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Proteomics/peptidomics tools to find CSF biomarkers for neurodegenerative diseases

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Methods
3.1. 2-DGE-MS
3.2. SELDI-TOFMS
3.3. IP-MS
4. Cerebrospinal fluid
5. Results
5.1. Alzheimer's disease
5.1.1. 2-DGE-MS
5.1.2. SELDI-TOFMS
5.1.3. IP-MS
5.2. Frontotempral dementia
5.2.1. 2-DGE-MS
5.2.2. SELDI-TOFMS
5.3. Multiple sclerosis
6. Discussion and conclusions
7. Acknowledgement
8. References

1. ABSTRACT

Neurodegenerative diseases are characterized by premature neuronal loss in specific brain regions. During the past decades our knowledge on molecular mechanisms underlying neurodegeneration has increased immensely and resulted in promising drug candidates that might slow down or even stop the neuronal loss. These advances have put a strong focus on the development of diagnostic tools for early or pre-clinical detection of the disorders. In this review we discuss our experience in the field of neuroproteomics/peptidomics, with special focus on biomarker discovery studies that have been performed on CSF samples from well-defined patient and control populations.

2. INTRODUCTION

Neurodegenerative diseases, such as the cognitive disorders Alzheimer's disease (AD) and frontotemporal dementia (FTD) and the movement disorder Parkinson's disease (PD) are characterized by premature neuronal loss in specific brain regions. Apart from a small number of neural stem cells that are created daily, neurons of the brain and spinal cord are not readily regenerated. Therefore, drugs aimed at inhibiting neurodegenerative processes are likely to be most effective if the treatment is initiated as early as possible upon onset of the pathologic condition. At present there are no specific therapies against any of these disorders, although some symptomatic treatments exist, such as acetylcholine inhibitors against AD and levodopa against PD. However, during the past decades our

knowledge on molecular mechanisms underlying neurodegeneration has increased immensely and resulted in promising drug candidates that might slow down or even stop the neuronal loss. These advances have put a strong focus on development of diagnostic tools for pre-clinical detection of the disorders. Therefore, proteomics, the study of the qualitative and quantitative protein content of tissues and biological fluids could be a valuable tool. Moreover, besides proteins, there are number of bioactive peptides (peptidomics) that great interest in the global studies on the central nervous system.

Our group realised early the potential of mass spectrometry based proteomics/peptidomics in neuroscience and has been using it in different research projects since (1,2). We have for example in several studies used different kinds of mass spectrometric methods, such as surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOFMS) (3-8), matrix-assisted laser desorption/ionisation (MALDI) TOFMS, and electrospray ionisation quadrupole-TOF tandem MS (ESI-QTOFMS/MS) where the later two have been frequently used in combination with analytical and preparative two-dimensional gel electrophoresis (2-DGE) for identification of disease specific up-and down-regulation of proteins in cerebrospinal fluid (CSF) (9-13). Recently we have also used a targeted proteomic approach combining immunoprecipitation and mass spectrometry (IP-MS) to study disease specific peptide patterns (14). In this review we discuss our experience in the field of neuroproteomics/peptidomics, with special focus on studies that have been performed on CSF samples from well-defined patient and control populations.

3. METHODS

3.1. 2-DGE-MS

In 2-DGE, proteins are separated in a two step approach according to their isoelectric point (pI), and molecular mass under denaturing conditions (15). Separation in the first dimension of analytical 2-DGE is carried out using immobilized pH gradient (IPG) strips, whereas the second dimension separation is performed using sodium dodecylsulphate polyacrylamid gel electrophoresis (SDS-PAGE). Staining of the gels with for example organic or fluorescent dyes enables detection of the protein spots under visible or ultraviolet light. (In our studies the highly sensitive, flourecent SYPRO-ruby protein stain has been used). Following digitization of the gels, software-based quantification is typically performed, comparing staining intensities of spots between gels. Then differentially expressed proteins between groups can be excised and subjected to enzymatic (tryptic) in gel digestion. Extraction of the resulting peptides enables mass spectrometric analyses by MALDI-TOFMS or ESI-QTOFMS/MS resulting in a peptide mass fingerprint or peptide fragment pattern of the protein. Identification of the proteins is possible when comparing the peptide mass fingerprint or peptide fragment pattern to theoretical lists of peptides generated by *in silico* digestion of proteins from a sequence database.

A major advantage with 2-DGE is that it provides valuable information of the intact proteins, e.g. approximate mass and pI. Furthermore, protein isoforms with post-translational modifications changing their net charge, are often well separated and can be independently quantified. Disadvantages include discrimination against certain classes of proteins such as hydrophobic, very basic and small (less than 10 kDa) proteins. Furthermore, the limited loading capacity of 2-DGE often necessitates enrichment and pre-fractionation steps in order to detect low-abundance proteins even in less complex samples. In our 2-DGE studies intact CSF proteins were separated either by analytical 2-DGE or a combination of analytical 2-DGE with an initial pre-fractionation step, consisting of preparative isoelectric focusing (IEF) in the liquid phase (9-12). The pre-fractionation step enabled enrichment of CSF proteins, from a ten time larger volume than direct 2-DGE, into fractions that were further analyzed by analytical 2-DGE.

3.2. SELDI-TOFMS

A parallel proteomics workflow to study intact proteins in biological fluids that have been used frequently in our lab is the SELDI technique. The technique was introduced in 1993 (16) and during the following years it was developed into a sensitive and reproducible high-throughput proteomic profiling technique (17,18). The SELDI-TOFMS technology is a two step process in which complex protein mixtures are initially fractionated into sub proteomes by retentate chromatography. Retentate chromatography is performed on ProteinChip Arrays with varying orthogonal chromatographic properties (i.e. anion exchange (Q10), cation exchange (CM10), metal affinity (IMAC) or reverse phase (H50)). By utilizing arrays with differing surface chemistries in parallel or in series we have resolved the CSF proteome into subsets of proteins with common properties. In the second step of the SELDI process bound proteins were detected in the ProteinChip reader, a highly sensitive MALDI-TOFMS. Since the sample molecules are bound to the arrays without any pre-treatment the procedure requires minute amounts of starting material, 5 microliter of CSF, and the sensitivity reaches the low-femtomole level. Unfortunately, the identities of the analytes (peptides or proteins) are not revealed by the analysis. In order to understand the biological role of putative novel biomarkers and to develop diagnostic and clinical assays their identity needs to be determined. The SELDI analysis supplies information on the molecular mass of the sample components and from the binding characteristics to different array surfaces conclusions can be drawn regarding the basic physicochemical properties of the proteins. This information greatly facilitates the subsequent identification process which consists of classical methods of protein purification combined with tandem mass spectrometry.

In addition to the chromatographic ProteinChip arrays described above the SELDI technology also includes arrays with preactivated surfaces designed to capture specific bait molecules, such as antibodies, receptor molecules or nucleic acids. A covalent bond is formed through a reaction between reactive groups on the array

surface and primary amino groups on the bait molecule. Once the bait molecule is coupled to the surface the arrays are incubated with biological samples to capture specific interactors (ligands, antigens, etc). Compared to traditional interaction assays the SELDI method adds an additional dimension since isoforms of the captured antigen can be resolved by their mass-to charge ratio (*m/z*) in the TOF analyser. The SELDI immuno-capture application has been used extensively to study the pattern of amyloid-beta (Abeta) peptides released from cultured cells or in CSF (19-25). It was first described in 1999 (26) and has since been developed into a commercially available product.

Even though the SELDI technique has found its application in a widespread area it does have some limitations. The most severe limitation is the absence of sequence information for the analysed sample components. Protein identification by purification of single protein components from biological samples is a labour-intensive and time consuming process. The technique is most suitable for analysis of peptides and proteins in the molecular mass range from 1-30 kDa. Sensitivity decreases for higher molecular mass species as does the resolution. However, the SELDI technique plays an important role as a complement to other well established profiling techniques such as 2D-gel electrophoresis or liquid chromatography (LC) MS/MS.

3.3. IP-MS

Immunoprecipitation with antibodies, also called immunoaffinity capture, in combination with mass spectrometry involves selective capture of desired analytes from solution, e.g. plasma, CSF, cell culture media and brain tissue extracts, prior to mass spectrometric analysis. The immunoprecipitation is generally conducted to reduce the complexity of a mixture of proteins and peptides and since proteins usually must be fragmented into peptides. The capture can be very selective towards a specific protein/peptide or towards a class of proteins/peptides such as phospho-proteins or isoforms of amyloid precursor protein (APP) and Abeta.

Our group have earlier used an IP-MS method to study different neuropeptide isoforms in human brain tissue (27). Here the method was based on immunoprecipitation followed by analysis using MALDI-TOFMS and the aim was to investigate neurotensin-like immunoreactivity and quantification of neurotensin isoforms in human brain tissue. Recently, we developed an IP-MS method for the analysis of Abeta peptides in CSF where Abeta-specific antibodies were added to magnetic Dynabeads and incubated over night on a rocking platform at +4 °C allowing binding of the antibody to the magnetic beads (Figure 1). (28,29). The antibody-coated beads was then further incubated with CSF allowing immuno-capture of the Abeta peptides. The beads were washed followed by elution of the peptides using formic acid (FA). The collected supernatant was dried down in a vacuum centrifuge and redissolved in 0.1% FA in 20% acetonitrile (ACN) and then analyzed using MALDI-TOFMS and nanoflow LC-ESI Fourier transform ion cyclotron resonance (FTICR) MS (14,28,29). Two of the biggest advantages using MALDI-TOFMS and FTICRMS are the very good mass resolution and the precise mass accuracy (low parts per million). Furthermore, using nanoflow LC-ESI will also increase the sensitivity.

4. CEREBROSPINAL FLUID

CSF is a valuable diagnostic window to neurodegenerative diseases for many reasons. Its molecular composition reflects metabolic processes in the brain in a direct manner, due to the free exchange of substances, e.g. peptides and proteins, between the brain and the CSF (30). The CSF biomarkers total tau, hyperphosphorylated tau, and the 42 amino acid form of Abeta (Abeta1-42) are now established markers for AD and can identify AD in the early, mild cognitive impairment (MCI) stage of the disease with high accuracy (31,32). In contrast, tau proteins are not detected in peripheral blood and when analyzing Abeta1-42 in plasma, no diagnostically meaningful information can be retrieved (33). There are, however, also some problems with CSF as a source of biomarkers. First, the total protein content in CSF of around 250 mg/L is dominated by albumin, immunoglobulins, and beta-trace protein (prostaglandin-H2 D-isomerase), representing approximately 80% of the total protein content, whereas many other proteins are found in nanomolar concentrations (30). Although it is possible to analyze proteins directly in neat CSF with MALDI-TOFMS, it is only the most abundant and relatively low molecular mass proteins that are detected (34). The problem with high-abundance proteins and how to deal with it will be discussed in detail below. Second, CSF studies necessitate spinal taps that have a bad reputation in some countries, in spite of a large body of evidence demonstrating the safety of the procedure (35-37). The only recorded complication in these studies was post-lumbar puncture headache. With the use of a small diameter needle (0.7 mm), the rate of mild headache (duration less than one day, not affecting daily life) was less than 4%, and the rate of moderate or severe headache (duration more than one day and/or affecting daily life) was less than 1%.

5. RESULTS

5.1. Alzheimer's disease

Alzheimer's disease is the most common cause of dementia and affects approximately 10% of the population older than 65 years. Research advances have enabled detailed understanding of the molecular pathogenesis of the hallmarks of the disease – i.e., plaques, composed of Abeta, and tangles, composed of hyperphosphorylated tau. Both tau and Abeta protein levels in CSF have become established diagnostic tools for AD, but additional markers would be of great value, both to facilitate early or pre-clinical diagnosis and to monitor disease progression or treatment efficacy in clinical trials. Finally, novel biochemical markers of AD might give important clues on the pathogenesis of the disease.

5.1.1. 2-DGE-MS

We have in two separate studies used three different combinations of analytical and preparative 2-DGE followed by mass spectrometric analysis for screening of

Table 1. Proteins identified in 2-DGE-MS studies as up- or down- regulated in AD compared to controls

CSF protein	Levels in AD vs. control			References	Comment
	2-DGE	"narrow" 2-DGE	IEF+ 2-DGE		
albumin			down	up (48,49)	
alpha1-antitrypsin		up	up	up (48,49), no change (84)	2 isoforms
alpha1beta-glycoprotein		down	down	down (50), no change (48)	2 isoforms
alpha2-HS glycoprotein			down	up (48)	
apolipoprotein A-I	down	down		no change (84)	2 isoforms
apolipoprotein A-IV	down			up (49)	
apolipoprotein E	down	down			5 isoforms
apolipoprotein J		down			2 isoforms
beta-trace protein		down		down (84), up (50), no change (48,84,85)	2 isoforms
cell cycle progression 8 protein		down			
kininogen		down			
retinol binding protein	up	down		up (48,49)	
transferrin			down	up (48)	
transthyretin	up		down	up (48), down (84), no change (49,84)	2 isoforms
ubiquitin	down				
beta2-microglobulin	up		up	up (48,49,50,52), down (8)	2 isoforms
zn-alpha2-glycoprotein	up				

disease-influenced CSF proteins. In our first 2-DGE-MS study, protein patterns from individual CSF samples from 15 AD patients and 12 controls were compared (10). We found that the levels of three different apolipoproteins, A-I, A-IV and E, were significantly reduced in the AD patients compared to the control group (Table 1 and Figure 1). Also the level of ubiquitin was significantly reduced in the CSF of the AD patients while the levels of beta2-microglobulin, retinol binding protein (RBP), and transthyretin (TTR) were increased (Table 1 and Figure 2a). Apolipoprotein E (apoE) is synthesized within the brain and has been implicated in the pathogenesis of AD. Inheritance of the epsilon4 allele of the *APOE* gene is strongly associated with increased risk of AD, mainly affecting the age of onset, with each allele copy lowering the age at onset by almost 10 years (38). ApoE and apoJ, another member of the same protein family, have been shown to bind soluble Abeta, and both are associated with senile plaques in the AD cortex. *APOE* epsilon4 has also been associated with increased Abeta deposition in the cerebral cortex (39) and reduced levels of CSF Abeta1-42 (40). Measurements with Enzyme-Linked ImmunoSorbent Assay (ELISA) have found a significant reduction of CSF apoE in AD compared with controls (41). TTR has been shown to bind the Abeta peptide and accordingly has been suggested to protect against Abeta deposition (42). Resent data also suggests that TTR preferentially binds to aggregated rather than monomeric Abeta and arrests further growth of the aggregates (43). Vitamin A (retinoid) is required in the adult brain to enable cognition, learning, and memory. While brain levels of retinoid diminish over the course of normal ageing, retinoid deficit is greater in late onset AD brains than in normal-aged controls (44). Both TTR, and RBP are retinol binding transport proteins and have been suggested as treatment targets for AD (44) Serum RBP and retinol have also been found to be reduced during acute infection and the decrease is proportional to the extent of the infection (45).

In the second 2-DGE-MS study the aim was to increase the sensitivity for the more low-abundance proteins (12). Our first approach was to use narrow pH range IPG strips (also referred to as ultra-zoom gels) in the first dimension separation. This method has earlier been shown to significantly increase the number of protein spots that can be resolved (46, 47). An additional advantage with narrow pH range strips is that higher sample loads can be used in comparison to the wide range gels. In this study the CSF protein patterns from 7 individual AD patients was compared with 7 healthy controls. The levels of 8 proteins were found to be significantly altered in the AD group compared to the controls. Moreover, in this study the levels of several apolipoproteins (J, E, and A-I) were found to be reduced in AD. Other proteins that were found to be significantly up- or down-regulated were, alpha1beta-glycoprotein, alpha1-antitrypsin, beta-trace, RBP, kininogen, and cell cycle progression 8 protein (Table 1). Significantly increased levels in of alpha1-antitrypsin in AD patients compared to controls were also found in two later proteomic studies using ICAT technology and LC-MS (48) and iTRAQ-labelling followed by 2D-LC-MS (49). The latter study also confirmed our finding of reduced levels of apoE in AD (49). Similarly, decreased levels of alpha1beta-glycoprotein in CSF from AD patients were found in a later study that employed multiaffinity depletion, 2-D differential gel electrophoresis (DIGE), and tandem MS (50).

In an attempt to further increase the sensitivity of the proteome analysis we combined pre-fractionation of the CSF proteins by liquid phase IEF with first dimension separation with medium pH range IPG strips. The combination of preparative IEF followed by analytical 2-DGE has earlier been shown by us to facilitate detection of less abundant proteins by reducing sample complexity and allowing for increased protein loads (51). In this study the 2-DGE CSF protein patterns from 5 (of the previous 7) AD patients were compared with 5 (out of 7) non-demented controls. Proteins that were found to be significantly up-or down-regulated in AD compared to controls included alpha1beta-glycoprotein, alpha2-HS-glycoprotein, alpha1-antitrypsin, beta2-microglobulin, transferrin, albumin, and TTR (Table 1). It is noteworthy that, although the pre-fractionation with IEF increased the amount of identified

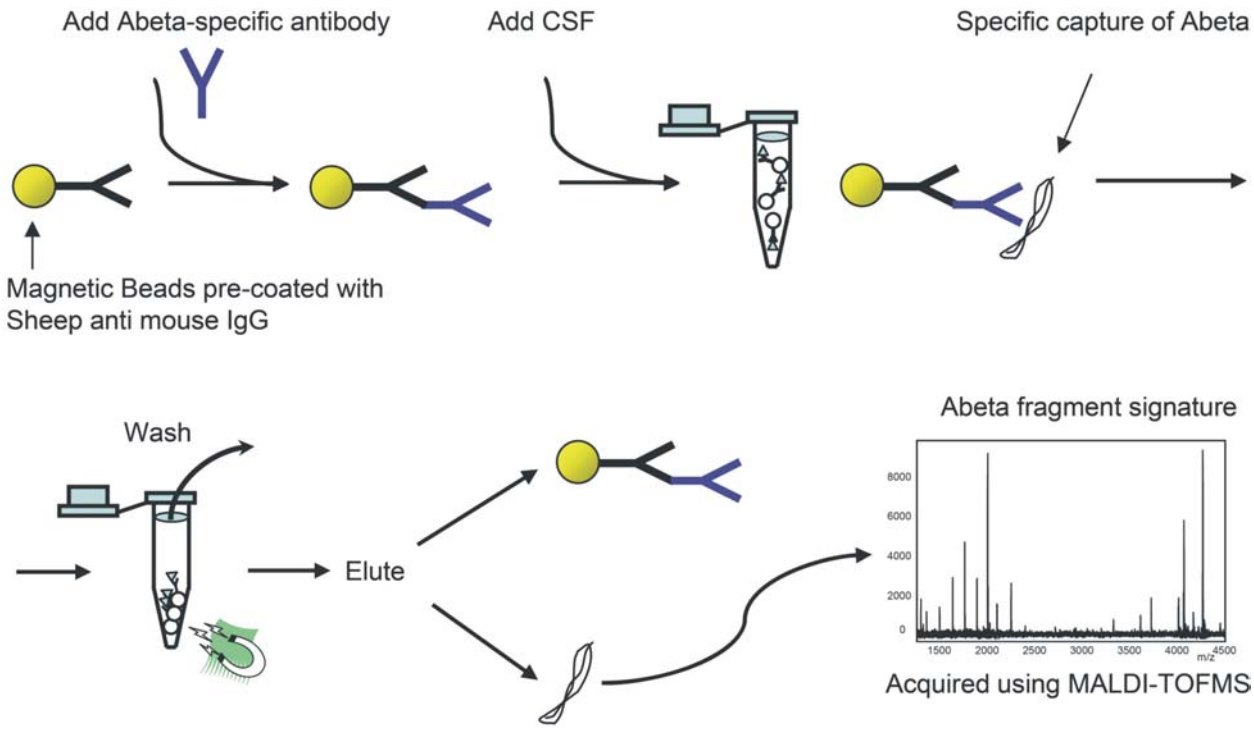

Figure 1. Schematic of the IP-MS methodology.

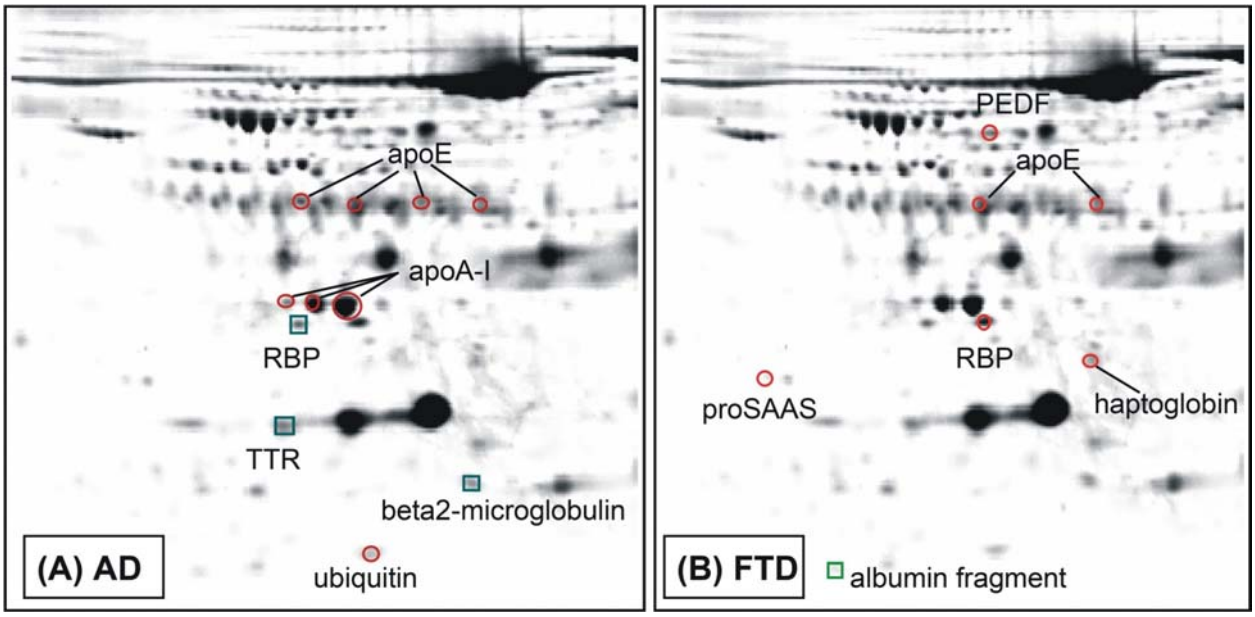

Figure 2. Typical image of SYPRO-Ruby stained mini-gel of CSF proteins. Circles denote the protein spots significantly decreased in the intensity, and squares denote the protein spots significantly increased in the intensity in CSF of patients compared to controls. A) AD B) FTD.

CSF proteins (51), we simultaneously lost most of the apolipoproteins. This is unfortunately a common problem with different kinds of pre-fractionation or other attempts to increase the sensitivity in many proteomics methods which can lead to loss of specific proteins or groups of proteins.

5.1.2. SELDI-TOFMS

In an explorative study we used SELDI-TOFMS to analyze of CSF from 95 AD patients and 72 non-demented healthy controls sourced from multiple

institutions to find putative biomarkers for AD (Simonsen *et al.* Neurobiology of aging 2007). The mean peak intensities from duplicate samples were used for statistical analysis. A Mann-Whitney test was used to calculate p-values for individual peaks across each group and the area under the receiver operator characteristics curve (ROC AUC) was also calculated producing a list of 30 candidate biomarkers. Twelve of the proteins were elevated in the AD patient CSF while 18 were decreased. Fifteen out of the 30 candidate biomarkers peaks were purified and positively identified. We detected increased levels of Abeta1-40, Abeta1-38, and C3a-anaphylatoxin des-Arg as well as decreased levels of several protein fragments such as an osteopontin fragment, a chromogranin A fragment, and a neurosecretory protein VGF fragment. We also detected decreased levels of intact cystatin C and increased levels of the eight amino acid N-terminal truncated cystatin C form in the CSF of AD patients. A similar investigation performed by Carette *et al.* showed partially overlapping results (52). In this study SELDI-TOFMS was used to generate CSF protein profiles from nine AD patients and ten healthy controls. Carrette *et al.* detected statistically significant differences regarding four over expressed and one under expressed polypeptide in the CSF of AD patients as compared to healthy controls. Four of them were further purified and identified as cystatin C, two beta2-microglobulin isoforms, an unknown 7.7 kDa polypeptide, and a 4.8 kDa VGF polypeptide. A decreased expression in AD CSF of the 4.8 kDa VGF polypeptide is in agreement with our data and one can speculate that the 7.7 kDa polypeptide detected by Carrette *et al.* corresponds to one of the osteopontin fragments identified in our study.

The finding that Abeta1-40 was increased in AD samples was not in agreement with findings from previous studies using ELISA for Abeta1-40 quantification (53, 54). To examine this further we analyzed the levels of Abeta1-40 in CSF from 23 AD patients and 13 healthy controls by three independent methods for quantification (6). Two of the methods used were immuno-based, ELISA and SELDI-TOFMS with antibody coated arrays, while the third method was based on direct binding of proteins to IMAC-Ni arrays. Abeta1-40 was detected on IMAC-Ni arrays as a 4328 Da peak and its identity was confirmed by tandem mass spectrometry on the purified peptide. Both the ELISA method and the SELDI immunoassay showed comparable levels of Abeta1-40 in the CSF of AD patients and healthy controls. The SELDI chromatographic assay, however, showed a statistically significant increase of Abeta1-40 in CSF of AD patients as compared to controls. The reason for the discrepancies between results from antibody-based methods and the SELDI chromatographic method might be explained by the analytical differences between these techniques. In CSF, Abeta1-40 has been shown to be present not only in monomeric but also in aggregated (Abeta oligomers) forms. It may also be bound to carrier proteins such as albumin and lipoproteins, which are also present in CSF. In such oligomers or complexes, the epitope recognized by the primary Abeta antibody may be masked and thus be unavailable for detection by the antibody. Antibody-based methods may therefore be better suited for detection of free Abeta but less suitable for measurement of total Abeta (both free and multimeric forms).

We also used SELDI-TOFMS for proteomic analysis of CSF to discover novel proteins and peptides that could be used to differentiate between patients with stable MCI and those with progression to AD (7). The study included 56 cognitively stable patients with MCI and 57 patients with MCI who developed AD.

The patients were followed up clinically at least until they developed a certain type of dementia or had been cognitively stable for more than 4 years (mean follow-up, 5.2 years; range, 4.0-6.8 years). Moreover, 28 healthy controls were included, who were followed up over 3 years. For biomarker selection the primary comparison was between MCI-AD and MCI-stable and a panel of 17 putative biomarkers for the progression from MCI to AD was discovered. Of these, 4 were down-regulated and 13 were up-regulated in the MCI-AD group. Five proteins were identified: a phosphorylated C-terminal fragment of osteopontin, ubiquitin, C4a-anaphylatoxin des-Arg, C3a-anaphylatoxin des-Arg, and beta2-microglobulin. Among the proteins with increased expression level in the CSF of patients with MCI progressing to AD were C3a-anaphylatoxin des-Arg and C4a-anaphylatoxin des-Arg. C3a and C4a are part of the complement system implicated in the inflammatory processes of AD. Notably is also that Abeta directly activates the complement cascade by binding to C1q-anaphylatoxin des-Arg,, which can produce the anaphylactic peptides C3a, C4a, and C5a (55), a process that is potently inhibited by complement factor H, encoded by a novel susceptibility gene for AD (56). C3a anaphylatoxin des-Arg was upregulated in patients with AD in our study described above. We also found increased levels of ubiquitin in the CSF of patients with MCI progressing to AD. This is in agreement with previous studies on AD using ELISA, which found increased CSF levels of both free and conjugated ubiquitin (57, 58). In the brain, ubiquitin is covalently associated with the insoluble neurofibrillary material of neurofibrillary tangles and senile plaques (59). The most well known function of ubiquitin is to target proteins for proteasomal degradation. Furthermore, we described the discovery of a candidate cytokine-related biomarker that was increased in the CSF of patients with MCI progressing to AD, a phosphorylated C-terminal fragment of osteopontin. Osteopontin is a pleiotropic integrin-binding protein and proinflammatory cytokine with functions in cell mediated immunity, inflammation, tissue repair, and cell survival. It has been identified as the most prominent cytokine- encoding gene expressed within multiple sclerosis lesions. beta2-microglobulin constitutes the small constant component of the class I major histocompatibility complex, and its presence in biological fluids represents the balance between membrane protein turnover and elimination. Partially folded beta2-microglobulin is a key intermediate in the generation of amyloid fibrils *in vitro*.

5.1.3. IP-MS

Pathogenic events in Alzheimer's disease involve an imbalance between the production and clearance of the

Table 2. Proteins identified in 2-DGE-MS studies as up- or down- regulated in FTD compared to controls

CSF protein	Levels in FTD vs. control		Comment
	2-DGE	IEF+ 2-DGE	
albumin	up	down	6 isoforms
apolipoprotein A-I		up	
apolipoprotein E	down		2 isoforms
beta2-microglobulin		up	
proSAAS	down		
PEDF	down		
retinol binding protein	down	down	
haptoglobin	down		
transthyretin	up		2 isoforms
zn-alpha2-glycoprotein		up	

neurotoxic Abeta peptide, especially the 42 amino acid peptide Abeta1-42. While much is known about the production of Abeta1-42, many questions remain about how the peptide is degraded. To investigate the Abeta isoform pattern, we have developed a method based on immunoprecipitation combined with mass spectrometry that determines the Abeta fragment pattern in CSF. By using different antibodies with different epitopes both N- and C-terminally truncated Abeta peptides were detected with MALDI-TOFMS and/or nanoflow LC/MS/MS with a hybrid linear ion trap FTICR mass spectrometer. By using IP-MS, we have performed a detailed characterization of different Abeta isoforms in CSF and detected 18 different truncated forms and using a multivariate method by means of the partial least squares discriminant analysis we showed that a relative abundance pattern of Abeta1-16, Abeta1-33, Abeta1-39, and Abeta1-42 in CSF distinguishes sporadic AD patients from non-demented controls with a sensitivity of 89%, specificity of 83% and a overall accuracy of 86%. Furthermore, we have increased the quantitative performance of the Abeta IP-MS method by using two different isotopically labelled Abeta peptides (Abeta1-15 $Arg^{13}C^{15}N$ and Abeta1-34 $Arg^{13}C^{15}N$). Our data support the view that an imbalance in Abeta homeostasis that causes plaque formation in sporadic AD may be primarily due to impaired degradation and clearance of the peptide and that enhancement of the activity of Abeta-degrading enzymes may be a viable strategy in the treatment of the disease. Further, the Abeta fragment pattern may be useful as a diagnostic marker for AD.

5.2. Frontotemporal dementia

Frontotemporal dementia (FTD) is a neuropathologically distinct neurodegenerative disorder that involves atrophy of the frontal and anterior temporal cortex regions with prominent tau pathology. The typical clinical picture is a slowly progressive dementia with disinhibition, early loss of insight, changes of oral/dietary behaviour and stereotypy of speech and behaviour, but these symptoms are not unique to FTD and are often seen in the more common neurodegenerative disorder, AD (60). Among the CSF candidate markers previously investigated in relation to FTD (total tau, hyperphosphorylated tau, Abeta1-42, S100b, and neurofilament) none has had the sensitivity and specificity needed for clinical use (61-67). Hence, studies designed to discover novel sensitive and specific markers for FTD are required.

5.2.1. 2-DGE-MS

In a 2-DGE-MS study (9) protein patterns from CSF were compared between 15 FTD patients and 12 controls. Six proteins were significantly altered in FTD compared to controls, including granin-like neuroendocrine precursor (proSAAS), pigment-epithelium derived factor (PEDF), RBP, apoE, haptoglobin, and albumin. ProSAAS, PEDF, and RBP had not been shown earlier to be involved in the FTD pathology (Table 2 and Figure 2b). In a following study we used pre-fractionation by liquid phase IEF to increase the sensitivity for low-abundance proteins. In this study 2-DGE patterns from CSF proteins from 5 individual FTD patients were compared with 5 non demented controls. 26 protein spots were changed at least two fold. Using mass spectrometry, 13 of these protein spots were identified, including RBP, Zn-alpha2-glycoprotein, apoA-I, beta2-microglobulin, TTR, and albumin. (Table 2). It is, interesting to note that there are distinct differences between the results for AD and FTD indicating that there might be different chemical pathologies in the two diseases.

5.2.2. SELDI-TOFMS

In an attempt to further explore the differences in protein profiles between clinically diagnosed FTD patients and non-demented controls a SELDI-TOFMS study was undertaken (5). In this study CSF protein profiles from 16 FTD patients were compared to the CSF protein profiles from 12 controls. CSF samples were analysed on four different SELDI array surfaces (Q10, CM10, H50 and IMAC-Cu). In total around 2000 protein peaks were detected and by single marker statistics on the normalised peak intensities 42 protein peaks were differentially expressed in FTD compared to controls. After extensive data reduction ten protein peaks were selected, five of which were increased in FTD and five were decreased. Using partial least square discriminant analysis (PLS-DA), the combination of these biomarkers discriminated FTD from non-demented controls with a sensitivity of 94%, a specificity of 83% and an accuracy of 89%. Five of the peaks were purified further and identified by tandem MS as a fragment of neurosecretory protein VGF, TTR, S-cysteinylated TTR, truncated cystatin C and a fragment of chromogranin B. It is noteworthy that TTR is the only CSF protein that can be identified as differentially expressed in FTD by independent proteomics methods including our own SELDI-TOFMS and 2-DGE-MS studies.

5.3. Multiple sclerosis

Multiple sclerosis is an autoimmune inflammatory disease of the nervous system (68). Despite intense research the identity of the primary antigen(s) initiating the autoimmune reaction is unknown and much is uncertain regarding the disease mechanism at the molecular level. Since early initiated treatment is likely to have a beneficial influence on disease progression, methods improving correct early diagnosis is of highest importance (69, 70). At present diagnosis is made on the basis of clinical status, the presence of CSF oligoclonal IgG-bands and specific lesions visible on magnetic resonance imaging

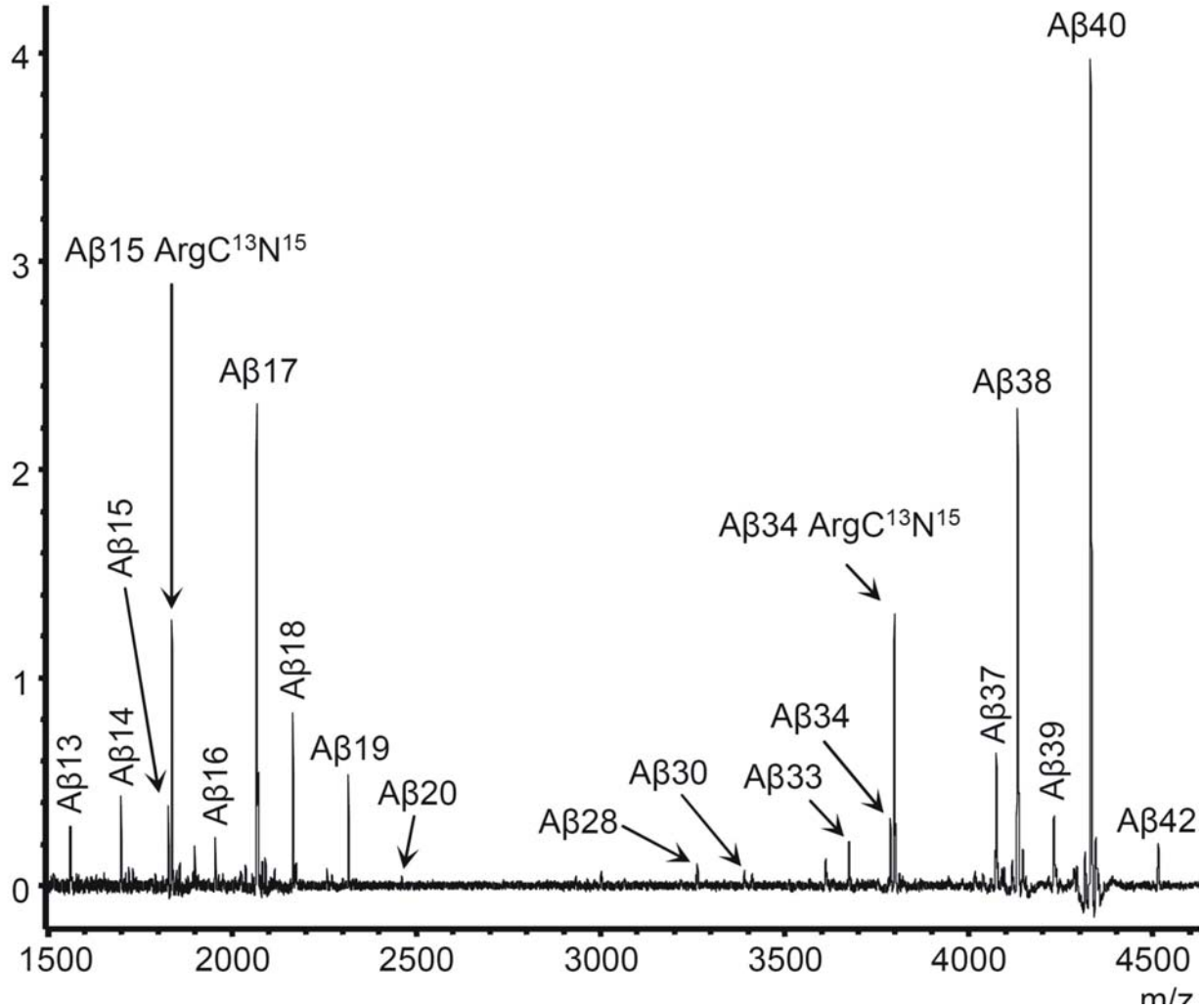

Figure 3. MALDI-TOF mass spectrum showing immunoprecipitated Abeta peptides from CSF.

(71). More specific disease markers, for example CSF proteins enhancing the accuracy of the diagnosis in early uncertain cases would be valuable. Disease-specific CSF proteins could also generate important information regarding multiple sclerosis pathogenesis.

5.3.1. SELDI-TOFMS

We have performed a global proteomic analysis to find proteins differentially expressed in CSF of multiple sclerosis patients as compared to healthy controls (4). SELDI-TOF was used to analyze protein expression in CSF from 46 multiple sclerosis patients, 46 healthy siblings and 50 unrelated healthy controls. In the mass range 2-10 kDa we found 24 proteins that were either up- or down-regulated in multiple sclerosis. Three proteins were purified and identified by MS/MS as two fragments derived from chromogranin B and one fragment from secretogranin II. The identity was confirmed by immuno-precipitation experiments (Figure 4) and the expression levels were confirmed by fragment specific radioimmuno assays (RIA). Proteins in the granin family are acidic soluble proteins widely distributed in neuroendocrine and nervous-system tissues (72). The granins are characterised by numerous pairs of basic amino acid residues, potential cleavage sites for prohormone convertases I and II that are likely to be involved in processing of granins into active peptides with numerous intracellular and extracellular functions (73). The chromogranin and secretogranin fragments identified in our study conforms well to the rule of proteolytic processing at di-basic amino acid motifs. The SELDI methodology war also used by Irani *et al.* to study protein profiles in CSF samples from patients with multiple sclerosis (74). Out of the four peaks found to be differentially expressed in multiple sclerosis, one with decreased levels in CSF was identified as intact cystatin C and one with increased levels was identified as an eight amino acid truncated form of cystatin C. The truncated form of cystatin C distinguished multiple sclerosis from other neurological disorders with 100 % specificity. However, the truncated 12.5 kDa cleavage product of cystatin C has been shown to be produced from full length cystatin C *in vitro* during suboptimal storage conditions (3, 75). Moreover, the Irani results have been refuted by several independent research groups (3, 76, 77). It has not been possible to reproduce the

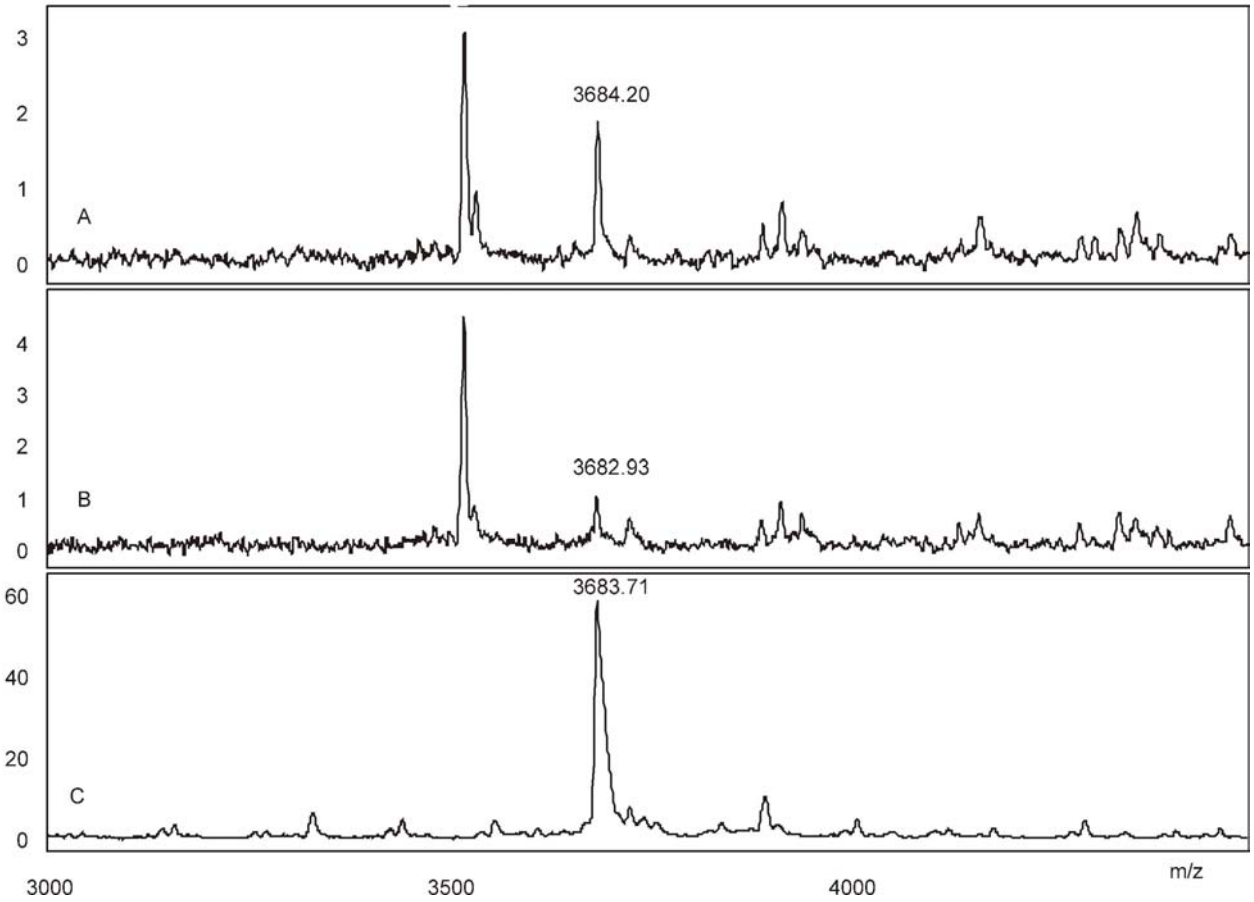

Figure 4. Immunoprecipitation experiment for validation of the secretogranin II peak in CSF. SELDI-TOF mass spectra of A) original CSF, B) CSF after immunoprecipitation with an antibody specific for amino acids 154-165 in the secretogranin II sequence, and C) the precipitated antigen.

finding of truncated cystatin C as a specific marker for multiple sclerosis. This further emphasizes the impact of pre-analytical variables on the analysis of biological fluids in proteomic studies.

6. DISCUSSION AND CONCLUSIONS

When comparing data from independent proteomics studies of neurodegenerative disease specific up-and down-regulation of CSF proteins, it is clear that the overlap in identified possible biomarkers is relatively low (49). To a certain extent this probably reflects the inherent strengths and weaknesses of the vast collection of methodologies used in a proteomics workflow to cover subsets of the proteome. For example, the optimum mass range of the SELDI technique where it has its highest resolving power and sensitivity, is in the mass range 1-30 kDa-range. Meanwhile, 2-DGE experiments are preferably used for the analysis of higher molecular mass proteins since the protein fraction below ~10 kDa is usually lost. Another potential source of the discrepancies is the variety of methods used to interpret proteomics data and to validate the protein identifications. Unfortunately, even high-quality data can produce false positive results and the accuracy of the experimental data as well as the rules and parameters for the database search has a profound impact on the results generated. Altogether, the field of proteomics is in a great need of guidelines to assess the quality of proteomics data. To address this problem the Human Proteome Organization (HUPO) has started to develop guidance documents to facilitate data comparison, exchange and verification within proteomics (Proteomics Standards Initiative http://www.psidev.info) (78, 79). An additional analytical challenge is that in proteomics the amount of variables, e.g., protein concentrations, usually greatly exceeds the number of observations/cases. Classical univariate statistics, which still dominates the scene, has its limitations for this kind of data structure. First, information embedded in combinations of variables is lost when the variables are analyzed one-by-one. In contrast, multivariate discriminant analysis utilizes all variables at the same time to build a model that best separates the predefined classes. Second, the risk of spurious findings increases with the number of variables. The quality of a multivariate classification model can be tested by randomly divide the observations into training and prediction sets prior to analysis. Only the training set is used for constructing a model, which is then utilized for classification of the prediction set. The quality

of the model is judged from the number of correctly classified observation in the prediction set. There are a number of different multivariate algorithms that can be used for classification. Some of these are evaluated in a paper by *et al.* (80), where references to the theoretical foundation for each technique can also be found.

Apart from the complications due to the proteomic methodologies involved one must also be aware that differences in CSF sampling and handling as well as differences in progression of the disease influence the results. Other factors such as classification criteria of patients (and controls) and patients medications may also vary between different studies. Clinical proteomics is also particularly susceptible to such pre-analytical factors as degradation or loss of the protein and peptides *in vitro* after sampling, influence of repeated freeze-thaw cycles, time and condition of sample storage, and adhesiveness of some molecules to the test tube wall. Our group, as well as other researchers, has previously shown that measured CSF tau and Abeta1-42 concentrations depend on test tube material and other storage conditions (81-83). Below we list 6 crucial factors that have to be taken into account when designing proteomics studies involving CSF samples:

1. Lumbar and ventricular CSF samples are not comparable.
2. A standardized collection volume should be used, preferably 10-12 mL of lumbar CSF to avoid gradient effects.
3. The lumbar punctures in a study should be performed at approximately the same time of day, to avoid the risk of detecting changes due to spontaneous diurnal variation.
4. Samples need to be screened for blood contamination.
5. CSF must be handled in a standardized way after the spinal tap.
6. Store samples in aliquots in polypropylene cryogenic vials at –80 °C without additional freeze-thaw cycles. Do not use different freezers for cases and controls.

Here it is important to note that the optimal sampling and storage conditions may vary between different proteins in the studied subproteome. Nevertheless, it is absolutely vital that samples from the disease and control groups are treated identically in all respects to avoid false positive results. Moreover, by targeting a well defined subproteome, e.g. isoforms of APP and Abeta, it is more straightforward to find and retain the optimum pre-analytical conditions.

We believe that proteomic findings should, whenever possible, be replicated in independent populations and preferably using independent techniques, e.g. immunochemical methods. Such a consensus would most likely decrease the number of spurious positive disease associations considerably and increase the likelihood of discovering diagnostic tools for pre-clinical detection of neurodegenerative disorders.

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Abbreviations: AD: Alzheimer's disease; FTD: frontotemporal dementia; PD: Parkinson's disease; SELDI: surface-enhanced laser desorption/ionisation; TOF: time-of-flight; MS: mass spectrometry; MALDI: matrix-assisted laser desorption/ionisation; ESI: electrospray ionisation; Q: quadrupole; 2-DGE: two-dimensional gel electrophoresis; CSF: cerebrospinal fluid; IP: immunoprecipitation; IPG: immobilized pH gradient; SD-PAGE: sodium dodecylsulphate polyacrylamid gel electrophoresis; IEF: isoelectric focusing; IMAC: metal affinity; Abeta: amyloid-beta; LC: liquid chromatography; APP: amyloid precursor protein; FA: formic acid; ACN: acetonitrile; FTICR: fourier transform ion cyclotron resonance; MCI: mild cognitive impairment; TTR: transthyretin; RBP: retinol binding protein; apo: apolipoprotein; ELISA: enzyme-linked immunosorbent assay; DIGE: differential gel electrophoresis; ROC AUC: area under the receiver operator characteristics curve; proSAAS: granin-like neuroendocrine precursor; PEDF: pigment-epithelium derived factor; PLS-DA: partial least square discriminant analysis; RIA: radioimmuno assays; HUPO: Human Proteome Organization

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