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Blood and plasma-based proteomic biomarker research in Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is the most important cause of dementia in the elderly. The molecular alterations preceding this neurodegenerative pathology may take place even 20 years before its clinical appearance. In this context, the discovery of biomarkers in biological fluids enabling an early presymptomatic diagnosis as well as discrimination from other types of dementia is eagerly awaited. In particular, since the traditional markers obtained both from cerebrospinal fluid inspection and neuroimaging approaches have not achieved a broad clinical application, research efforts have been focused on the development and validation of biomarkers in blood. The benefit of searching for blood-based candidate biomarkers is evident due to the easiness and non-invasiveness nature of blood samples collection compared with any other body fluid. As a result, blood may constitute a rich source of disease biomarkers. Interestingly, among the technological platforms used to perform research into the biomarker discovery arena, proteomics has attained more recent consideration. In the present review, we provide a comprehensive assessment of patterns of biomarkers detected in plasma and serum specimens for the diagnosis of AD by employing proteomic approaches. Currently, growing evidence suggests that blood protein signatures are helpful to increase the likelihood of successful diagnosis of AD. Accordingly, this area of research promises to yield exciting results in the next future.

Contents

1. Introduction . . . 2
2. Technologies for proteomic analysis . . . 3
 2.1. Proteomic approaches for plasma and serum analysis . . . 3
  2.1.1. Two-dimensional polyacrylamide gel electrophoresis and mass spectrometer analysis . . . 5
  2.1.2. Protein profiling . . . 5
  2.1.3. Shotgun proteomics . . . 6
  2.1.4. Validation of biomarker candidates . . . 7
 2.2. Detection of patterns of biomarkers in blood for AD diagnosis . . . 7
  2.2.1. Proteome-based biomarkers of AD in plasma . . . 7
  2.2.2. Oxidative stress and proteomics of oxidized plasma proteins in AD . . . 11
  2.2.3. Proteome-based biomarkers of AD in serum . . . 12
  2.2.4. Proteome-based biomarkers in blood and treatment efficacy of AD . . . 12
  2.2.5. Depiction of plasma and serum in AD proteomic studies . . . 13

Abbreviations: AD, Alzheimer's disease; ApoA-I, apolipoprotein A-I; CNS, central nervous system; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; EMA, European Medicines Agency; ESI, electrospray ionization; F2-IsoPs, F2-isoprostanes; FDA, Food and Drug Administration; GC, gas chromatography; HCs, healthy controls; HPLC, high-performance liquid chromatography; HUPO, Human Proteome Organization; HUPO BPP, HUPO Brain Proteome Project; HUPO PPP, HUPO Plasma Proteome Project; ICAT, isotope coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; *m/z*, mass-to-charge ratio; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MCI, mild cognitive impairment; MS/MS, tandem mass spectrometry; NFTs, neurofibrillary tangles; PET, positron emission tomography; PiB, Pittsburgh Compound B; p-tau, hyperphosphorylated tau; PTMs, post-translational modifications; SELDI-TOF, surfaced-enhanced laser desorption/ionization time-of-flight; t-tau, total tau; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis.

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3. Concluding remarks . . . 14
Acknowledgements . . . 14
References . . . 14

1. Introduction

Alzheimer's disease (AD) – the main cause of dementia in the elderly – results in extracellular beta-amyloid (Aβ) plaques deposition and hyperphosphorylation of tau protein with formation of neurofibrillary tangles (NFTs). These modifications lead to neuronal cell death, vascular dysfunction, and inflammatory processes (Blennow et al., 2006). AD diagnosis is time-consuming and challenging, particularly for physicians who lack dedicated training in this area, since it involves the integration of psychological tests, imaging technologies, and exclusion of other neurological pathologies. This problem is aggravated in patients coming from rural communities and lower socio-economic background, who may not easily obtain access to state of the art diagnostic methods for AD such as imaging or other systems existing in university research settings, which amplify the sensitivity and reliability of AD diagnosis. Actually, recent investigations indicate that about two-thirds of dementia cases may be undiagnosed (Ho et al., 2010). It is estimated that the first AD-typical molecular alterations may occur in subjects even several decades years before the manifestation of clinical symptoms of dementia. Thus, owing to the long duration of such an asymptomatic phase, during which pathophysiological mechanisms are building up, it is of paramount significance to detect biological markers reproducing accurately features of AD pathophysiology, before the expression of dementia (Sperling et al., 2011). Accordingly, new diagnostic criteria for AD have recently been proposed, which define pre-dementia stages (''prodromal AD'' (Dubois et al., 2010, 2007)); Mild Cognitive Impairment (MCI) due to AD (Albert et al., 2011) and even presymptomatic stages of AD (''asymptomatic AD'' (Sperling et al., 2011), ''asymptomatic at-risk for AD'' (Dubois et al., 2010, 2007)). These new diagnostic concepts are necessary prerequisites and legitimation for prevention and early intervention trials in AD and may help to identify early preclinical stages which might be more responsive to potential disease-modifying therapies than later clinical stages of AD.

In recent years, progresses in the use of biomarkers have aroused a significant interest of regulatory agencies throughout the globe. In Europe and the US, the use of biomarker methods is a high priority on the work programs and listings both of the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) (Broich et al., 2011). In particular, the Biomarkers Definitions Working Group of the US National Institutes of Health (2001) described a biological marker as an objectively measured feature that is assessed as an indicator of normal biological or pathogenic processes or pharmacological responses to a therapeutic intervention. Such biomarkers should be reliable, non-invasive, simple to perform, inexpensive, and employed to exactly categorize the population in line with the disease (Schneider et al., 2009).

Advances in the knowledge of the natural history of AD might have a central function in determining the real efficacy of new drugs: the early diagnosis of AD in association with new classes of disease-modifying drugs may slow down the neurodegenerative traits of the pathology. Unfortunately, an accurate, early diagnosis of AD is still difficult because early symptoms are shared by various disorders, reflecting common neuropathological properties.

The levels of amyloid β peptide 42 ($A\beta_{1\text{-}42}$), total tau (t-tau) protein, and hyperphosphorylated tau (p-tau) protein in cerebrospinal fluid (CSF) have been comprehensively investigated and have shown strong association with clinical AD (Shoji, 2002; Shoji et al., 2002). In particular, $A\beta_{1\text{-}42}$, t-tau, and p-tau measurement in CSF of AD patients, as well as individuals with other dementing pathologies and normal elderly controls emphasized sensitivity and specificity levels between 80% and 90% to identify AD versus normal elderly (Blennow et al., 2010; Hampel et al., 2008). Moreover, CSF amounts of $A\beta_{1\text{-}42}$, t-tau, and p-tau were described to be able to discriminate between those subjects with MCI who are likely to develop AD (MCI-AD) and those who do not convert (MCI-MCI) (Hampel et al., 2008; Mattsson et al., 2009). At the present time, these CSF markers represent one of the best validated diagnostic biomarkers of AD. Nevertheless, lumbar puncture, the procedure required to collect CSF samples, is still considered in several countries a relatively invasive practice that may cause patient discomfort and present some side effects such as the post-lumbar puncture headache (De Almeida et al., 2011). Despite the fact that large numbers of studies show marginal incidence of lumbar puncture associated headaches and virtually non-existent further neurological or clinical complications in a memory clinic setting (Zetterberg et al., 2010), CSF sampling still suffers from a negative public reputation, accompanied by high rates of reservation among patients (Schneider et al., 2009).

Similarly, some radiolabeled ligands, with the capability of binding to fibrillar Aβ deposits in the brain, have been expansively studied. Up to now, the most commonly utilized amyloid tracer is the ''Pittsburgh Compound B'' (PiB) (Herholz and Ebmeier, 2011; Prvulovic and Hampel, 2011). Positron emission tomography using PiB (PiB-PET) can be employed as a tool for monitoring changes in the Aβ plaque burden (Klunk et al., 2003). Interestingly, post-mortem autopsy studies disclosed an important correlation between PiB binding and histological Aβ plaque load (Bacskai et al., 2007; Ikonomovic et al., 2008). Unfortunately, a major drawback is the fact that PiB is radiolabeled using the ^{11}C isotope, which has an extremely short half-life time (about 20 min), making PiB-PET technology bulky, uncomfortable and expensive. As a result, the use of this imaging modality in primary healthcare settings and in routine screening of individuals at risk of AD currently appears to be impractical (Ho et al., 2010; Klunk and Mathis, 2008).

Given that, to date, the recognized and traditional biological markers obtained from both CSF examination and neuroimaging approaches have not achieved an extensive clinical application, research efforts have been focused, ultimately, on the development and validation of biomarkers in blood, plasma, or serum (Schneider et al., 2009).

Blood is considered a complex liquid tissue that encompasses cells and extracellular fluid. It comprises, as a source for biomarkers, a number of dissimilar molecules. Proteins, nucleic acids, lipids, and other metabolic products can be observed in plasma, serum, and cellular compartments. The latter includes erythrocytes, leucocytes, and platelets, separated either crude (e.g., buffy coat after density gradient centrifugation) or isolated by flow cytometry into distinct cell subsets. Given the existence of different unique compartments, the variety of potential candidate biomarkers in blood is significant and may embrace: protein concentrations, protein isoforms, and post-translational modifications (PTMs); a vast group of metabolic products (such as lipids and

carbohydrates) that are subject to substantial alterations; DNA, comprising single nucleotide polymorphisms, and RNA, the complexity of which is only beginning to be recognized (Thambisetty and Lovestone, 2010). In this connection, RNA, besides being extracted from cells, has recently been discovered to be included in plasma exosomes, a fascinating and interesting new potential source of biomarkers (Hunter et al., 2008; Simpson et al., 2009). Such observations are useful to stress that, unlike CSF and other body fluids, blood is a multifaceted tissue (Thambisetty and Lovestone, 2010).

The prospective benefit in searching for blood-based biomarkers appears evident as blood samples are easier to collect than CSF and can present a potentially rich source of disease biomarkers. Moreover, disruption of the blood-brain barrier, which takes place throughout AD, may increase the exchange of proteins and peptides between blood and CSF in both directions (Zipser et al., 2007) and potentially provide stage- or endophenotype-specific diagnostic information.

Blood-based biomarkers could be attained routinely in the community in primary care as well as in the patient's home. They lend themselves to frequent measurement – even in elderly people and critical patients – and to high-throughput and cheap inspections (Thambisetty and Lovestone, 2010). Accordingly, a blood-based testing would be broadly accessible, non-invasive, easy and fast to execute as well as economic (Schneider et al., 2009).

2. Technologies for proteomic analysis

Among the emerging platforms developed to carry out research into biomarkers for neurodegenerative diseases, with AD being studied most extensively, proteomics has surely gathered more recent consideration. The word proteome was utilized first in 1994 to designate a set of all proteins expressed by a particular genome (Wasinger et al., 1995). Since its composition may differ from tissue to tissue and from cell to cell, the proteome refers to a set of proteins in a certain time and space, which underlines its dynamic nature (Tambor et al., 2010).

The essential unit of a proteome is represented by proteins, the functional entities of the cell, highlighting biochemical reactions, enzymatic processes, and signal transduction pathways (Rai and Merlini, 2009). Proteins consist of an amino acidic sequence encoded by a specific gene. Nevertheless, the genetic code alone is not able to provide comprehensive information about a protein: whereas the genomic information, dependent on the arrangement of four nucleotides, is static, the information contained in proteins is not merely restricted to their amino acid sequence (Tambor et al., 2010). Proteins display characteristics due to PTMs, conformational alterations, and alternative splicing processes that reveal the extraordinary variability of the proteomic information. Thus, all these observations account for the high amount of proteins that is much greater than the number of their corresponding genes, mainly in the eukaryotic reign. For these reasons, proteins are believed to have a high potential as dynamic biomarkers for use in the diagnosis, prediction and monitoring the development of a given pathology (Thambisetty and Lovestone, 2010).

Since proteomics was first established to have the ability to characterize a great number of differences in both protein quality and quantity, it has been utilized on a global scale in neuroscience to analyze and unravel the molecular "bar code" of the brain. Such a system-based approach is defined as neuroproteomics, i.e., the large-scale profiling and functional annotation of brain proteins (Becker et al., 2006). The depiction of the Central Nervous System (CNS) proteome under normal and pathological conditions is one of the central initiatives sponsored by the Human Proteome Organization (HUPO), the largest international, non-profit consortium supporting proteomic research and analysis of human tissues. Specifically, the target of the HUPO Brain Proteome Project (HUPO BPP) is the understanding of the pathologic mechanisms of the CNS proteome in ageing and neurodegenerative disorders. This aim will be accomplished by decoding the normal brain proteome, correlating the expression pattern of brain proteins and mRNA, and detecting proteins responsible for neurodegenerative diseases (Hamacher and Meyer, 2005).

The most important problem related to the search for protein biomarkers is the enormous dynamic range – more than 10 orders of magnitude – of blood proteins (Anderson and Anderson, 2002; Corthals et al., 2000) that makes it unlikely that methods capable of detecting all blood proteins will be established in the near future. This wide concentration range, together with the well-known complexity of proteins compared to other types of molecules, suggests that any proteomic technology presently adopted is only studying a small fraction of potential biomarker-relevant changes (Thambisetty and Lovestone, 2010).

In spite of these challenges, a substantial improvement in proteomic exploration of complex tissues – including blood – was made possible by advances in analytical instrumentation, particularly in the mass spectrometry (MS) arena with the development of new, cutting-edge types of mass spectrometers and enhancement of ionization techniques. Moreover, progresses in technologies associated with proteins separation and sample complexity reduction – such as electrophoretic and chromatographic methods – have to be considered. Ultimately, bioinformatics, including the capacity to gather, store, and process huge amount of data, gave valuable contribution to the execution of proteomic studies. Thanks to such advances, the HUPO Plasma Proteome Project (HUPO PPP), whose objective is the mapping of all proteins in plasma, was able to classify, in its first phase, more than 9,000 proteins with some confidence and about 900 with very high certainty (Omenn et al., 2005; States et al., 2006).

2.1. Proteomic approaches for plasma and serum analysis

An assortment of technological approaches is employed in the discovery of biomarkers. The term discovery refers to the unbiased and semiquantitative process by which the differential expression of specific proteins between conditions is documented. Discovery can make use of a diversity of human biological materials as well as mouse models or cell lines and, basically, encompasses a comparison between normal and unhealthy states. The outcome of the discovery phase is represented by a list of proteins shown to be differentially expressed between the normal and the affected tissue according to the semiquantitative estimation of protein/peptide relative abundances. Such lists classically include tens to hundreds markers. Some of them may return false positives: this means that these molecules, after a further testing step, are not differentially expressed anymore with reference to the distinction of interest. For this reason, they should be referred to as "candidate biomarkers" and not just biomarkers (Rifai et al., 2006). At the present time, the systems that have been characterized in biomarker discovery projects show distinctive advantages but may also suffer from specific and significant problems. As a result, a careful examination before the selection of the final technology should be critically considered. A common denominator for such proteomic platforms is the use of MS, an analytical method enabling precise measurements of molecular weights of single constituents of a sample. A mass spectrometer can be divided into three parts: an ion source, an analyzer, and a detector. In a classic MS experiment, sample molecules are converted into gas phase and ionized in the ion source, separated in relation to their mass-to-charge ratio (m/z) in the analyzer, and then detected. The ion

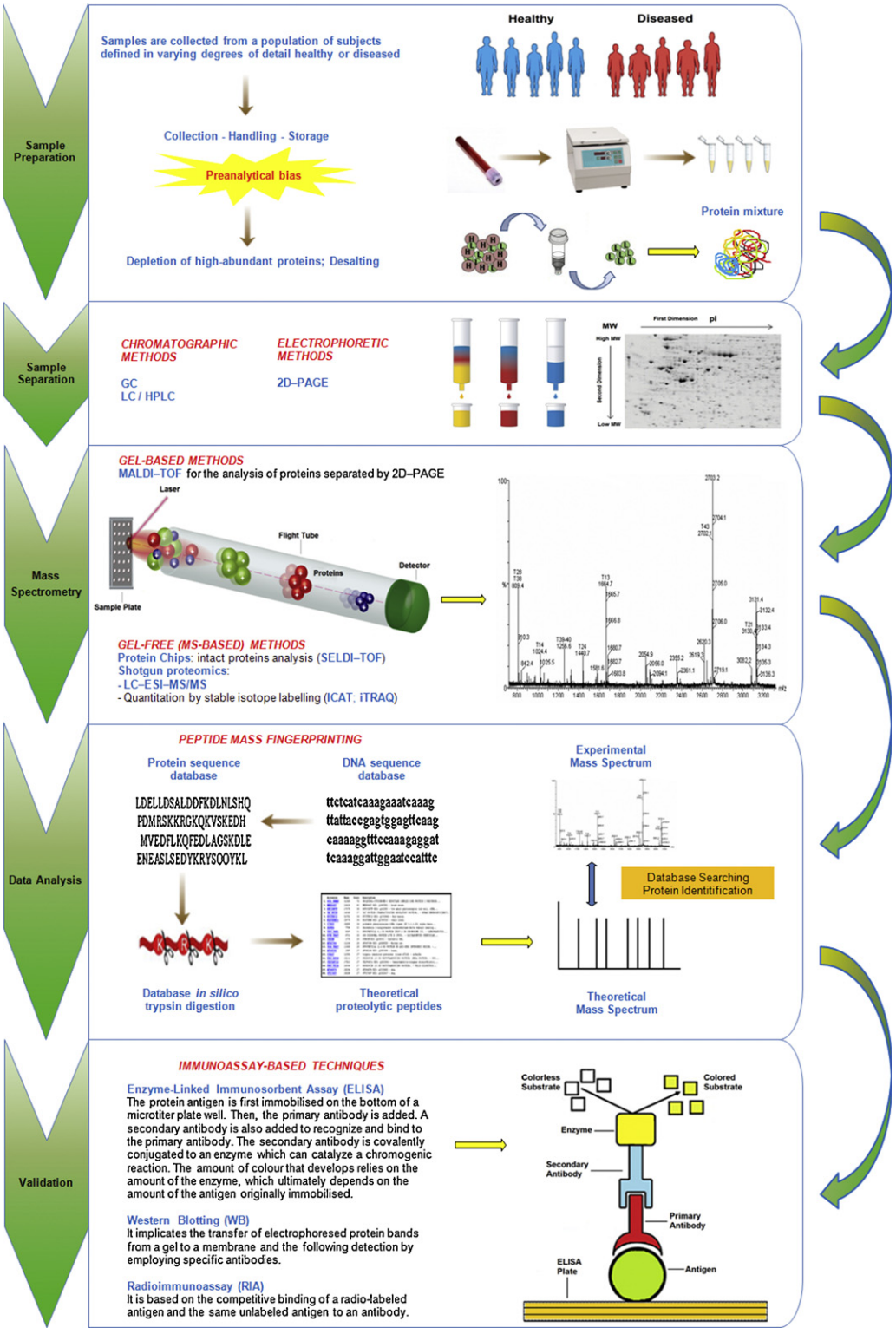

Fig. 1. Overview of a global mass spectrometry-based biomarker-discovery workflow in plasma/serum samples.
Usually, such a workflow includes sample collection, handling, proteins separation through electrophoretic or chromatographic methods, mass spectral analysis, data examination, bioinformatics tools employment, and ultimately validation. Once the samples have been obtained, they are normally processed to remove high-abundant proteins or fractionated into more convenient sizes. After processing, samples are directed towards one or more mass spectrometry platforms for protein identification and relative quantitation to detect proteins that are differentially expressed in the diseased samples vs. the healthy samples.
Once a panel of biomarker candidates has been designated in the discovery phase, a validation phase must be performed with the aim of selecting the ones with highest potential for the clinical diagnosis. While the biomarker candidates are typically identified based on mass spectrometry methods, it is often appreciated to develop an independent analytical process to validate these potential markers. For instance, immunoassays-based techniques are often utilized for validation and development for clinical diagnosis owing to their high sensitivity and throughput. In particular, Enzyme-Linked Immunosorbent Assay (ELISA) and Western blotting (WB) are commonly used immuno-based methodologies to confirm that the concentration of the prospective biomarker differs significantly between the control and the diseased state.
ELISA assay: an unknown amount of a certain antigen is attached to the surface of a microtiter plate well; next, a specific antibody is applied on the plate surface in order to be bound to the antigen. The antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The successive enzyme-substrate

sources generally exploited are matrix-assisted laser desorption/ionization (MALDI), surfaced-enhanced laser desorption/ionization (SELDI), and electrospray ionization (ESI). MALDI and SELDI sources make use of a pulsed laser beam to sublimate analyte molecules – i.e. to directly convert them from the solid state to the gaseous one – and promote their ionization (Tambor et al., 2010). The mechanism of ESI implicates the release of ions, from a droplet, into the gas phase at atmospheric pressure. Next, the gaseous ions are moved into the mass analyzer (Lovestone et al., 2007). The most frequently utilized mass analyzers include the time-of-flight (TOF), ion trap, quadrupole, and Fourier transform ion cyclotron resonance. They are dissimilar in the mechanisms accountable for ion separation, mass accuracy and resolution, but, when used together, they are complementary in the process of protein identification (Aebersold and Mann, 2003; Caudle et al., 2008). An overview of a general mass spectrometry-based workflow for the discovery of biomarkers in plasma/serum samples is documented in Fig. 1.

2.1.1. Two-dimensional polyacrylamide gel electrophoresis and mass spectrometer analysis

Gel electrophoresis indicates the technology in which charged molecules are forced through a gel medium conducted by an electrical current. Electrodes positioned at both ends of the gel supply the driving force. The physiochemical features of the particles as molecular weight, structure, electric charge, and ionic strength regulate how rapidly an electric field can move them across the gel (Luque-Garcia and Neubert, 2007). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) was the first approach applied in full proteomic experiments and is still considered the cornerstone separation technique for complex protein mixtures. The two dimensions, through which the high-resolution separation of proteins occurs in a gel medium, are represented by two physicochemical features: isoelectric point (p*I*) and molecular weight (Carrette et al., 2006; Gorg et al., 2004; O'Farrell, 1975). The final outcome consists of a protein map where different proteins are separated into individual spots. Protein spots are then visualized through several conventional staining protocols (Candiano et al., 2004; Chevallet et al., 2008) and fluorescent dyes (Berggren et al., 2000). Stained gels are digitalized and then evaluated via dedicated software to estimate the amount of specific proteins by comparing the intensities of stained spots (Berth et al., 2007). After separation, protein spots are subjected to the analysis by MS (see Box 1).

2D-PAGE supports the separation of hundreds to a few thousands of protein spots. However, this procedure presents some shortcomings. Proteins with either low (<5000 Da) or high (>150,000 Da) molecular weight and with specific physicochemical properties, as hydrophobic membrane proteins, are problematic to separate and reveal. Also, individual spots can include two or more proteins that differ minimally in their pI and molecular weight; therefore, such proteins are not fully separated. Furthermore, proteins present at very low abundance in composite specimens like plasma/serum or cell extracts cannot be discovered because they are masked by highly abundant molecules (Apweiler et al., 2009).

Box 1. Mass spectrometry analysis of protein spots.

A piece of gel containing an isolated protein of interest is excised and enzymatically digested by a sequence-specific protease, as trypsin, leading to a mixture of peptides ready for a MALDI-TOF-based analysis (Tambor et al., 2010). In MALDI, analyte solutions are left to dry onto target plates in the presence of a matrix substance that induces the formation of crystals. When the target is struck by a laser beam, a set of gaseous ions is produced because the energy of the beam is absorbed by the matrix and transmitted to the analyte molecules which become charged and can be accelerated into the TOF analyzer upon application of a voltage differential. This electrical voltage supplies all the ionized analytes with the same energy. The TOF analyzer separates ions based on the differences in their mass-dependent transit time (i.e., time of flight) from the ion source to the detector along the tube under vacuum. Once the protein ions strike the detector, it records the TOF of the analyte (expressed as *m*/*z*) and the intensity of the response. A MS spectrum is thus obtained, plotting the measured signal intensities (*y* axis) as a function of *m*/*z* values (*x* axis) (Lovestone et al., 2007; Tambor et al., 2010).
The experimentally recorded *m*/*z* measurements are then compared with theoretical *m*/*z* values: the latter are obtained by *in silico* translation of DNA sequences of genes into proteins, from which theoretical proteolytic peptide masses are then calculated (Thiede et al., 2005). In case of a successful matching, a specific protein is assigned to the spectrum with a definite likelihood (score) in line with the MOWSE (MOlecular Weight SEarch) scoring algorithm (Pappin et al., 1993). The probability-based MOWSE score is at the origin of the birth of Mascot, a powerful search engine for protein identification from mass spectrometry data (http://www.matrixscience.com/). If a spectrum does not provide adequate data to identify a protein, the tandem mass spectrometry (MS/MS) technique should be employed. In a MS/MS, two mass spectrometers are placed in sequence to enable direct acquisition of a peptide sequence. One peptide species is selected out of a mixture in the first mass spectrometer and dissociated by collision with an inert gas, as argon or nitrogen. After that, the final fragments are divided in the second component in order to generate the tandem (MS/MS) mass spectrum (Domon and Aebersold, 2006; Mann et al., 2001).

2.1.2. Protein profiling

Direct MS examination of a sample may offer fast and full comprehension of its protein profile since it enables the acquisition of a vast range of *m*/*z* values. Therefore, protein profiles of samples can be rapidly compared, resulting in a list of different peak intensities (Tambor et al., 2010). Gel-free approaches as SELDI-TOF have been introduced to surmount some 2D-PAGE weaknesses; however, these have their own limitations and need extensive bioinformatics and biostatistics efforts (Lovestone et al., 2007; Tambor et al., 2010) (see Box 2). A major limitation of SELDI is the lack of the ability to rigorously identify proteins; consequently, the information about a prospective biomarker protein is restricted to its *m*/*z* value. Characterizing proteins or peptides solely by their *m*/*z* is not sufficient for diagnostic purposes because their

reaction gives place to a signal – generally represented by a color change in the substrate – that can be measured.
WB (or protein immunoblotting): it makes use of a gel electrophoresis approach to separate proteins. These proteins are then transferred to a membrane (usually nitrocellulose or polyvinylidene fluoride, PVDF), where they are detected ("probed") using antibodies specific to the target protein.
Radioimmunoassay (RIA): a very sensitive *in vitro* assay method for estimating the concentrations of antigens. The principle of RIA implicates the competitive binding of a radio-labeled antigen with the same unlabeled antigen towards a high-affinity antibody. Since radioactive substances are exploited, RIA requires specific precautions and licensing.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; ICAT, isotope coded affinity tags; iTRAQ, isobaric tags for relative and absolute quantitation; LC, liquid chromatography; LC-ESI-MS/MS, liquid chromatography electrospray ionisation tandem mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; RIA, radioimmunoassay; SELDI-TOF, surfaced-enhanced laser desorption/ionization time-of-flight; 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; WB, western blotting.

Box 2. Surfaced-enhanced laser desorption/ionization technology.

SELDI platform can be considered a variant of MALDI technique (Hutchens and Yip, 1993; Kiehntopf et al., 2007). What makes this system distinctive is its association with protein chip arrays, peculiar chips whose surfaces are coated with a protein-fractionating resin. Based on the chemistry of the resin, the chip surface can result hydrophobic, hydrophilic, cationic, anionic, or present metal-chelating groups, in such a way that whole classes of proteins are selectively captured. Alternatively, the array can be made immunospecific, by covering the surface with monoclonal antibodies that are selective for only a few proteins. Once bound, the samples are washed, using a buffer to remove all non-specific bindings, and the retained proteins are then analysed by a TOF-MS (Issaq et al., 2002; Merchant and Weinberger, 2000).
This system is a hybrid technology allowing protein separation (by solid-phase chromatography) and analysis (by mass spectrometry) to be carried out using the same analyzer, thus making the investigation easier (Gretzer et al., 2003; Shi et al., 2009).

unknown identity impedes supplementary validation with independent methods (Tambor et al., 2010). Problems of reproducibility and the need for a standardization of preanalytic and analytic factors prevent this technology from becoming a routine proteomic test (Banks, 2008; Bruegel et al., 2009; Callesen et al., 2009). In spite of these shortcomings, SELDI has been fruitfully applied to the investigation of potential biomarkers in the CSF of AD and frontotemporal dementia patients (Carrette et al., 2003; Ruetschi et al., 2005; Simonsen et al., 2008).

2.1.2.1. Array-based proteomics. Protein microarrays, also called protein chips, are typically prepared by depositing and immobilizing molecular probes, designed to capture specific proteins at specific sites, on a solid support. A large catalogue of solid supports based on glass-, plastic-, or silicon-slides are available (Apweiler et al., 2009). Once proteins are immobilized on the slides, they can be probed for a multiplicity of functions and activities. The resulting signals are measured by using fluorescent or radioisotope labels. Two categories of protein microarrays presently exist: analytical and functional protein microarrays. The former, used in clinical diagnosis both for biomarker identification and to monitor protein expression levels, comprise the antibody microarrays, in which antibodies are arrayed on glass surfaces at high density. Functional protein microarrays have several uses: (a) to investigate various types of protein activities, like protein-protein, protein-lipid, protein-DNA, and protein-drug interactions; (b) to identify enzyme substrates; (c) to profile immune responses (Chen and Zhu, 2006). Since high-density DNA microarray technology has played a key role in the analysis of the whole genome and gene expression patterns, protein arrays are correspondingly developing to follow DNA microarrays as a potential research and diagnostic tool for equivalent processing of complex samples (Apweiler et al., 2009).

2.1.3. Shotgun proteomics

Shotgun proteomics denotes the direct analysis of complex protein mixtures to quickly produce an overall profile of the proteome within the mixture (Wu and MacCoss, 2002). It recognizes proteins through cooperation between chromatographic methods and mass spectrometry. Chromatographic processes can be defined as separation methods characterized by mass-transfer between stationary and mobile phases. According to the physical state of the mobile phase it is possible to distinguish between gas chromatography (GC) and liquid chromatography (LC) (Heftmann, 2004). When analytes in the liquid phase are forced to flow through a chromatographic column under high pressure conditions, the separation technique is called high-performance liquid chromatography (HPLC) (Heftmann, 2004). Once in the column, the mixture is resolved into its single components. Further discussion on HPLC and its interaction with mass spectrometry can be found in Box 3.

Box 3. High-performance liquid chromatography and mass spectrometry.

HPLC associated with MS has recently become a system extensively used for proteomic purposes. The separation of samples is based on biophysical features: surface charge (ion-exchange chromatography); hydrophobicity (hydrophobic interaction chromatography); affinity for metals (immobilized metal affinity chromatography); polarity (reversed-phase liquid chromatography), in which the mobile phase is significantly more polar than the stationary phase (Cristea et al., 2004; Martosella et al., 2005; Roe and Griffin, 2006; Stasyk and Huber, 2004; Thiele et al., 2007).
The combination between LC, a liquid-phase based technique, and MS, a gas phase approach, has been made possible by the advent of ESI (see paragraph 2.1). The liquid containing the analytes of interest is dispersed by ESI into a fine aerosol that is next introduced into a mass spectrometer. As a result, ESI is the ion source of choice to associate LC with MS. The LC-MS system enables the study of proteomes with greater depth and improved accuracy of quantification than when using one-dimensional profiling techniques that record all ions in a single mass spectrum (Apweiler et al., 2009; Tambor et al., 2010).
In a standard shotgun experiment, a protein mixture is, first of all, digested by a sequence-specific protease as trypsin that has the ability to cleave a protein into multiple peptides so that proteomic specimens usually comprise hundreds of thousands of peptides. This considerable amount of molecules does not permit a direct MS-based analysis: numerous peptides flowing into the mass spectrometer at any given time may overwhelm the instrument detector. This would result in a decreased number of peptide identifications, and critically increase the LC-MS analysis variability (Cargile et al., 2004). To minimize the problem, the mixture of peptides has to be separated prior to MS analysis. In this connection, a number of different fractionation and separation methods have been integrated to develop a more sensitive ''multidimensional'' liquid chromatography system, exhibiting an elevated peptide separation power. In multidimensional separations, two or more forms of LC techniques are associated in the attempt to better fractionate peptides before they enter the mass spectrometer (Zhang et al., 2010).

The amount of proteins disclosed in proteomics experiments has grown rapidly since LC-based technologies have been used to describe the CSF proteome (Abdi et al., 2006; Maccarrone et al., 2004; Pan et al., 2007; Ramström et al., 2004; Wenner et al., 2004). Of interest, the HUPO PPP unequivocally documented that shotgun methods result in a much higher number of identified proteins relative to 2D-PAGE (Omenn et al., 2005).

2.1.3.1. Stable isotopes and quantitative proteomics. An explanatory step in protein biomarker discovery is represented by quantitative proteomics. It enables quantitative variations of a protein to be assessed under different disease and control conditions. In the past several years, many MS-based quantitative proteomics approaches have been established (Aebersold and Mann, 2003). These embrace the use of chemical reactions to introduce stable isotopic tags at definite functional groups on peptides or proteins, like the isotope coded affinity tags (ICAT) (Gingras et al., 2007; Gygi et al., 1999;

Shiio and Aebersold, 2006) and isobaric tags for relative and absolute quantitation (iTRAQ) (Gingras et al., 2007; Ross et al., 2004). Isotopic labeling methods rely on the use of "heavy" stable isotopes (such as ^{2}H, ^{13}C, ^{15}N, and ^{18}O) in order that mass discrepancies between peptides from control and sample cases are generated and the ratio of ion intensity can then be correlated back to protein expression (Gingras et al., 2007). ICAT technology has been employed to inspect modifications in the CSF proteome related to AD (Zhang et al., 2005), identifying almost 400 proteins, many of which were changed in relative abundance in aging and/or AD patients. The iTRAQ approach is based upon chemically tagging the N-terminus of peptides resulting from protein digests which are isolated from samples in different disease and control groups. This system has been employed to compare the CSF proteome of patients having neurodegenerative disorders (AD, Parkinson's disease, and dementia with Lewy body) with age-matched controls (Abdi et al., 2006).

2.1.4. Validation of biomarker candidates

When a panel of prospective biomarkers has been identified in the discovery phase, a validation (or confirmatory) phase should be followed with the aim of selecting from the list of markers the ones with the highest potential for clinical diagnosis of the pathology. This last step necessitates a clinical assay to be developed and largely tested on thousands of samples that closely reflect the population where the clinical test would be applied. It is well-established that biomarker candidates are usually identified via MS technologies; nevertheless, MS is not able to accomplish the combination of high throughput with high measurement accuracy and precision (Rifai et al., 2006). In addition, MS is not broadly available and is not yet routinely accepted for these tests by the FDA (Kingsmore, 2006). Therefore, setting up an independent analytical system to validate such putative markers is highly appreciated. Immuno-based assays are frequently considered ideal for validation and development for clinical diagnosis owing to their high sensitivity and throughput. Western blotting, enzyme-linked immuno-sorbent assay (ELISA), and radioimmunoassay are immunological methods commonly employed to verify that the concentration of the candidate biomarker significantly differs between the control and the diseased state (Wei and Li, 2009). For example, assuming that a given protein has a plasma or serum concentration in the range of ng/mL to pg/mL, ELISA or radioimmunoassay should be performed. They provide a greater level of sensitivity compared with more sophisticated non-immuno approaches, like LC and MS, and are immediately accessible in research and development setting of the clinical laboratory (Vitzthum et al., 2005). In particular, since the use of radioisotopes is discouraged in the clinical laboratory, ELISA has many advantages over radioimmunoassays and can represent an attractive option.

However, substantial attention should be taken during the development and optimization of an ELISA because a multiplicity of variables are recognized to influence its performance, such as the avidity and concentration of capture and detection antibodies, incubation time and temperature, sample volume and dilution, enzyme and substrate types, and the quality of the detector (Wild, 2005). It is also essential to take into account that, although general guidelines are proposed for choosing the suitable antibody concentrations, sample dilutions, incubation time and temperature, the optimal conditions can only be determined empirically (Rifai et al., 2006). Moreover, transferring MS-derived data to a validated immunodiagnostic platform may result challenging because of the absence of commercially available antibodies, technical problems, or high costs related to the development of high-quality antibodies (Wei and Li, 2009). Once validated, biomarkers are then selected for commercialization, in which the research immunoassay is refined to meet the rigorous standards needed for clinical tests (Rifai et al., 2006).

2.2. Detection of patterns of biomarkers in blood for AD diagnosis

In the context of translational research, the development of AD biomarkers may be accomplished in two different ways. The first approach is designated as "knowledge-based" since it relies on the present knowledge of AD biology. It is related to the direct comprehension of the neuropathological mechanisms responsible for the development and advance of AD. In practical terms, it consists of the implementation of biochemical assays to evaluate candidate biomarkers based on the neuropathology of AD (Ghidoni et al., 2011). This strategy can be useful in providing mechanistic information on the pathology processes. Nevertheless, it is intrinsically biased because it inevitably has to begin from a model, speculated by the researcher, which is dependent on the currently available data (Lopez et al., 2005).

With the introduction of innovative MS technologies offering the option to inspect proteins and peptides in a wide range of molecular weights, interest arose in developing diagnostic platforms founded on unbiased quantification of differences in global patterns of protein/peptide masses, especially in blood from normal and diseased subjects. This second approach, named "unbiased", has a number of benefits: it does not deduce any mechanism and, consequently, should be unbiased regarding the identification of disease-associated proteins. Therefore, it gives the opportunity to include proteins in the diagnostics that are not known to be implicated in the pathogenic process. The advantage of this methodology lies in its capacity to potentially disclose previously unpredicted metabolic networks in a specific disease. Disadvantages include the possibility that inaccuracies in data collection, processing, or analysis may generate artifacts rather than true discriminating features. These shortcomings should be avoided by using: (1) appropriate sample collection and handling procedures and a robust, reproducible processing and analysis protocol to identify and reject poor-quality data; (2) exhaustive assessment of individuals to be included in the investigations; (3) suitable attention to statistical methods (Lopez et al., 2005). In summary, the "unbiased" approach incorporates proteomic and bioinformatic tools enabling unbiased analyses of several prospective markers that may be informative and helpful concerning AD (Ghidoni et al., 2011).

To date, few studies have used proteomic techniques to detect plasma and serum biomarkers for AD (Table 1). However, some authors propose that blood-based signatures coupled with the primary neuropathological features of AD are evident and may be beneficial to both disease severity and progression (Ewers et al., 2010; Fehlbaum-Beurdeley et al., 2010; Ray et al., 2007; Schneider et al., 2009; Song et al., 2009).

2.2.1. Proteome-based biomarkers of AD in plasma

From this perspective, one of the most influential studies on plasma AD biomarkers was carried out by Ray and coworkers (2007), who exploited protein array technology – specifically, cytokine antibody arrays (Huang, 2004) – to examine the concentrations of 120 secreted cell-signaling proteins in plasma samples of AD patients and non-demented controls. These proteins were chosen given that immune and inflammatory pathways are progressively associated with AD both in the CNS and in the periphery (Britschgi and Wyss-Coray, 2007; Wyss-Coray, 2006). Interestingly, the plasma levels of 18 proteins were reported to differentiate AD specimens from non-demented controls with nearly 90% accuracy. In addition, baseline plasma amounts of the examined proteins were able to distinguish MCI individuals who later progressed to AD from those unchanging or converted to

Table 1
Overview of proteomic studies in blood for the diagnosis of AD.

Identified protein pattern	Function	Method	Patients and subjects	Diagnostic value (as reported by the studies)	Study
Proteome-based biomarkers of AD in plasma					
Ang-2 ↑; CCL5 ↓; CCL7 ↓; CCL15 ↓; CCL18 ↑; CXCL8 ↑; EGF ↓; G-CSF ↓; GDNF ↓; ICAM-1↑; IGFBP-6 ↑; IL-1α ↓; IL-3 ↓; IL-11 ↑; M-CSF ↓; PDGF-BB ↓; TNF-α ↓; TRAIL-R4 ↑	Immune response; hematopoiesis; apoptosis; neuroprotection and neurotrophic activity	Antibody arrays	AD ($n=85$) NDCs ($n=79$) ODs ($n=11$) MCI ($n=47$) ONDs ($n=21$) Rheumatoid arthritis ($n=16$)	Accuracy close to 90% (AD vs. NDCs)	Ray et al. (2007)
Complement factor H precursor ↑; α-2 macroglobulin ↑; Serum albumin precursor ↑; CD5 antigen-like precursor ↓; Ig lambda chain C regions ↑; Desmoplakin ↑	Immune regulation; inflammatory pathways	2D-PAGE; LC-MS/MS	Discovery phase AD ($n=50$) vs. NDCs ($n=50$) Validation phase AD ($n=111$) vs. Non-AD group ($n=400$) [=other neurodegenerative diseases ($n=196$) and NDCs ($n=204$)]	Sensitivity = 56% Specificity = 80%	Hye et al. (2006)
α-1 antitrypsin ↑; DBP ↑; IHRP ↓; Apo J precursor ↓; PKAC1↓; Unknown protein migrated at about 45 kDa ↑	Regulation of the activation of CNS microglia; actin metabolism; fibrinolysis	2D-PAGE; LC-MS/MS	AD ($n=10$) vs. NDCs ($n=10$)	AUC = 0.89 (95% CI, 0.74–0.99) for α-1 antitrypsin in detecting AD Sensitivity = 77% Specificity = 88% (optimum cut-off level of α-1 antitrypsin = 1.5 mg/mL)	Liao et al. (2007)
Complement C1 inhibitor ↓; PEDF ↓	Neuroprotection against apoptotic signals; modulation of glial proliferation and activity; immune regulation	2D-PAGE; LC-MS/MS	Discovery phase Probable AD ($n=47$) vs. NDCs ($n=47$) Validation phase Probable AD ($n=100$) vs. NDCs ($n=100$)	Discovery set: Sensitivity = 60% Specificity = 63% Validation set: Sensitivity = 58% Specificity = 58%	Cutler et al. (2008)
Clusterin (Apo J) ↑	Association with atrophy of hippocampus and entorhinal cortex; role in AD pathogenesis	2D-PAGE; LC-MS/MS	Discovery phase Mild-to-moderate AD ($n=27$) vs. MCI ($n=17$) Validation phase AD ($n=119$) MCI ($n=115$) NDCs ($n=110$)	Hazard ratio for a 10-ng/mL rise in plasma clusterin concentration for risk of becoming a rapid AD decliner = 1.071 (95% CI, 1–1.147; $p=0.05$)	Thambisetty et al. (2010)
Oxidative stress and proteomics of oxidized plasma proteins in AD					
Isoprostane 8,12-iso-iPF2α-VI ↑	Lipid peroxidation	GC-MS	AD ($n=50$) MCI ($n=33$) NDCs ($n=40$)	Higher isoprostane 8,12-iso-iPF2α-VI: in plasma of AD vs. MCI ($p<0.03$) in plasma of MCI vs. NDCs ($p<0.001$)	Praticó et al. (2002)
Isoprostane 8,12-iso-iPF2α-VI ↑	Lipid peroxidation	GC-MS	Probable AD ($n=25$) Possible AD ($n=10$) NDCs ($n=25$)	Higher isoprostane 8,12-iso-iPF2α-VI: in plasma of Probable AD vs. NDCs ($p<0.0001$) in plasma of Possible AD vs. NDCs ($p<0.0001$)	Praticó et al. (2000)
Isoprostane iP2α-IV (F2A) ↔	Lipid peroxidation	GC-MS	AD ($n=49$) MCI ($n=47$) Non-demented PD ($n=47$) NDCs ($n=48$)	Clinical value not reported for the diagnosis of AD or MCI	Irizarry et al. (2007)
α-1 antitrypsin↑; Transferrin ↑; Hemopexin ↑[a]	Heme/iron/redox homeostasis; activation of the acute phase response	2D-PAGE; MALDI-TOF/MS	AD ($n=10$) vs. NDCs ($n=9$)	Clinical value not reported; α-1 antitrypsin, transferrin, and hemopexin were 5-, 6.5- and 107-fold higher in AD than NDCs	Yu et al. (2003)
α-1 antitrypsin precursor ↑ fibrinogen γ-chain precursor ↑[a]	Inflammatory pathways	2D-PAGE; MALDI-TOF/MS	AD ($n=9$) vs. NDCs ($n=9$)	Clinical value not reported; α-1 antitrypsin precursor and fibrinogen γ-chain precursor had a 2- to 6-fold greater specific oxidation index in plasma from AD patients compared to NDCs	Choi et al. (2002)
Proteome-based biomarkers of AD in serum					
Thrombopoietin ↑; Tenascin C ↑; TNF-β ↑; Eotaxin-3 ↑; PP ↑; α-2 macroglobulin ↑; VWF ↑; IL-15 ↑; β-2 microglobulin ↑; VCAM-1 ↑; IL-8 ↑; IGFBP2 ↑; Fas ligand ↑; Prolactin ↑; Resistin ↑; IL-1ra ↓; PAP ↓; CRP ↓; TNF-α ↓; SCF ↓; MIP-1α ↓; CK-MB ↓; G-CSF ↓; IL-10 ↓; S-100B↓	Inflammatory pathways	Multiplex fluorescent immunoassay	AD ($n=197$) vs. NDCs ($n=203$)	AUC = 0.91 (95% CI, 0.88–0.95) Sensitivity = 80% Specificity = 91% AUC = 0.95 (95% CI, 0.92–0.98) Sensitivity = 94% Specificity = 84% After addition of age, sex, education, and APOE status to the algorithm	O'Bryant et al. (2010)

ApoA-I ↓	Inhibition of Aβ peptides formation and aggregation	2D-PAGE; MALDI-TOF/MS	AD ($n=59$) vs. NDCs ($n=74$)	Clinical value not reported; ApoA-I significantly lower in AD vs. NDCs ($p<0.0002$)	Liu et al. (2006)
α-1 acid glycoprotein ↓; Apo E ↑; Apo B-100 ↑; Complement C3 ↑; Transthyretin ↑; Factor H ↑; Haptoglobin α-2 chain ↑; Hemoglobin α chain ↑; Hemoglobin β chain ↑; HRG ↑; Vitronectin precursor ↑; α-2 macroglobulin ↑; Complement C4 ↑	Inflammatory pathways; modulation of complement cascade pathways; coagulation and fibrinolysis	MDLC; SELDI-TOF/MS; 2D-PAGE; MALDI-TOF/MS	AD ($n=41$) CHF ($n=35$) IR/D2 ($n=49$) NDCs ($n=90$)	AUC = 0.922 (95% CI, 0.833–0.972) for hemoglobin to distinguish AD patients from NDCs Sensitivity = 90.2% (for hemoglobin with respect to AD patients) Specificity = 90.0% (for hemoglobin with respect to NDCs) (optimum cut-off level of hemoglobin = 19.2 μg/mL)	Zhang et al. (2004)
Albumin-bound protein fragments and peptides	N/A	Affinity chromatography; MALDI-TOF/MS	AD ($n=62$) vs. Non-AD group ($n=240$) [= MCI ($n=33$) and NDCs ($n=207$)]	Sensitivity = 83% Specificity = 96% Test efficiency = 47%	Lopez et al. (2005)
Four unknown albumin-bound protein fragments or peptides: 1690.93 Da ↓; 1777.95 Da↓; 1864.98 Da ↓; 2021.09 Da ↓	N/A	Affinity chromatography; MALDI-TOF/MS	AD ($n=40$) PD ($n=55$) NDCs ($n=47$)	Sensitivity = 70% Specificity = 38.3% Test efficiency = 52.9% (AD vs. NDCs) Sensitivity = 78.2% Specificity = 92.5% Test efficiency = 84.2% (AD vs. PD)	German et al., 2007
Proteome-based biomarkers in blood and treatment efficacy of AD					
Complement C1 inhibitor (complexed form) ↑; complement C1 inhibitor (free form) ↓; α-2 macroglobulin ↓; Apo E ↓; Complement factor H precursor ↑[b]	Modulation of complement cascade pathways	2D-PAGE; LC-MS/MS	AD ($n=41$)	No clinical value reported: the authors suggested that the identified proteins could serve as markers of drug efficacy	Akuffo et al. (2008)
14-3-3 protein ε ↑; Aip1 ↓; Peroxiredoxin-2 ↑; MAPK1 ↓; Beta-actin ↓; Annexin A1 ↓; GAPDH ↓; Transforming protein RhoA ↓; ANP32B ↓[c]	Cell signaling pathways; cytoskeleton; apoptosis; intracellular redox status functions	2D-PAGE; MALDI-TOF/MS	Probable AD ($n=15$)	No clinical value reported: the authors suggested that the identified proteins could serve as markers of drug efficacy	Mhyre et al. (2008)

Abbreviations: 2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; AD, Alzheimer's disease; Aip1, actin-interacting protein 1; Ang-2, cngiopoietin-2; ANP32B, acidic (leucine-rich) nuclear phosphoprotein 32 family member B; ApoA-I, apolipoprotein A-I; Apo B-100, apolipoprotein B-100; Apo E, apolipoprotein E; Apo J, apolipoprotein J; AUC, area under the receiver operating characteristic curve; CCL, chemokine containing a C-C motif; CHF, congestive heart failure; CI, confidence interval; CK-MB, creatine kinase MB; CNS, central nervous system; CRP, C-reactive protein; CSF, cerebrospinal fluid; CXCL, chemokine containing a C-X-C motif; DBP, vitamin D-binding protein; EGF, epidermal growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GC–MS, gas chromatography coupled to mass spectrometry; G-CSF, granulocyte-colony stimulating factor; GDNF, Glial-derived neurotrophic factor; HPLC, high-performance liquid chromatography; HRG, histidine-rich glycoprotein; ICAM-1, Intercellular adhesion molecule-1; IGFBP-2, insulin-like growth factor-binding protein-2; IGFBP-6, Insulin-like growth factor-binding protein-6; IHRP, inter- α-trypsin inhibitor family heavy chain-related protein; IL, interleukin; IL-1ra, interleukin-1 receptor antagonist; IR/D2, insulin resistance/type-2 diabetes; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MAPK1, mitogen activated protein kinase 1; MCI, mild cognitive impairment; MDLC, multidimensional liquid chromatography; MIP-1α, macrophage inflammatory protein-1α; NDCs, non-demented controls; ODs, other dementias; ONDs, other neurological disorders; PAP, prostatic acid phosphatase; PD, Parkinson's disease; PDGF-BB, platelet-derived growth factor BB; PEDF, pigment epithelium-derived factor; PKA-C1, cAMP-dependent protein kinase catalytic subunit α isoform 1; PP, pancreatic polypeptide; S-100B, S-100 calcium binding protein B; SCF, stem cell factor; SELDI-TOF/MS, surfaced-enhanced laser desorption/ionization time-of-flight mass spectrometry; TNF-α, tumor necrosis factor-α; TNF-β, tumor necrosis factor-β; TRAIL-R4, Tumor necrosis factor-related apoptosis-inducing ligand receptor-4; VCAM-1, vascular cell adhesion molecule-1; VWF, von Willebrand factor.

[a] Oxidation levels.

[b] Following rosiglitazone administration.

[c] Following valproic acid administration.

different dementing pathologies with 81% accuracy. As a result, such distinctive alterations in the concentration of blood signaling proteins may represent a prominent AD-specific molecular phenotype (Ray et al., 2007). These discoveries have raised the attention of the scientific community because they revealed a well-documented peripheral signature from blood cell-signaling proteins which might be significant for the comprehension of AD pathological mechanisms. Moreover, this was the first attempt to employ the array technology in AD biomarker discovery research (Thambisetty and Lovestone, 2010). In more detail, the biological analysis of the 18 plasma proteins successfully selected revealed both a global decrease in the amount of factors coupled with hematopoiesis and inflammation in the course of AD and deficiencies related to neuroprotection, neurotrophism, phagocytosis, and energy homeostasis. Intriguingly, such 18 biological markers encompassed cytokines, chemokines, and growth factors all belonging to a network of molecules through which cells are able to communicate with each other. This immense set of protein factors that allow the communication among cells has been defined "communicome", in line with other established system wide groups, (i.e., genome, transcriptome, and proteome) (Britschgi and Wyss-Coray, 2009). Correlation networks have been utilized to study the relationship between proteins of the "communicome" (Ideker et al., 2001); the application of this analysis to the 18 plasma proteins of the AD signature, showed that the link among these molecules was extensively modified in AD patients compared to non-demented controls (Britschgi and Wyss-Coray, 2009). Thus, if the correlation network in the non-demented controls group is equivalent to a homeostatic network, then the correlation network in the AD patients set is thought to be imbalanced. Since the 18 cell-signaling factors are co-regulated with other molecules, this imbalance may affect additional areas of the "communicome", interrupting other signaling networks. Therefore, the identified AD signature designates an imbalance in the network of intercellular signaling proteins. The comprehension of the reasons underlying the alterations detected in the "communicome" may supply useful information concerning AD mechanisms and help plan innovative therapeutic intervention (Britschgi and Wyss-Coray, 2009).

A rigorous biomarker discovery program should consist of an initial discovery phase study where the major aim is to obtain protein signatures that are dissimilarly expressed in cases compared to controls followed by a validation step using a higher-throughput methodology. Hye et al. (2006) carried out a case-control study on plasma samples using a proteomics-based approach. In the discovery phase of proteomic analysis, 50 people with AD and 50 healthy controls (HCs) were recruited; in the validation stage, samples from 511 patients with AD and other neurodegenerative disorders and HCs were inspected. AD patients and HCs samples were compared by 2D-PAGE to analyze the profile of plasma proteins: 15 protein spots were found to be considerably different in AD cases compared with age-matched HCs. Statistical analysis of 2D-PAGE data found 56% sensitivity and 80% specificity. After that, spots were excised and analyzed by liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) for the identification of such proteins.

Of the 15 spots revealed, 13 included components of immunoglubulin or serum albumin precursor, nine displaying a growth and four a reduction in AD. Of interest, albumin and immunoglobulins have been scrutinized, in the past, in CSF and in plasma/serum as markers of the integrity of the blood–brain barrier with variable findings; some exhibiting a relative increase of immunoglubulins and/or albumin in CSF but other studies have not replicated these results (Hampel et al., 1999, 1995). Besides albumin and immunoglobulin fragments, a number of 11 proteins differing between cases and controls have been identified. The function of almost all of these molecules is related to immune regulation, thus convincingly supporting the hypothesis of an early inflammatory process in AD.

In order to confirm the proteomic data, independent experiments employing specific and sensitive methods directed against the protein targets of interest have to be performed. In such validation studies, careful attention must be paid to delineate the groups of interest. To this aim, the inclusion of plasma samples from non-AD dementias or other neurodegenerative diseases besides HCs, is expected to offer significant information regarding the specificity of the potential biomarkers for diagnosis of AD. In this proteomic study, confirmatory experiments made use of immunoblotting assays specifically for complement factor H precursor and α-2 macroglobulin proteins, the two molecules exhibiting the greatest alteration in expression between AD and HCs plasma samples. These experiments were carried out in an independent group of 511 individuals that, besides AD and HCs, comprised plasma specimens from patients with vascular dementia, Huntington's disease at various stages (pre-symptomatic, mild, moderate/advanced), motor neuron disease, multiple systems atrophy, and progressive supranuclear palsy. Since plasma levels of complement factor H precursor and α-2 macroglobulin were remarkably higher in AD patients compared with HCs and did not show any change in other neurodegenerative pathologies, such validation studies designated these two proteins as biomarkers specific for AD (Hye et al., 2006). Moreover, these molecules have been previously demonstrated to be involved in AD pathology. The protein α-2 macroglobulin has been disclosed to have a function in modulating the immune response (Armstrong and Quigley, 2001) being stimulated by inflammatory cytokines (Strauss et al., 1992) and may be a marker of injury to the blood-brain barrier (Cucullo et al., 2003). Interestingly, the presence of α-2 macroglobulin has been revealed in amyloid plaques (Bauer et al., 1991). Complement factor H precursor is an important inhibitor of activation of the alternative complement pathway (Rodríguez de Córdoba et al., 2004) and, like α-2 macroglobulin, has previously been found to exist in amyloid plaques in AD (Strohmeyer et al., 2002). In addition, complement factor H precursor and α-2 macroglobulin have also been observed to be positively associated with the hippocampal metabolite ratio N-acetylaspartate/myo-inositol, a biochemical measure strictly related to cognitive decline in AD and MCI individuals (Thambisetty et al., 2008).

The combination between 2D-PAGE and LC–MS/MS has also been exploited in a study by Liao and colleagues (2007) who recognized a number of possible plasma biomarkers by comparing AD patients with non-demented control subjects. Three proteins resulted overexpressed: α-1 antitrypsin, vitamin D-binding protein, and an unknown protein migrated at around 45 kDa in the gel; the others were underexpressed: inter-α-trypsin inhibitor family heavy chain-related protein, apolipoprotein J precursor, and cAMP-dependent protein kinase catalytic subunit α-1. Some of these molecules have been identified to have a critical function in the CNS microglia activation, while others are implicated in actin metabolism and fibrinolysis in the periphery (Liao et al., 2007). Since the occurrence of α-1 antitrypsin has been described in NFTs as well as amyloid plaques (Gollin et al., 1992), its high plasma levels detected in AD patients via 2D-PAGE were additionally confirmed using an ELISA assay. The assessment of the efficacy of plasma α-1 antitrypsin for AD diagnosis gave 77% sensitivity and 88% specificity (Liao et al., 2007). The protein α-1 antitrypsin contributes to the control of proteinases activity during inflammation, coagulation, and fibrinolysis processes (Potempa et al., 1994). Furthermore, elevated amounts of this marker have been detected in AD proteomics studies of CSF (Sihlbom et al., 2008). In addition, its oxidized form has also been identified in AD patients

by means of plasma proteomics analyses (see Section 2.2.2) (Choi et al., 2002; Yu et al., 2003).

Two new biomarkers were recently described in a gel-based proteomic study integrated with LC–MS/MS: pigment epithelium-derived factor and complement C1 inhibitor have been observed to be down-regulated in plasma of AD patients; these remarks were then confirmed using ELISA commercial kits and Western blotting assays (Cutler et al., 2008). Pigment epithelium-derived factor, an extracellular serine protease inhibitor with neurotrophic, gliastic, antiangiogenic and antitumourgenic features (Fernandez-Garcia et al., 2007) is a neurotrophic factor that protects neurons and microglial against apoptotic signals, modulates glial proliferation and activity, and protects against neuronal damage triggered by Aβ (Sanagi et al., 2005; Storozheva et al., 2006; Sugita et al., 1997). It has also been proposed as a potential marker of AD in CSF (Davidsson et al., 2002). Complement C1 inhibitor is a basic factor of the complement cascade that may be dysregulated in AD brains (Veerhuis et al., 1998).

In recent research, the already mentioned 2D-PAGE followed by LC–MS/MS proteomic system was used in conjunction with a multimodal neuroimaging approach to identify plasma proteins associated with AD pathology and to demonstrate that plasma levels of clusterin (apolipoprotein J) are associated with *in vivo* pathology, disease severity, and rate of clinical progression in AD patients (Thambisetty et al., 2010). As a measure of the *in vivo* pathology, the structural magnetic resonance imaging was employed to detect atrophy in the hippocampus and entorhinal cortex. These are two components of the medial temporal lobe typically characterized by early pathological modifications in AD (Braak and Braak, 1991).

First of all, a discovery-phase proteomics experiment was carried out to recognize proteins associated with hippocampal volume in AD and MCI patients. Eight spots, including complement C3, γ-fibrinogen, serum albumin, complement factor I, clusterin (in 2 spots), serum amyloid-P, and α-1 macroglobulin, were identified. After that, a second discovery-phase proteomics test was executed in an independent group of AD individuals which have been divided into fast and slow progressors according to their annual rate of cognitive decline. At the end of the procedure, eight distinct spots, comprising complement C4a (in 3 spots), complement C8, clusterin, apolipoprotein A1 (in 2 spots), and transthyretin, were detected. Given that the only protein common to both discovery-phase investigations was represented by clusterin, validation studies were performed in a larger cohort of subjects to confirm this finding. The final outcome revealed that clusterin was significantly linked both with hippocampal atrophy in AD and MCI individuals and with fast progressing, i.e. more aggressive, AD (Thambisetty et al., 2010).

Interestingly, in addition to apolipoprotein J, other plasma and CSF proteins have been described as extracellular chaperones that are theoretically able to affect Aβ aggregation and toxicity, for example α-2 macroglobulin, haptoglobin, and serum amyloid-P (Wilson et al., 2008).

2.2.2. Oxidative stress and proteomics of oxidized plasma proteins in AD

The link between peripheral oxidative stress and neurodegeneration has been extensively studied. Some authors theorize that oxidative stress originally develops in the periphery leading to a decrease of antioxidants in the CNS and, ultimately, to oxidative injury and neurodegeneration (Praticò, 2005). Others speculate that oxidative stress originates in the CNS and then several metabolic end-products are produced and conveyed into the periphery (Praticò, 2005). Lipid peroxidation, protein carbonyls, and DNA oxidation processes in the brain of AD patients have been described as indicators of oxidative damage (Solfrizzi et al., 2006). Isoprostanes are compounds resulting from free radical-mediated peroxidation of poly-unsaturated fatty acid (Lovell and Markesbery, 2007). Even if these molecules do not exhibit neurotoxic properties in physiological conditions, elevated concentrations of F2-isoprostanes (F2-IsoPs) have been reported in the brain and CSF of AD individuals (Montine et al., 2007; Praticò et al., 2000). For instance, in a longitudinal study, CSF F2-IsoPs amounts in AD patients were significantly augmented during the follow-up period as well as noticeably reduced in those treated with anti-oxidants (Quinn et al., 2004). The importance of F2-IsoPs in AD and MCI plasma is still debated. Praticò et al. (2002) disclosed high levels of F2-IsoPs in plasma, CSF, and urine of MCI patients by using GC followed by MS technology; analogous results were observed in AD subjects (Praticò et al., 2000). On the other hand, in another study, plasma F2-IsoPs concentrations, determined in HCs as well as in AD, MCI, and Parkinson's disease patients through the same proteomic approach, did not result to be significantly dissimilar (Irizarry et al., 2007). Moreover, another study described that plasma (and urine) levels of F2-IsoPs were not correlated to CNS levels in AD patients (Montine et al., 2002). Variations in the collection methods, assay factors, and cohort dissimilarities may account for such discrepancies (Irizarry et al., 2007). As a result, additional work is requested to make secure decisions concerning the validity of F2-IsoPs as plasma biomarkers.

The importance of oxidative damage in the pathogenesis of AD is also emphasized by the accumulation of oxidized proteins in the AD brain at sites of neurodegeneration. A proteomic approach founded on affinity column chromatography in cooperation with 2D-PAGE and subsequent MALDI-TOF analysis was exploited to elucidate probable discrepancies in the expression of native and oxidized glycoproteins using pooled plasma samples obtained from sporadic AD patients and from normal elderly controls. This analysis led to the discovery that levels of oxidatively-modified proteins investigated were significantly higher in whole AD plasma compared with the non-demented controls. More correctly, nine spots – representing glycoproteins that exhibited a larger abundance or a higher specific oxidation index or both features in AD plasma relative to controls – were documented to be isoforms of α-1 antitrypsin, hemopexin, and transferrin, after MALDI-TOF analysis. These discoveries support the conclusion that plasma glycoproteins are believed to be abnormally expressed and susceptible to anomalous oxidation in sporadic AD (Yu et al., 2003).

The protein α-1 antitrypsin, one of the major serine protease inhibitors (serpins), protects tissues from enzymes of inflammatory cells. Increased levels of α-1 antitrypsin in AD plasma are involved in systemic activation of the acute phase response in such pathology (Scacchi et al., 2001).

Hemopexin is another crucial acute phase protein that binds the free heme prosthetic group in the circulation and presents it as hemopexin-heme complexes to hepatocytes (and other cells expressing hemopexin receptors) for internalization and disposal, and prevent heme-mediated oxidative tissue damages (Delanghe and Langlois, 2001). Even though circulating hemopexin promotes the induction of oxigenase-1 – a protein implicated in AD pathogenesis (Schipper, 2011) – by supporting heme release to the cells in physiological conditions, enlarged or stoichiometrically atypical amounts of hemopexin have been demonstrated to suppress heme-mediated induction of oxigenase-1 gene transcription in cultured rat and embryonic chick hepatocytes (Sinclair et al., 1994). Therefore, increased concentrations of circulating hemopexin in AD, or unusual behavior originating from abnormal oxidation, may facilitate the reduction of oxigenase-1 protein expression as reported in plasma, CSF (Schipper et al., 2000) and choroids plexus epithelium (Anthony et al., 2003).

Transferrin is a glycosylated metal-carrying plasma protein. It is mainly involved in the transport of circulating ferric iron and its

release to tissues bearing surface transferrin receptors (Schipper, 1999). The *TfC2* allele of transferrin, located on chromosome 3, has been disclosed to be related to late-onset AD (Namekata et al., 1997). Furthermore, uncommonly glycosylated (5- and 6-sialo) transferrin isoforms have been observed in AD plasma (Van Rensburg et al., 2004). In summary, this important study emphasizes the activation of the acute phase response in patients with early sporadic AD as well as global disruption of glycoproteins expression and heme/iron/redox homeostasis (Yu et al., 2003).

In another investigation, plasma samples from AD subjects and age-matched HCs were subjected to 2D-PAGE. Seven main protein spots were reported to have different levels of oxidation; however, the extent of oxidation was noticeably greater in the AD specimens. After the excision of spots from the gel, the following MALDI-TOF approach revealed that the oxidized proteins were isoforms of fibrinogen γ-chain precursor protein (in 3 spots) and α-1 antitrypsin precursor (in 4 spots). Both proteins, displaying a two- to six-fold higher specific oxidation index in AD patients relative to HCs, are implicated in AD inflammatory pathways (Choi et al., 2002). Fibrinogen oxidation may be significant to AD etiology given that all six fibrinogen chains (α_2, β_2, γ_2) are required to be intact to have physiological fibrinogen. Oxidative alterations of the γ-chain may generate an augmented activation of plasminogen thus contributing to fibrinolysis and proteolysis in the areas of inflammation (Stief et al., 1989). Blood coagulation and fibrinolysis have been shown to play a critical function in inflammation of extravascular tissue in AD (Strohmeyer and Rogers, 2001). Interestingly, α-1 antitrypsin oxidized isoforms elicit inflammatory responses by stimulating primary human monocytes (Moraga and Janciauskiene, 2000) and may therefore sustain the inflammation in AD and other neurodegenerative diseases (Vivien and Buisson, 2000).

2.2.3. Proteome-based biomarkers of AD in serum

Proteomics technologies have also been used to search for biomarkers in human serum. A very important investigation was carried out by O'Bryant and coworkers (2010) who made use of a multiplex fluorescent immunoassay to identify a panel of 25 proteins that were either differentially overexpressed ($n = 15$) or underexpressed ($n = 10$) in AD patients relative to HCs and established a serum protein-based algorithm to discriminate AD individuals from HCs. The sensitivity and specificity values for biomarkers were equal to 80% and 91%, respectively. Several of the total markers detected were primarily represented by inflammatory and vascular factors as macrophage inflammatory protein-1α, eotaxin-3, tumor necrosis factor-α, C-reactive protein, von Willebrand factor, and some interleukins. Given the inflammatory nature of the up-regulated or down-regulated proteins disclosed, the existence of a pro-inflammatory endophenotype within AD subjects was proposed. Such biomarker profile may offer targeted therapeutic strategies for the treatment this specific subset of patients.

Liu and colleagues (2006) were able to select 9 differentially expressed spots on 2D-PAGE gel in serum samples from AD patients and sex- and age-matched controls. After MALDI-TOF analysis, decreased serum levels of apolipoprotein A-I (ApoA-I) were observed in AD patients relative to controls. ApoA-I, the main component of high-density lipoproteins, is synthesized in the liver and small intestine (Sliwkowski and Windmueller, 1984). However, the existence of ApoA-I mRNA has also been documented in spinal cord neurons (Fujii et al., 2002); moreover, cerebral endothelial cells were discovered to release ApoA-I (Möckel et al., 1994). It is implicated in cholesterol transport from peripheral tissues to the liver to remove the excess of cholesterol from the body and preserve lipid homeostasis. Cholesterol homeostasis is an important modulator of the proteolytic processing of amyloid precursor protein, from which Aβ peptides originate (Grösgen et al., 2010). In addition, ApoA-I was also revealed to inhibit the aggregation of Aβ oligomers in an *in vitro* study (Koldamova et al., 2001). Altogether, ApoA-I might not only to decrease the formation of Aβ, but also diminish their accumulation, therefore inhibiting the development of senile plaques. However, it is necessary to take into account that apolipoproteins are difficult to identify in prefractionation proteomics studies, probably because of the inclination of lipoproteins to adhere to plastic vials, thus resulting in loss during sample transfer (Davidsson and Sjogren, 2006).

In the paper by Zhang and coworkers (2004), the traditional 2D-PAGE approach was replaced by the use of multidimensional liquid chromatography systems (see Box 3) to separate proteins, followed by MS examination. A series of molecules including some acute response proteins such as haptoglobin and hemoglobin were reported to be elevated in comparison with HCs. In contrast, levels of α-1 acid glycoprotein were lower. The concentrations of other proteins as apolipoprotein B-100, fragment of factor H, histidine-rich glycoprotein, vitronectin, and α-2 macroglobulin were demonstrated to be elevated in AD patients.

Two independent proteomic studies have used MS-based methods to scrutinize carrier-protein-bound signatures obtained from serum in AD. The low-molecular-weight part of the human blood proteome includes peptides as well as fragments of proteins. Such "fragmentome" fascinated researchers because it is a rather unexplored portion of the proteome (Mehta et al., 2003–2004). A fraction of the low-molecular-weight species in blood are bound to high-abundance carrier proteins (as albumin), thus extending their circulating half-lives in serum by avoiding their renal excretion.

In the first work, albumin-bound peptides were isolated from serum samples of AD, MCI, and no-cognitive impairment subjects by using affinity chromatography. In particular, the dye-ligand affinity separation method was employed. Such approach is intended to capture high-abundance carrier proteins in blood and then enrich for peptides and protein fragments bound to these circulating carriers. Next, MALDI-TOF technology was performed to acquire the peptide mass spectra; the resulting m/z profiles were utilized to build predictive models in order to detect AD (Lopez et al., 2005).

The second investigation followed an equivalent procedure and led to the discovery of four spectral peaks (at 1690.93 Da, 1777.95 Da, 1864.98 Da, and 2021.09 Da) as potential serum biomarkers, appropriate to discriminate AD patients from HCs and individuals with Parkinsońs disease (German et al., 2007). The signal intensities of such peaks were significantly lower in AD samples compared with HCs. Of interest, three of the four peaks were common to the study by Lopez and coworkers (2005) indicating that MS exploration of carrier protein-bound signatures may be a reliable approach for the discovery of new AD biomarkers in blood.

2.2.4. Proteome-based biomarkers in blood and treatment efficacy of AD

Few studies pertaining to the use of proteomic platforms to link peripheral protein concentrations to treatment response after drug administration in AD patients have been carried out. Akuffo and colleagues (2008) performed 2D-PAGE and LC–MS/MS to define a panel of proteins related to a dose response to the rosiglitazone in a clinical trial in AD patients. A significant association between plasma levels of complement factor H precursor, α-2 macroglobulin, apolipoprotein E, and complement C1 inhibitor and Assessment Scale-Cognitive (ADAS-Cog) score in AD patients administered with the highest dosages of rosiglitazone (4 or 8 mg), compared with the placebo, was reported. Specifically, a complexed form of complement C1 inhibitor was identified; such

high-molecular weight form was found to be increased at the 4 mg dose; the free form resulted reduced at the 8 mg dose. The levels of α-2 macroglobulin as well as those of apolipoprotein E were shown to be lowered at the 4 mg dose; the concentration of complement factor H precursor was disclosed to be augmented at the 8 mg dose. Three of the four proteins identified in this study play a role in the modulation of complement cascade (Davis et al., 2007). Neuroinflammation is known to play a significant role in pathology of AD and, therefore, it is likely that the alterations in these proteins or complexes may directly or indirectly reproduce the disease process (Bonifati and Kishore, 2007). However, further examinations are needed to elucidate the role of the complement cascade and its regulation in AD pathophysiology and therapy.

In an analogous study design, the proteomic investigation (2D-PAGE coupled with MALDI-TOF analysis) of leucocytes isolated from peripheral venous blood was executed in AD patients before and after 4-weeks administration with valproic acid (divalproex sodium formulation). At the end of the therapy, 10 proteins differentially expressed were observed. In particular, two proteins were significantly up-regulated following valproic acid treatment: 14-3-3 protein ε and peroxiredoxin-2. The other proteins, considerably down-regulated, encompassed: actin-interacting protein 1, mitogen activated protein kinase 1, beta actin, annexin A1, glyceraldehyde 3-phosphate dehydrogenase, transforming protein RhoA, and acidic (leucine-rich) nuclear phosphoprotein 32 family member B. All these markers, linked to cell signaling pathways, cytoskeleton, apoptosis, and intracellular redox status functions, may be significant to both AD development and mechanisms of action of valproic acid (Mhyre et al., 2008). However, the therapeutic function of divalproex sodium is not fully understood in AD; it may decrease agitation and anxiety related to dementia (Porsteinsson et al., 2003; Sival et al., 2002), but these findings are not entirely accepted (Tariot et al., 2005). For this reason, supplementary studies are needed.

In conclusion, there are relatively few plasma/serum proteomics analyses of AD, and new proteome-based plasma/serum biomarkers need to be validated independently by several groups to increase confidence in the reproducibility of the data. The multiplicity of the experimental strategies and the wide heterogeneity of the technologies employed may account for the degree of the variability. Certain studies have been carried out to investigate the up- or down-regulation of protein expression levels; other examinations detected qualitative modifications, for example PTMs. It's important to emphasize that – after the discovery of a panel of proteins – additional confirmatory tests are mandatory to assess the specificity and sensitivity of the identified proteins as prospective biomarkers, and their predictive value in large numbers of samples and in longitudinal studies.

2.2.5. Depiction of plasma and serum in AD proteomic studies

From the discussion of the proteomic studies reported above, it is evident that blood is not the matrix directly examined; rather, it is represented by the plasma or serum fraction of blood.

Plasma is the liquid portion of unclotted blood that is left behind after all cell types are removed. To obtain plasma, blood is withdrawn from the patient using venipuncture in the presence of an anticoagulant and the specimen is centrifuged to remove cellular components (Issaq et al., 2007). Serum is prepared by collecting plasma in the absence of any anticoagulants. Under these circumstances, a fibrin clot is generated. The clot is then removed by centrifugation, leaving behind serum. The coagulation process makes serum qualitatively dissimilar from plasma. The subtraction of a large portion of the fibrinogen content of plasma in the form of the fibrin clot makes serum protein concentration lower than that of plasma. Other proteins results also to be eliminated by specific or non-specific interactions inside the fibrin clot (Issaq et al., 2007).

The major goal of the HUPO PPP is the comprehensive characterization of the plasma/serum proteome. In particular, an important issue that the HUPO PPP is trying to address is the comparison between serum and plasma specimens in relation to the human proteome.

Historically, serum samples are predominant in the archives; however, in the past they were accumulated owing to requirements of conventional assays and not necessarily because they represent the most suitable specimens for protein analyses (Rai et al., 2005a). Specifically, some issues should be addressed:

(1) The issue of platelet contamination should be carefully considered by detecting peptide contents in various samples. During processing of peripheral venous blood into serum, several *ex vivo* processes take place and they lead to the neogeneration of many peptides. Since serum formation is dependent on a biochemical process, it is realistic to hypothesize that numerous parameters such as temperature after sample collection, time for sample processing/clot formation or medication of patients, can modify the peptide content of serum. These issues are complicated to standardize in routine clinical practice, in cohorts, and among different centers (Rai et al., 2005a).
(2) Certain proteins may bind to the clot in an uncontrolled way; this may lead to a parallel reduction in the amount of free proteins during clot formation.
(3) Ultimately, serum displays many highly concentrated peptide signals, which prevent the detection of endogenous peptides.

Therefore, according to these considerations, the use of serum samples, especially for peptidomic biomarker discovery, should be avoided.

In contrast, the acquisition of plasma, as stated above, requires the use of anticoagulants. Plasma collection tubes include different anticoagulants such as ethylenediaminetetraacetic acid (EDTA), heparin, or sodium citrate. Each of these stabilizers can affect the protein composition in plasma and, therefore, the use of these specimens in proteomic experiments (Rai et al., 2005a). With reference to the selection of the more suitable sample type for proteomic investigations, we suggest that this should be dependent on the downstream analysis that is carried out. Each of the following individual sample types: (a) serum, (b) EDTA-plasma, (c) heparin-plasma, and (d) citrate-plasma has its own advantages and disadvantages and should not be employed under specific circumstances (Banks et al., 2005; Haab et al., 2005; Rai et al., 2005a,b; Tammen et al., 2005). The HUPO PPP recognized that there is an excessively high number of variables to contemplate in order to obtain a universal consensus on the best plasma standardized procedures for every anticoagulant. However, the consideration of these factors in study design, in association with the documentation of all steps of samples handling, may decrease some of these drawbacks (Tuck et al., 2009).

In addition, a specific working group, the Specimen Collection and Handling Committee (SCHC) was created with the aim of assessing all preanalytical variables that can theoretically influence the outcome of proteomic examinations. The goal of the SCHC would be the development of standard operating procedures (SOPs) for blood collection and handling for proteomic studies. Nevertheless, this seems to be a discouraging mission given the huge number of variables that should be methodically studied and the complication and difficulties in standardization, as demonstrated by the HUPO PPP efforts (Rai et al., 2005a). In conclusion, the proper selection of the optimal specimen between serum and plasma implicates that the researcher is conscious of all limitations

and takes the necessary precautions to ensure that the right specimen is selected (Rai et al., 2005a; Tuck et al., 2009). Alternatively, more than one type of sample – e.g. plasma *and* serum – may be collected and utilized for parallel analyses.

3. Concluding remarks

Plasma and serum, being the primary clinical samples due to the ease of availability and large amounts of other tissue proteins, have been studied extensively among the different biological fluids for disease biomarker discovery. Global initiatives as the HUPO PPP have been undertaken to elucidate pre-analytical variability, and to make efforts to standardize specimen collection, handling, and processing procedures (Omenn et al., 2005). Moreover, the importance of informatic tools for data management and collaboration with other disease-related initiatives of the HUPO to enlarge the area of plasma/serum biomarker discovery has been highlighted (Omenn, 2011; Omenn et al., 2009).

During the last decade, the development of MS-based technologies has designated proteomics as a major platform to interrogate the plasma/serum proteome for the discovery of next generation biomarkers that have diagnostic, prognostic or therapeutic efficacy. This would meet the need for an improved diagnosis and effective therapy (Ray et al., 2011).

MS-based methods, together with innovative tools in progress, are welcomed because they will significantly improve our ability to identify markers in blood. However, older technologies, like 2D-PAGE, remain of tangible meaning and it is unlikely that they will be completely replaced in the immediate future. Researchers should be flexible and employ a diversity of approaches to achieve the best results in their search for markers (Thambisetty and Lovestone, 2010).

Since it is clear that proteins and almost certainly other elements of blood, are subject to variations in AD individuals, a biomarker in blood should be conceivable; this is likely even because the most promising AD biomarkers – Aβ peptides and inflammation-related proteins – are not brain specific but are usually found in the periphery and may undergo alterations in response to AD. However, currently, there are no proteins, transcripts or metabolites in blood that have been satisfactorily replicated to be recognized as AD biomarkers. Based on the data from the literature, it would appear questionable that a blood marker alone will be in itself sufficient for the diagnosis of AD. On the contrary, it seems most likely to have a combination of markers: several proteins coupled with other blood-based or non-blood-based markers, as imaging. In addition, it is doubtful that there will be only one set of biomarkers for all possible uses in AD. It is possible that there will be a set of biomarkers to support AD diagnosis, a different group of molecular markers to predict outcome in AD patients or conversion in MCI, and probably another set used to monitor the progression of the disease (Thambisetty and Lovestone, 2010).

Given the increasing availability of large sample sets, the assortment of technologies available to scrutinize the blood proteome, and the collaborative studies between academy and industry, plasma/serum biomarkers are expected to become a key instrument for AD investigation.

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