and LC-MS/MS [20]. Recently it was shown in a meta-analysis that the CSF YKL-40 concentrations are moderately increased in AD compared to controls (effect size 1.29) [11].

Isobaric labeling

Quantitative proteomic analysis by isobaric labeling is based on labeling the sample proteins, after tryptic digestion, with a reagent that contains heavy isotopes (^{13}C, ^{15}N). The reagent exists in different isobaric forms in which the heavy isotopes are differently distributed between a *reporter group* and a *mass balance group*. Upon MS/MS fragmentation, the reporter group is released and detected at its specific mass-to-charge (*m/z)*. Because the reporter *m/z* is unique for each form of the reagent, differently labeled samples can be

combined in a multiplex. Using the tandem-mass tag (TMT) method with 10-plex analysis, isobaric labeling shortens analysis times and enables larger clinical studies to be performed.

Using isobaric labeling in a discovery proteomic study of patients suffering from delirium; a risk factor for development and progression of dementia, and patients with mild AD, changes in several protein family groups were reveled, including apolipoproteins, secretogranins/chromogranins and clotting/fibrinolysis factors [21].

Mer TMT/iTRAQ-studier???

Endopeptidomics

While most CSF proteomic biomarker studies to date follow the standard procedure of digesting the sample proteins with trypsin prior to LC-MS, increasing attention is paid to the low-molecular weight polypeptides in CSF – the CSF endopeptidome. Recent studies have revealed that CSF contains a large number of endogenous peptides potentially reflecting a multitude of processes in the brain, such as enzymatic activity and secretion. Thus, endogenous peptides may represent an important source of disease biomarkers [15,22,23]. In a recent study, over 18,000 endogenous CSF peptides were identified, among which were peptides derived from the core AD biomarkers tau and Aβ. Having molecular masses below 10 kDa, endogenous peptides can easily be separated from the larger, high-abundant proteins that make up the bulk protein mass in CSF, such as albumin and immunoglobulins, improving mass spectrometric detection sensitivity. They can be directly analyzed by MS, without prior tryptic digestion, simplifying sample preparation, and removing a potential source of variation.

Using a sample preparation protocol for fractionating endogenous peptides in CSF the endopeptidome of postmortem CSF from neuropathologically confirmed AD patients and non-demented controls was analyzed [24]. The protocol was based on a step-wise partial

protein- and peptide precipitation and resolubilization, and subsequent analysis by CE-MS. Peptides from several proteins that have previously been reported to be altered in AD were identified, including neurosecretory protein VGF, Comp C4 andAlfa-2HS Glycoprotein.

Using an integrated proteomic and endopeptidomic approach based on TMT labeling that enabled analysis of proteins and endogenous peptides to be performed on the same sample aliquot it was shown in a small pilot study comprising eight AD patients and eight non-demented controls that endogenous peptides derived from ineurosecretory protein VGF, integral membrane protein 2B and metallothionein-3 were decreased in AD [23]. The same method was used in an AD treatment study of a γ-secretase inhibitor [25], in which alterations in the CSF endopeptidome were measured longitudinally following administration of the drug. Several peptides were detected that changed in abundance in a dose-dependent manner. Among these were found a fragment of Aβ and APLP1; known γ-secretase substrates, demonstrating the applicability of the approach.

Targeted quantification by mass spectrometry

Multiple reaction monitoring (MRM) mass spectrometry is a highly sensitive and selective method which can be used for targeted quantitation of protein/peptide abundances in complex biological samples such as CSF. In an MRM experiment, the first mass analyzer selects a precursor ion (the protein or peptide of interest) which is fragmented. The fragment ions are then measured and recorded in the second mass analyzer. By adding isotopic labelled version (*e.g.*, ^{13}C and/or ^{15}N) of the peptide of interest prior to any sample preparation, absolute quantification can be obtained. In a MRM or SRM experiment several different precursor ions can be monitored enabling quantification of multiple targets in a panel format. This approach was used to quantify Aβ1-38, Aβ1-40 and Aβ1-42 in two clinical materials, each consisting of

15 AD patients and 15 cognitively normal controls using solid-phase extraction and isotopic labelled variants of the target peptides for absolute quantification. There was a moderate to good correlation between the SRM method and ELISA with similar sensitivities and specificities (80% and above). The LC-MS Aβ1-42 concentrations were however approximately twice as high compared to the ELISA measurements. Using the Aβ1-42/Aβ1-40 ratio showed an improved separation between AD and controls [26]. An optimized and thoroughly validated method for quantifying Aβ1-42 is now accepted as a reference measurement procedure (RMP) by The Joint Committee for Traceability in Laboratory medicine (JCTLM) [20]. A similar method for Aβ1-42 has also been accepted as a RMP [27]. In a cross validation study consisting of 100 non-demented patients with mild cognitive complaints, the RMP Aβ1-42 showed a good agreement with cortical Aβ deposition determined using amyloid positron emission tomography (PET). Sensitivities and specificities were more than 80% when using only Aβ1-42 , and more than 90% when using the CSF Aβ1-42/Aβ1-40 ratio [28].

A method to measure the metabolism of Aβ1-38, Aβ1-40 and Aβ1-42 *in vivo* was achieved by infusing stable isotope labeled leucine intravenously for nine hours. CSF was then collected every hour for 36 hours and the rate of metabolism was measured as the labeled leucines were incorporated in the newly synthesized Aβ. All three Aβ peptides had the same rate of incorporation of measurable labeled leucine (after four hours) as well as peak level (between 18 to 20 hours post infusion) before decreasing (21 to 32 hours post infusion) [29].

Tau is an emerging protein for MRM analysis and different approaches have been used. Using antibodies to first enrich tau from CSF followed by digestion, a tryptic peptide common for all six human forms of tau was quantified using LC-MS/MS. Results showed a significant difference in tau concentrations between AD (n=50) patients and healthy controls (n=50). The method correlated well with Meso Scale Discovery total tau immunoassay [30]. Another

approach is to enrich the tau proteoforms by SPE [31]. Seven tryptic peptides were measured using a triple quadrupole mass spectrometer, an instrument more common in clinical laboratories compared to high resolution instruments. While the results correlated well with ELISA methods the MRM concentrations obtained were 17-25 times higher. Only one of the seven peptides seems to reflect total tau, and the authors argue that the seven peptides can be used as independent biomarkers [32]. Recently, a PRM method to measure 18 peptides covering the entire tau protein using high resolution MS was developed in which protein precipitation followed by solid phase extraction (SPE) was used for sample cleanup [33]. The method was applied on a clinical cohort including of AD, progressive supranuclear palsy (PSP) and DLB. MS results showed a good correlation with ELISA measurements, while MS values were about 20 times higher. Results showed that not only the central core of the tau protein but also peptides along the entire protein was increased in AD.

Synaptosomal-associated protein 25 (SNAP-25) is a marker of functional synapses since it mediates communication by initiating fusion of synaptic vesicles and it has been hypothesized that soluble forms of brain SNAP-25 resembles SNAP-25 in CSF. Indeed, the levels of tryptic peptides of SNAP-25 were increased in AD compared to non-demented controls in three cohorts (including very early stages of AD). These findings imply that CSF SNAP-25 might be used to follow the progression of cognitive decline [34]. Similarly, in a study comprising patients with MCI due to AD, patients with dementia due to AD and controls the pre-synaptic protein synaptotagmin-1 was analyzed. The levels of synaptotagmin-1 were increased in the MCI and AD compared with controls, where the highest levels were in the MCI group (only in one of two sample set) and it was suggested that that the synaptic degeneration can be identified in preclinical AD [35].

The *APOE ε4*-allele is strongly associated with AD and is the best established genetic association with AD [36]. Despite intensive research has been conducted to shed light on how

ApoE contributes to the AD pathology, where the risk of developing AD is approximately 15 times higher in homozygotes of the ε4 allele compared with non-carriers [26], has the mechanism not yet been elucidated. In order to investigate if ApoE can be used as a biomarker for AD, an SRM method to measure the different ApoE isoforms in human CSF was developed. One peptide for each isoform was quantified. The extent of methionine oxidation, glutamine deamidation and cyclization of N-terminal carbamidomethylcysteine was also studied. Despite good validation results the correlation between SRM and ELISA was moderate [37]. The same method was later used to measure ApoE isoforms (ApoE2, ApoE3 and ApoE4) in CSF from AD patients and non-AD individuals. However, the results showed no difference in ApoE concentrations between the groups [38].

Ubiquitin has been associated with several diseases including AD. Recently a method to quantify free monoubiquitin in CSF was developed. The intact protein was measured since enzymatic digestion would not be useful as the information of the ubiquitin form would be lost. Results from the study showed an increased concentration of ubiquitin in CSF in AD (as well as Creutzfeldt–Jakob disease (CJD) compared to controls and other neurodegenerative diseases, which the authors use to argue that there might be an AD specific process for the increase [39].

Mutations in the *TREM2* gene have been shown to be associated with an autosomal recessive form of early-onset dementia [40]. In a discovery cohort including 37 AD and 22 controls and in a follow-up study comprising 24 AD and 16 controls, a statistically significant increase of CSF soluble TREM2 (sTREM2) in AD patients was observed, but with a large overlap between AD and controls. Thus, sTREM2 does not seem to be useful as a diagnostic tool but rather to study inflammation and glial activation, unrelated to Aβ pathology (since sTREM2 did not correlate with Aβ1-42) [41].

Based on the core CSF AD biomarker profiles (Aβ1-42, T-tau and P-tau measured with ELISA) a cohort of AD (14) and controls (14) were selected and analyzed using PRM for lysosome-associated membrane protein 2 (LAMP2). The levels of all three peptides were reported to increase in AD compared to controls. LAMP2 did not correlate with any of the AD core biomarkers [42].

Several highly multiplexed MRM methods have also been reported in attempt to narrow in on new potential biomarkers for AD. By analyzing 25 peptides corresponding to 16 candidate biomarkers, selected from previously published data from 2D gel electrophoresis and **matrix-**assisted laser desorption/ionization **(**MALDI)-time-of flight (TOF), it was shown that 12 of the 16 target proteins were consistent with the literature regarding up- or down regulation in AD [43]. However, due to the low patient number (three AD and three normal subjects) the study needs to be replicated in a larger independent cohort. This method was later modified to use micro LC instead of nano LC [44]. In another study, 39 peptides corresponding to 30 proteins (selected from published proteomic discovery experiments) were quantified longitudinally over four years. While APP, transthyritin, YKL-40, complement component C3, prostaglandin-d2 synthase and ApoE exhibited differences between AD and Co, AD and MCI or MCI and Co, longitudinal results showed a reduction for neuronal pentraxin receptor, NrCAM and chromogranin A overtime [45]. Finally, a method to measure 320 peptides from 142 proteins was used to analyze samples from the AD Neuroimaging Initiative consisting of AD (n=66), Co (n=85) and MCI (n=134). Peptides from hemoglobin A and B, superoxide dismutase, neuronal pentraxin-2, neurosecretory protein VGF and secretogranin-2 showed a significant difference between progressors and nonprogressors in the MCI group. [46].

Targeted enrichment by immunoprecipitation – mass spectrometry

Affinity purification or immunoprecipitation (IP) with monoclonal or polyclonal antibodies combined with characterization and quantification of eluted proteins and peptides with MS is one variant of targeted proteomics. The antibodies, directed towards a protein or family of proteins, can be bound to different media, *e.g.*, magnetic beads, plate surfaces or columns to selectively purify and concentrate the target analyte. The eluate is then analyzed most commonly with LC-MS but MALDI TOF/TOF has also been used. The advantage of IP-MS is that the purification often is highly selective making it possible to extract a particular protein or protein family from a very complex sample matrix such as plasma, tissue, or CSF. The results from IP-MS studies can also be utilized to guide development of ELISAs or other immunoassay that target a particular protein form. Moreover, IP-MS makes it possible to characterize and simultaneously quantify several different forms of the target protein. The main disadvantage is that the method depends on the existence of a suitable antibody aimed at the protein of interest. In addition, if antibodies directed towards a particular protein are available efforts are often directed towards developing an immunoassay instead of a hybrid method such as IP-MS.

A major drive in IP-MS has been to target proteins that are related to the AD pathology including different forms of Aβ and its precursor, amyloid precursor protein (APP) [47]. Several studies have identified and characterized many different forms of Aβ in CSF, brain tissue, and plasma. The CSF concentrations of several of these have been shown to be influenced by drugs and disease processes [48]. A recent example of an ELISA developed because of results from IP-MS is the study by Perez-Grijalba *et al.* where they developed an ELISA being able to selectivity measure Aβ1-17 both in CSF and plasma.

It has been known for 20 years that several synaptic proteins can be detected in CSF [49] and that synaptic dysfunction and loss are directly linked to memory disturbances at the early stages

of AD [50-53]. Several studies have used IP-MS to study synaptic pathology by characterizing and quantifying presynaptic proteins in CSF. Neurogranin, a postsynaptic protein, is an example of a hypothesis driven workflow. In 2010, it was shown by semi-quantitative western blotting that CSF neurogranin is increased in AD compared to healthy controls [54]. Recently, it was shown in two independent pilot studies by IP-MS that CSF neurogranin is expressed as a variety of small endogenous peptide fragments (in addition to full-length protein) of which the concentration of peptide Ng48-76 was significantly increased in AD compared to controls [55]. In addition, it has also been shown that some of the neurogranin peptides are unique for plasma and not detectable in CSF and the other way around [56]. Based on these findings, novel anti-neurogranin antibodies and an ELISA were developed. It was then shown that CSF neurogranin is increased already at the early clinical stages of AD and predicts cognitive deterioration and disease-associated changes in metabolic and structural biomarkers over time [57]. Today, several studies have confirmed the use of neurogranin to aid to the AD diagnosis and in Sweden the ELISA is currently introduced into the clinic for routine analysis. Recently it was shown by IP-MS in three separate cohorts that the CSF concentration of a fragment from the presynaptic protein SNAP-25 was significantly higher in AD compared to controls, also at the very early stages of the disease, showing its value as a marker for synaptic integrity [58]. Another synaptic protein, synaptotagmin-1, appears to be essential for the maintenance of an intact synaptic transmission and cognitive function. Using IP-MS is has been shown in two independent sample sets that the CSF concentration of synaptotagmin-1 was significantly increased in patients with dementia due to AD and in patients with mild cognitive impairment due to AD compared to controls [59]. In addition, in sample set I the synaptotagmin-1 concentration was significantly higher in patients with mild cognitive impairment due to AD compared with patients with dementia due to AD. Thus, CSF neurogranin, SNAP-25, and synaptotagmin-1 are promising biomarkers to monitor synaptic dysfunction and degeneration in AD.

Besides plaques, tangles, and synaptic pathology there is an increasing body of evidence suggesting that endo-lysosomal dysfunction is a pathogenic mechanism of AD [22]. Hence, proteins involved in the normal function of endo-lysosomal vesicles could be tentative biomarkers of AD. The lysosomal protein LAMP2 is involved in chaperone mediated autophagy and recently it was shown that the concentrations of three CSF LAMP2 peptides were increased in AD compared to non-AD controls [42]. Altered CSF LAMP2 concentrations may indicate endo-lysosomal dysfunction in AD, but further studies of larger cohorts also including patients with other neurodegenerative disorders are required.

Lately, several methodological studies focusing on reproducibility and co-IP in affinity-purifications have been published [60]. Quantitative affinity purification mass spectrometry: a versatile technology to study protein–protein interactions Front Genet. 2015; 6: 237.). Efforts have also been put into standardizing the methods and checking reproducibility across laboratories (Aebersold igen). However, even if IP-MS has been used in several studies showing potentially promising results, for AD there is still no IP-MS method that has been fully validated and introduced to clinical use.

Other targeted and functional proteomic approaches

Another approach to focus on a subset of the CSF proteome is to either investigate specific protein interactions or specific functional groups. There are, however, only a few published studies employing these approaches. For example, using IP to isolate binding partners to Aβ, seven proteins that bind specifically to Aβ1-40 were identified [61]. Of the seven proteins, the protein Dkk-3 was selected for further investigation with ELISA. However, no difference was found when comparing CSF from AD and control subjects.

Making use of a redox proteomics approach by using 2D-gels with immunoblotting of carbonyl-modified CSF proteins, proteins which may be linked to oxidative stress and (early) AD have been identified [62]. MALDI fingerprinting was used to identify the proteins and 2D Western blot was used for quantification. This investigation points at early protein oxidative events in AD compared to MCI and controls with several proteins upregulated in AD. However, the small N (6+6+6) means that the finding needs to be replicated.

A number of Aβ peptides were discovered to also occur in various *O*-glycosylated forms in CSF [63]. The *O*-glycosylated Aβ peptides belonged to two distinct groups; one consisting of peptides spanning the β-secretase cleavage site with C-termini ending at Gln15 of Aβ and one consisting of short Aβ peptides with N-termini at Asp1 of Aβ. The latter group exhibited a number of different glycans situated at Tyr10. Quantification was performed using label free LC-MS after IP with the anti-Aβ antibody 6E10. In a small cohort of 6 AD + 7 non-AD subjects higher CSF concentrations of the Tyr10 glycolsylated Aβ forms in AD compared to the non-AD controls was reported. This finding also needs replication in a larger material and with a method better suited for quantification. In another type of glycosylation study CSF samples were subjected to PNGaseF to cleave off N-glycans from their proteins. Subsequently a number glycans were identified that differed in concentration between AD/MCI and controls [64]. They used PCS analysis to make a model and then employed the model to divide the AD and MCI groups into subgroups. While one of the AD/MCI subgroup differed little from controls, the other exhibited good separation.

By a combined isobaric TMT labeling with subsequent IP using anti-Aβ antibodies, a cohort of 8 AD, 11 MCI, and 19 controls has been analyzed by LC-MS/MS operated in data dependent mode [65]. Although none of the detected peptides can be used as a biomarker for

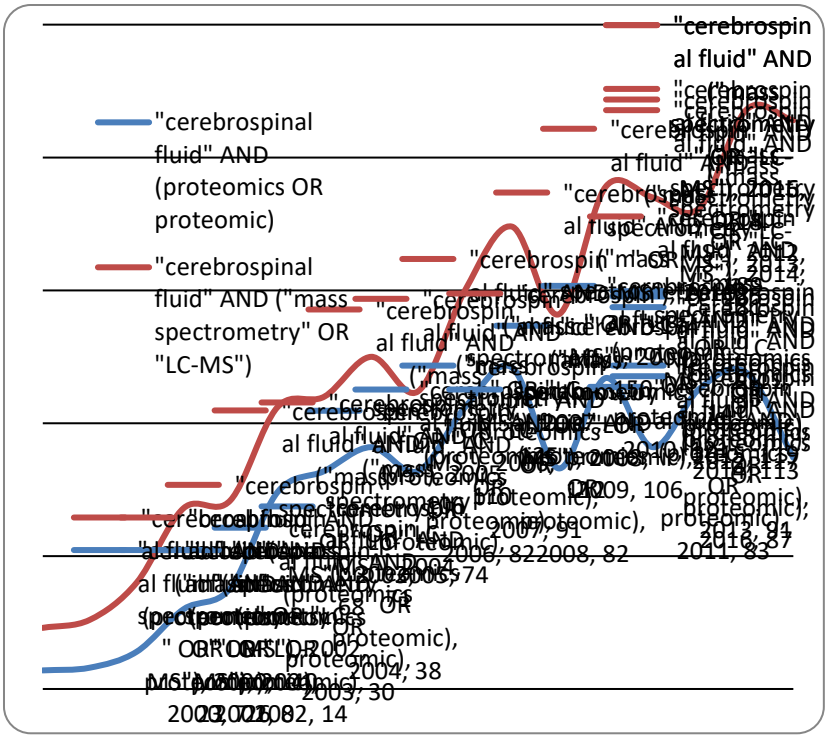
AD, this is a promising approach and allowed parallel relative quantification of 27 Aβ peptides. The described approach could also allow for analysis of post-translationally modified peptides such as glycans. Possible disadvantage is the labeling cost and the quality of the quantification compared to SRM/PRM, but it should work to perform the LC-MS/MS in a targeted way to improve robustness and sensitivity; of course at the expense of number of analytes measured.

In *in vitro* experiments on Aβ oligomerisation, auto-cleavage was observed to produce Aβ1-23, Aβ 1-24, and Aβ1-25 [66]. After development of an AβX-25 neospecific antibody, these fragments were proven to be present in both brain and CSF. CSF from a cohort of 16 AD and 14 non-demented control subjects was investigated by SRM but no difference between the groups was observed.

Expert commentary: the author's expert view on the current status of the field under discussion.

Despite nearly two decades of research, including explorative mass spectrometric studies and targeted approaches (e.g., SRM/PRM and immunoassays), proteomic analysis in CSF has failed to identify any new clinically useful biomarkers for AD. While several biomarker candidates have been identified, none have yet reached the clinic to aid to the AD diagnosis.

Discovering new CSF biomarkers has obviously turned out to be difficult, and the current trend is that while the use of LC-MS in CSF analysis is steadily increasing since the beginning of the century, the number of CSF proteomic studies per year has leveled out in the last five years and may in fact be decreasing (Figure 1).



What is lacking in several published studies is validation of the potential biomarker in an independent cohort using an independent method. However, it may be difficult for a proteomic laboratory to gain access to well characterized AD and control materials, and repeating a study in an independent cohort may be even more challenging. Thus, what is needed is closer collaboration between proteomic research groups and clinics.

What is evident in the panel approaches, including both mass spectrometry and immunoassay studies, is that the same “usual suspects” are interrogated over and over again. For example, apoE, clusterin, chromogranin, secretogranin, neurosecretory protein VGF, VEGF, transthyretin, and HFABP have been reported in several proteomics studies with varying results, and yet none of these potential biomarkers has reached the clinic. One reason may be lack of validation studies, as mentioned above. Also, many of these proteins have turned up as biomarker candidates of other neurodegenerative disorders, suggesting that they may reflect processes downstream to the core pathology, and thus not by highly specific to AD.

It is noteworthy that all these proteins are fairly high-abundant and have been identified in early discovery studies. Improved detection sensitivity of mass spectrometers, improved LC performance, and improved sample preparation methods are making it possible to detect lower-abundant CSF proteins, which may lead to the identification of low abundant proteins that might be more directly involved in the disease process, Recent examples include mapping of the phosphorylation sites of CSF tau (Russell CL et al. 2016), Lehmann, Anns Snare complex, CSF exosomes, the identification of endogenous tau peptides (Karl 2017).

Also evident from the literature is that, apart from highly targeted sets of biomarkers (*e.g.*, enzymes and metabolites that reflect liver function and a complete blood count that reflects various hematological conditions), there is actually no panel for multiple biomarkers in clinical use for any disease. Thus, AD is in good company. There are many possible explanations for this; the most obvious being that the biomarkers are not adding any additional disease specific information for the AD diagnosis; they still may separate AD from control statistically but the overlap between the groups investigated is too large for the biomarker to be of clinical usefulness. Another explanation could be that in proteomic and panel studies are often several analytes monitored in the same study setting. Thus, to find a significant change in the levels of a novel biomarker among several identified, the sample set has to be rather big in order to pass correction of the p-value for multiple testing. In order to identify new biomarker targets, validate them, and implement them into the clinic, the proteomic field may have reconsider the workflow from the discovery phase to the clinic. The work flow can divided into explorative driven work flow and hypothesis driven work flow. Even though the two work flows have different starting points, the two pathways will converge at the development of assays which are suitable for the clinic.

Several proteomic studies have applied digestion of the samples using, e.g., trypsin prior to mass spectrometric analysis and in order to detect as many tryptic peptides as possible, long

gradients are used. But what is gained in sensitivity by this approach is not unlikely lost in disease specific information regarding endogenous peptides. Similarly, when it was discovered that Aβ can be measured in CSF, no clear difference in the concentrations between AD and controls was obtained. This was due to that total Aβ was measured; however, when a selective method for measuring Aβ42 was developed, a marked decrease was evident in AD patients.

Today immunoassays are often the method of choice when introducing a method into the clinic. However, the ELISA methods used for AD are not yet fully standardized, which influences the apparent concentration measured. In addition, immunoassays suffer from matrix effects and differences in assay calibrations. There is ongoing work in different consortia. Both the Alzheimer's Association Quality Control program for CSF biomarkers and the International Federation of Clinical Chemistry Working Group for CSF proteins have the goal to harmonize these assays. Recently, a mass spectrometry-based reference measurement procedure (RMP) for absolute quantification of Aβ1-42 in CSF was developed which will be used by kit vendors to harmonize the different ELISAs on the market. However, what is gained in specificity, reduced matrix effects, and the possibility of using panels of many target molecules within a single analysis using the RPM is lost regarding sample throughput when comparing to state-of–the-art immunoassay methods.

Five-year view: a speculative viewpoint on how the field will evolve in 5 years' time.

It is well established that AD includes several pathologies besides plaques and tangles. Whereas we have quite good biomarkers for neurodegeneration and plaque and tangle pathology in AD, we currently lack biomarkers for other neuropathological changes that are common in neurodegenerative diseases. We encourage intensified efforts to find reliable fluid

biomarkers for TDP-43 and α-synuclein inclusions. In a five-year perspective we believe that there will be an extended panel of CSF biomarkers for neurodegenerative diseases that could be used to classify patients with cognitive symptoms into biomarker categories based on what pathology (or combination of pathologies) that is most likely to drive the symptoms. Such a panel could be immunochemical or based on targeted MS analysis of the sample and could also include markers that reflect microglial activation, synaptic dysfunction, apoE phenotype, as well as markers that may indicate resilience or protective factors. This will be of outmost importance when we have a treatment having a disease modifying effect since we need to know not only if the patient has AD; we also need to know the comorbidity so the patients are given proper treatment.

The knowledge gained from Aβ, tau, and neurogranin (and many other proteins) has driven the proteomics towards peptidomics since we know that many of the proteins are expressed as endogenous peptides which may hold more disease specific information than the full length protein itself.

Key issues: 8–10 bullet points summarizing the review.

- Proteomic analysis in CSF has failed to identify any new clinically useful biomarkers of AD.
- Closer collaborations between proteomic research groups and clinics are needed.
- Several of the identified proteins are most not likely not specific for AD
- In order to identify low abundant proteins improved detection sensitivity of mass spectrometers, improved LC performance, and improved sample preparation methods are required.
- Today there is no CSF panel for multiple biomarkers in clinical use for any disease.

- In order to identify new biomarker targets, validate them, and implement them into the clinic, the proteomic field may have reconsider the workflow from the discovery phase to the clinic.
- Endogenous peptides may hold more disease specific information than the full length protein itself.
- Comorbidity (the presence of more than one disease process) is present in more than half of the AD patients.

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References: target of 80 references **Reference annotations:** please highlight 6–8 references that are of particular significance to the subject under review as "* of interest" or "** of considerable interest" and provide a brief (1–2 line) synopsis.

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