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Review

Circulating biomarkers of protein oxidation for Alzheimer disease: Expectations within limits

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ABSTRACT

Alzheimer disease (AD), the most common dementing disorder, is a multifactorial disease with complex etiology. Among different hypotheses proposed for AD one of the most corroborated is the "oxidative stress hypothesis". Although recent studies extensively demonstrated the specific oxidative modification of selected proteins in the brain of AD patients and how their dysfunction possibly correlates with the pathology, there is still an urgent need to extend these findings to peripheral tissue. So far very few studies showed oxidative damage of proteins in peripheral tissues and current findings need to be replicated. Another limit in AD research is represented by the lack of highly specific diagnostic tools for early diagnosis. For a full screening and early diagnosis, biomarkers easily detectable in biological samples, such as blood, are needed. The search of reliable biomarkers for AD in peripheral blood is a great challenge. A few studies described a set of plasma markers that differentiated AD from controls and were shown to be useful in predicting conversion from mild cognitive impairment, which is considered a prodromal stage, to AD. We review the current state of knowledge on peripheral oxidative biomarkers for AD, including proteomics, which might be useful for early diagnosis and prognosis.

1. Introduction

Alzheimer disease (AD) is one of the most disabling dementing disorders in the elderly, affecting more than 20 million people worldwide, and the number is expected to double in the next 20 years [1]. AD is characterized by neuronal loss, deposition of senile plaques, formed by Amyloid beta peptide, and neurofibrillary tangles of hyperphosphorylated tau protein, particularly in the hippocampus, amygdala and frontal cortex, consistently with the cognitive and memory deficits in AD dementia [2]. AD is often preceded by a condition of mild cognitive impairment (MCI) that can be considered the intermediate phase between normal aging and the early form of AD [3]. Subjects with amnestic MCI show objective cognitive problems, involving memory, with no or low functional impairment in activities of daily living. Persons with MCI have an increased risk of developing dementia, and amnestic MCI very often evolves to AD [4]. Consequently MCI may be a useful AD prodromal phase in which to test putative biomarkers for their efficacy for early and accurate disease detection.

Although the pathogenesis of AD is not yet fully known, it is clear that the disease is caused by a combination of risk factors. Among several hypotheses, oxidative stress is considered to play a significant role [5–7]. In different areas of AD brain affected by neurodegeneration, increased levels of reactive oxygen species (ROS), the primary cause of oxidative stress, have been detected. The increased intracellular ROS, besides causing oxidative damage to the lipids and proteins also react with nitric oxide, produced by activated microglia, enhancing the formation of peroxynitrite and other reactive nitrogen species (RNS). In the conditions of oxidative/nitrosative stress, proteins are subjected to post-translational modifications, such as carbonylation, adducts formation with the products of lipid peroxidation, glutathionylation of cysteine residues, formation of mixed disulfide, and nitration of tyrosine residues [8]. If the oxidized proteins are not adequately repaired or removed, toxic cell damage occurs. Protein oxidation involves modifications of the three-dimensional structure and physiochemical properties that can result in fragmentation, aggregation and increased susceptibility to proteolysis [9]. Increased levels of protein aggregates in the form of fibrils together with increased lipid peroxidation have been shown, both in AD and MCI brain [10]. However, the cellular, molecular and biochemical mechanisms of neurodegeneration in AD are not yet clarified.

As well, one of the major problems concerning AD is the lack of a test or procedure having diagnostic power.

Diagnostic criteria currently used for AD are based on clinical features supporting a probabilistic diagnosis. These criteria are affected by low specificity, particularly when applied at very early stages or used to monitor effects of disease-modifying drugs in interventional studies. Similar problems occur with the diagnostic criteria of MCI. Moreover, the clinical symptoms required for the diagnosis are evident only a long time after the beginning of disease, as a consequence of

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substantial cell loss. Thus, therapies are initiated only after diagnosis; their modest benefit, in part, may be explained by the fact that some irreversible brain damage already has occurred by the time dementia is recognized [11].

Current AD biomarkers are two characteristic proteins, β-amyloid and tau protein, which are assayed in cerebrospinal fluid (CSF) [12]. Although CSF represents the most suitable biological fluid to study neurodegenerative diseases since it can reflect the biochemical changes occurring in brain, its analysis is not always easily feasible for a large scale screening, because the costs involved are enormous and procedures are invasive, uncomfortable and not without risk.

For a full screening and early diagnosis, biomarkers easily detectable in biological samples, such as plasma, are needed. Blood-based biomarkers, minimally invasive, could increase diagnostic accuracy, and could be useful for prognosis and in monitoring therapeutic interventions, especially for large scale studies and for repeated measures. Up to now, the search for reliable biomarkers for AD in peripheral blood is very challenging because of difficulties with the standardization of the methods of analysis and the low reproducibility of the results. Although a set of plasma markers that differentiated AD from controls have been shown to be useful in predicting conversion from MCI to AD [13], the study has not been yet verified by other researchers and the application of these candidate biomarkers have yet to achieve the diagnostic power, sensitivity, and reproducibility necessary for widespread use in a clinical setting. Oxidized proteins may represent potential candidate biomarkers for "oxidative stress diseases", such as AD. The present paper reviews the current knowledge on protein oxidation in biological fluids, which could potentially discriminate AD from non-AD cases and/or support early diagnosis.

2. Markers of protein oxidation

Several hypotheses have been proposed to explain AD pathogenesis and recently accumulating evidence suggests a key role of oxidative stress [6,14,15]. However the precise mechanism that increased levels of oxidative damage play in mediating the onset and progression of AD remains controversial. Oxidative stress results from the perturbation of the natural balance between pro- and anti-oxidant systems that further leads to the damage of biomolecules, loss of their functionality and consequently to cell loss [16]. Proteins are major targets of ROS, as a result of their abundance in cells, plasma, and most tissues, and their rapid rates of reaction both with many radicals and with other oxidants. Common markers of oxidative stress are: protein oxidation indexed by protein carbonyls, 3-nitrotyrosine and protein glutathionylation [17], lipid peroxidation indexed by thiobarbituric acid-reactive substances (TBARS), free fatty acid release, iso- and neuro-prostane formation, 2-propen-1-al (acrolein), and 4-hydroxy-2-trans-nonenal (HNE) [18], DNA oxidation (8-hydroxy-2-deoxyguanosine) [19] and advanced glycation end products detection (AGEs) [20].

Oxidative modifications of protein often promote unfolding or conformational changes that can lead to loss of specific protein function [21] and formation of cross-linked protein aggregates, which are resistant to removal by proteinases. The accumulation of oxidatively modified proteins may disrupt multiple cellular functions including protein turnover, cell signaling pathways, induction of apoptosis and necrosis suggesting that protein oxidation could have both physiological and pathological significance.

2.1. Protein carbonylation

Protein carbonyl groups are generated by direct oxidation of several amino acid side chains (i.e., Lys, Arg, Pro, Thr, His and others), backbone fragmentation, hydrogen atom abstraction at alpha carbons and Michael addition reactions of His, Lys, and Cys residues with products of lipid peroxidation [22]. Protein carbonyls are also produced by glycation/glycoxidation of Lys amino groups, forming advanced glycation end products (AGEs) [16,23]. Because protein carbonyls are chemically stable compared with the other products of oxidative stress, they are generally used as markers to determine the extent of oxidative modification both in in vivo and in vitro conditions.

The most common way to measure carbonylated proteins is the DNPH-based detection method, where samples are derivatized with 2,4-dinitrophenylhydrazine (DNPH), and are immunochemically detected with an antibody against the resulting protein hydrazone adduct; however spectrophotometric and HPLC quantitation of the DNPH adduct also could be used. Protein carbonyl enzyme immuno-assay kit has recently become available [24]. Enrichment and purification of carbonylated proteins can be achieved with biotin labeling of carbonyl moiety followed by affinity selection of protein mixtures [25].

Another method for measurement of protein carbonylation implies the biotinylation of carbonyl groups with biotin hydrazide and the reduction of the resulting Schiff bases. The detection of the protein-bound imine can be assessed with enzyme- or fluorophore-linked avidin or streptavidin [26]. Fractionation or tryptic digestion of the avidin-selected fraction of carbonylated proteins followed by proteolysis and MS can also be performed.

2.2. Protein nitration

Protein nitration is another widely recognized marker of protein oxidation and numerous studies support the idea that nitrosative stress contributes to neurodegeneration in AD [27–29]. Cysteine, methionine, phenylalanine, and tyrosine residues of proteins are particularly susceptible to RNS. The addition of nitrite to the protein tyrosine residues affects its role in redox cell signaling and oxidative inflammatory response and impedes protein phosphorylation, which is crucial for regulation of protein function [30].

To measure the levels of protein nitration several methods of analysis are employed. These include chemical analysis using HPLC and GC coupled to a mass spectrometer and the immunochemical detection of 3-nitrotyrosine using specific antibody [28,31]. Levels of nitrotyrosine can be assessed also using a Nitrotyrosine ELISA test kit [24]. Dityrosine is analyzed by MS with electrochemical detection [32].

2.3. HNE-modified proteins

Lipid peroxidation is one of the major sources of free radical-mediated injury that directly damages membranes and generates a number of secondary products [33–35]. The lipid peroxidation process involves the interaction of oxygen-derived free radicals with polyunsaturated fatty acids and results in a variety of highly reactive electrophilic aldehydes that are capable of easily attaching covalently to proteins by forming adducts with cysteine, lysine, or histidine residues. Among the aldehydes formed, Malondialdehyde (MDA), 4-hydroxynonenal (HNE) and acrolein represent the major products of lipid peroxidation [36]. In addition, fatty acids esters such F2-isoprostanes (F2-IsoPs) and F4-neuroprostanes (F4-NPs) are also formed [37].

Several methods have been developed for detection of either free HNE, its metabolites or its conjugation products with biomolecules. Briefly, free HNE can be quantified by high performance liquid chromatography, however, due to the relative instability of HNE, the determination of biological adducts is preferred [34].

Currently, mass spectrometry (MS)-based techniques play a key role in elucidating the protein covalent adduction by HNE, stoichiometry and sites of modification [38]. In recent years analytical possibilities have been greatly expanded because of the availability of polyclonal and monoclonal antibodies directed against protein-bound cysteine, lysine or histidine adducts of HNE [39]. Rapid and simple kits with monoclonal antibodies are already commercially available.

2.4. Protein glutathionylation

Protein glutathionylation is the formation of a mixed disulfide between protein cysteinyl residues and a small-molecular-weight thiol [40]. The reversible glutathionylation of proteins is important in the response of cells to oxidative damage and may also be significant in redox signaling [41]. This modification apparently occurs mainly under oxidative stress conditions but may also be important under normal conditions, especially for regeneration of several thiol peroxidases. Glutathionylation can protect cysteine thiols against irreversible oxidation but can also alter, either positively or negatively, the activity of many proteins. Therefore, glutathionylation could be an important redox signaling mechanism, allowing cells to sense and signal harmful stress conditions and trigger appropriate responses [42].

Considering the potential importance of glutathionylation, a number of methods have been developed for identifying proteins undergoing glutathionylation. Protein glutathionylation can be both visualized and quantitated using radiolabeled GSH detected by fluorography [43], or analyzed with immunochemical methods that exploit monoclonal antibody anti-GSH bounded to the proteins [44].

2.5. Advanced glycation and advanced oxidation end products

AGEs, during AD, colocalize with neurofibrillary tangles, senile plaques, microglia, and astrocytes and have been also measured in plasma. Non-enzymatic glycosylaton is a common post translational modification of proteins in vivo, resulting from reactions between glucose and amino groups on proteins; this process is coined the "Maillard reaction" and results in the formation of AGEs.

Measurement of AGEs can be performed or by competitive ELISA kit or by quantitative fluorescence spectroscopy [45]. ELISA kit uses either a monoclonal antibody directed against imidazolone, an advanced glycation end-product formed by reaction of arginine with 3-deoxyglucosone, or a polyclonal antibody against hemocyanin-advanced end-product.

Advanced oxidation protein products (AOPPs), a relatively novel marker of oxidative damage, are considered as reliable markers to estimate the degree of oxidant-mediated protein damage. They are formed during oxidative stress by the action of chlorinated oxidants, mainly hypochlorous acid and chloramines (produced by myeloperoxidase in activated neutrophils). They are supposed to be structurally similar to AGE-proteins and to exert similar biological activities as AGEs, i.e. induction of proinflammatory cytokines and adhesive molecules.

Determination of AOPPs in plasma is based on spectrophotometric detection, calibrated with chloramine-T solutions that in the presence of potassium iodide absorb at 340 nm, according to method proposed by Witko-Sarsat et al. [46].

Another protein oxidation marker is represented by the oxidation of hydrophobic amino acyl residues to hydroxy and hydroperoxy (P-OOH) derivatives that are measured by the guanidine-perchloric acid-ferric-xylenol orange method (G-PCA-FOX) [47]. Protein sulphydryl groups (−SH) also are indicative of altered redox status as well as altered capacity to maintain correct structures of the proteins during stress condition. Protein –SH levels are a good reflection of excess free radical generation that cause oxidation of P-SH. Sulphydril groups are estimated spectrophotometrically, based on the ability of the –SH group to reduce 5, 5′-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product [47,48].

2.6. Redox proteomics in biofluids

Recent researches are focused on the possibility to develop new proteomics platforms for the screening and assessment of groups of proteins that show altered structure or concentration in association with disease pathogenesis and progression and may therefore offer specific molecular signatures that are more diagnostic than single proteins. Among different proteomics approaches, our laboratories have extensively developed and applied to the analysis of human samples a "redox proteomics" technique, which allow the identification of oxidatively modified proteins [17]. Redox proteomics offers a broad spectrum of information that provides insights into the mechanisms of diseases, identification of disease-associated markers and may also help to identify selected target for specific therapies [49]. To date, numerous studies reported the identification of oxidized proteins in almost all the known neurodegenerative diseases.

Redox proteomics analysis of post-mortem brain from AD and MCI subjects, revealed the presence of oxidative modifications of several proteins involved in different cellular functions including energy metabolism, antioxidant defense, proteasome function, neuronal communication, cytoskeletal integrity among others. These results contributed to identify which selective metabolic network is impaired and how it possibly translates into clinical symptoms. In addition, data obtained by the analysis of progressing stages of the disease suggest that oxidative stress is an early event in the pathogenesis of AD.

A recent challenge is represented by the application of this technique to biological fluids, especially CSF and blood, for the identification of peripheral oxidatively modified proteins potentially involved with the progression of the disease. Redox proteomics approach combines two-dimensional gel electrophoresis with immunochemical detection of oxidized proteins followed by mass spectrometry for protein identification. Proteins containing reactive carbonyl groups/3-NT/HNE are detected by 2D Western blot analysis using specific antibodies. The mass spectrometric analysis basically uses two different approaches that include peptide mass fingerprinting (PMF) using MALDI-TOF and sequence tags using nano-ESI tandem mass spectrometry (MS/MS). The identification of a protein is then performed using proteomics databases [26]. However, proteomics has a number of limitations linked to the methodology and to complexity of samples nature of the sample. The most common limitations include the solubilization of membrane proteins, the mass range and detection limits, and the proteins with high Lys/Arg content (which produce very low molecular weight tryptic peptides) [17]. An alternative method of detection, 2D-gel based, is based on the use of fluorescent tags into the modified proteins. With this technique protein carbonyls are derivatized with biotin hydrazide and then probed with avidin-fluorescein isothiocyanate (FITC) in the gel [50].

Although 2D-PAGE represents currently one of the most-used technique for protein separation, non-SDS-PAGE methods such as 2D-high performance liquid chromatography (2D-HPLC) [51,52], isotopically coded affinity tags (ICAT) [53], or multiple dimensional protein identification techniques (MuDPIT) can be also employed [54]. The 2D-HPLC technique involves the elution of digested peptides from a strong cation exchange column followed by reversed phase separation, and identification by MS [55]. The ICAT method has been used extensively in quantitative proteomics to evaluate the abundance of expressed proteins. The ICAT approach is based on the tag of different population samples with different ICAT reagents that have dissimilar mass (light and heavy version). Samples are then combined, digested with a protease and subjected to avidin affinity chromatography to isolate peptides labeled with isotope-coded tagging reagents, then analyzed by liquid chromatography-mass spectrometry. For measurement of oxidatively modified proteins, the affinity tag contains a iodacetamide moiety that reacts with free cysteines, hence when reactive cysteines are exposed to oxidants under non-reducing conditions this method analyze the decrease in ICAT labeling. The change in labeling can be successively quantified from the ratio of ICAT labeling, and the cysteine residue can be identified from the LC-MS/MS derived sequences of the peptides obtained by proteolysis [53]. Recently, an improved approach analogous to ICAT, called isobaric tag for relative and absolute quantitation (iTRAQ), has been developed [55] and also used to search for 3NT modifications. This method is based upon chemically tagging the N-terminus of peptides generated from protein digests. Labeled samples

are then combined, fractionated by nanoLC to generate reporter ions that show up at low mass-to-charge (m/z) values in the analysis by tandem mass spectrometry. Database searching, for the fragmentation data of the peptides, results in the identification of the labeled peptides and hence the corresponding protein.

The MuDPIT technology consists of a 2D-chromatography separation, prior to ESIS. The first dimension is normally a strong cation exchange and the second dimension is a reverse phase chromatography, which complements the strong cation exchange because it is efficient at removing salts and is compatible with ESI [54]. Another interesting enrichment steps for protein carbonyls detection involve the use of an Oxidation-Dependent Element Coded Affinity Tags (O-ECAT) reagent, the ((S)-2-(4-(2- aminooxy)-acetamido)-benzyl)-1,4,7,10-tetraazacyclododecane-N,N′,N′′,N′′′-tetraacetic acid that serves to chelate a rare earth metal such as Tb or Ho. Treating samples with different rare earth metals according to sample origin allows differential coding of samples. Coded peptide fragments were then selected with an immunosorbent column and analyzed by nano RPC-FTICR mass spectrometry [56,57].

3. Circulating biomarkers: limits and prospects

Up to now, a definite diagnosis of AD can be made by postmortem neuropathological examination, but brain tissue is inappropriate for early diagnosis of cognitive decline. AD treatment is symptomatic and starts at advanced stages of the disease, when dementia has been clinically established with a battery of probabilistic neuropsychological, cognitive and functional tests. The treatments approved for AD are palliative and considered to have marginal efficacy while new drugs are administered only at late stages and therefore proved to be poorly beneficial. In this view the diagnosis of AD at earlier stages represent a key step for the administration of preventive and disease-modifying therapies that is the only way to protect brain neurodegeneration. The diagnostic markers currently used or being tested include neuroimaging, genetic testing and neurochemical testing for body fluid, such as CSF, plasma, serum, urine and blood cells.

Neuroimaging is currently gaining high interest for the possibility to test several promising markers, as suggested by the funding of the Alzheimer's Disease Neuroimaging Initiative (ADNI) project through the use of MRI or PET employed to analyze hippocampal volumetry and brain flow or in the scan of amyloid plaques in the living human brain [58,59].

However a big limitation concerning the use of these diagnostic methods is represented by the cost and availability of the instruments of analysis, impeding the routinely use of these technique for the diagnosis of the asymptomatic early stages of AD.

It is generally recognized that body fluids from living subjects represent the best potential source of information about proteins related to human diseases. Thus, growing interest is still devoted in the search of putative body fluid biomarkers for early diagnosis and stage progression of AD with particular attention to blood-derived markers. So far, CSF biomarkers reflecting the amyloid cascade hypothesis and cytoskeletal degeneration (total tau, and phosphorylated-tau) have been extensively studied and found to be promising and reliable biomarkers of AD pathology. Increase in total tau and p-tau, and decrease in Aβ42 level and Aβ42/Aβ40 ratio have been documented in CSF from AD patients and from MCI subjects [60–62]. The usefulness of these four parameters has been confirmed with large prospective studies, including the ADNI project [59].

The lumbar punctures used to obtain CSF, though less invasive than surgery, still remains an invasive procedure that can be followed by several side effects including headache, back pain, nausea and vomiting and still carry a small risk of infection and damage to the spinal cord. In addition, patient consent is far from being easily obtained and this aspect still remains the major limit for clinical routine analysis.

In this view and ideal biomarker would show up in a simple blood test and the majority of research has focused on biomarker parameters in blood, which is relatively easy to collect. Nevertheless only few studies were well correlated with disease and the development of methods for measuring biomarkers in blood is complicated by the potential influence of the blood brain barrier on the dynamics of release of these proteins into blood. Indeed, the blood–brain barrier prevents unrestricted transfer of plasma constituents across the CNS endothelium to the extracellular space. A major difference between the CSF, which is in equilibrium with the fluids of the extracellular space, and plasma is that the CSF concentration of protein is about 200-fold less than that in plasma. This raises the question as to whether exposure to some plasma proteins might modulate properties of neuronal or non-neuronal components of the CNS in homeostasis, or following injuries that result in increased permeability of the blood–brain barrier. For example, ischemic stroke is associated with pathophysiological changes affecting both glial and neuronal brain tissue. These changes are mirrored in the release of specific proteins into peripheral blood. Neurone-specific enolase, protein S100B and glial fibrillary acidic protein are those proteins investigated most often as peripheral surrogate markers of brain damage after stroke in humans [63,64].

Other limitations are the influence of other pathologies at systemic level, the standardization of the methods and the reproducibility of the results.

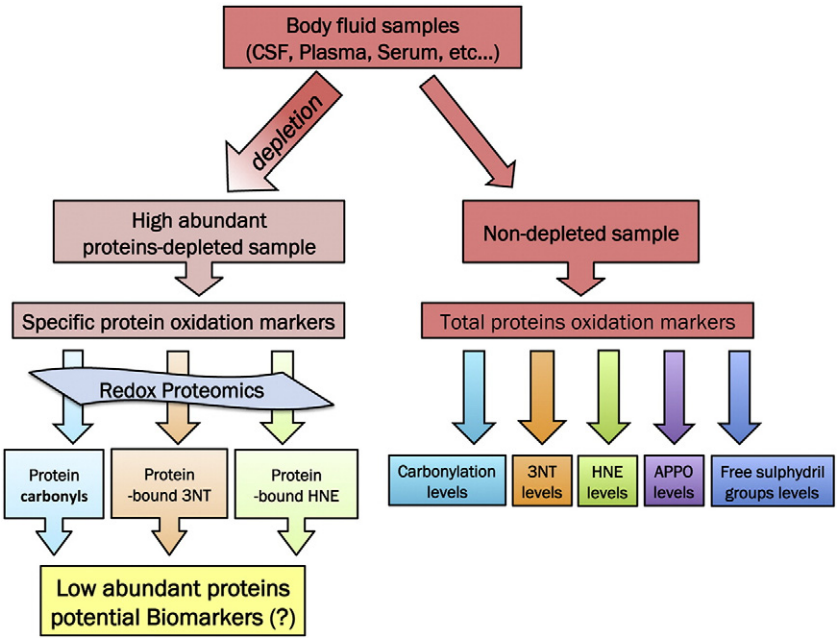
Studies evaluating the diagnostic performance of a biomarker for AD should include determination of the molecule's sensitivity and specificity and reproducibility of results [65]. 1) Sensibility represents the ability of a biomarker to identify patients who have disease; 2) Specificity represents the ability of a biomarker to identify patients who do not have disease. According to the criteria for an ideal Alzheimer disease diagnostic biomarker, outlined by the Ronald and Nancy Reagan Research Institute—National Institute on Aging Working Group, sensitivity and specificity should exceed 80%. 3) Reproducibility is the variations in biomarker levels between different centers probably result from variations in clinical procedures– such as the protocols for sample processing and other laboratory practices–as well as batch-to-batch variation in the biomarker assays. These types of variations are well known in clinical chemistry, and are routinely controlled by external control programs. Standardized protocols should minimize variation caused by differences in preanalytical and laboratory procedures and, thus, allow direct comparisons of biomarker levels between laboratories and between publications [11].

To overcome batch-to-batch variation in CSF biomarker assays, biomarker kit vendors should implement new standards for quality control. Assays should exhibit low overall variability in calibration curves and strict limits of variability across batches. To achieve these goals, stringent quality control of critical reagents, including antibodies and calibrators, is needed.

OS is present in AD since the early stages [6,66] of the disease and the search for anomalous levels of free radical by-products or oxidatively modified molecules might facilitate the selection of biomarkers of early AD diagnosis. Several studies have shown that free radical products are present in the cerebrospinal fluid (CSF), serum and plasma of AD patients [67–69].

The study of plasma, serum and CSF "oxidative biomarkers" for AD has been conducted by considering two different methodological approaches. The first one analyzes non depleted samples and allows the measurement of total protein oxidation markers, while the second one, essentially for proteomics studies, entails a purification step based on depletion of the most abundant proteins and thus allowing the detection of low protein fraction (Scheme 1). This latter is considered a limiting step in proteomics research and there is still an urgent need of further methodological refinement.

Recently NIH coordinated the Biomarker of Oxidative Stress Study (BOSS), a study to determine which biochemical products of



Scheme 1. Representative scheme for analysis of biological fluids. Non-depleted samples are analyzed for total oxidation markers (protein carbonyls, 3NT, HNE, APPO and free thiol groups). Samples undergoing high abundant protein depletion are analyzed for potential biomarker discovery by redox proteomics approach (protein carbonyls, 3NT and HNE).

oxidative damage represent a satisfactory in vivo biomarkers in an experimental model of oxidative injury to rat liver [70]. Of these, MDA, F2-IsoPs and 8-OHdG were acceptable quantitative in vivo biomarkers of oxidative damage.

In the past years the analysis of body fluid composition by proteomics and redox proteomics has been performed by several groups [71–79] However, as previously stated, the discovery and validation of protein biomarkers from body fluids is disadvantaged by the enormous difference in quantity between the high and the low abundant proteins with a dynamic range of about 12 orders of magnitude. Serum albumin, the most abundant circulating protein, represents about two-thirds of the entire protein content of plasma, and with IgG and few other high-abundance proteins constitute greater than 90% of total protein mass, interfering with the detection of lower-abundance proteins that might represent the biologically interesting population [80,81]. Depletion of abundant plasma proteins prior to analysis is a common strategy for increasing the number of proteins detectable with mass spectrometry [82–84]. There are several methods for removing proteins based on their biochemical and biophysical features, such as molecular weight, hydrophobicity and isoelectric point [85–87]. Among these techniques, the most common ones rely on antibody-based retention of a chosen set of the most abundant proteins [88]. Commercial removal kits are available, and the clearing of high-abundant proteins ranging from 2 to 20 allowing the depletion of about 85–95% of the total protein content depending by the number of high-abundant protein considered. Several comparative studies concerning the various depletion approaches show improved quality of proteomics separation and quantitation applied to biological fluids after removal of high-abundant proteins [82,83,89]. Fig. 1 shows a 2D gel of human plasma after depletion of albumin and IgG (Panel a) and depletion of 14 high abundant protein (Agilent) (Panel b) obtained in our laboratory. By following this approach, we were able to successfully identify an increased number of proteins after depletion of albumin and IgG in amniotic fluid from women carrying Down syndrome fetus compared with healthy controls [90]. However the downsides of any depletion step is represented by the probability of removing some low-abundance proteins along with the abundant species as well as removing proteins non-covalently bound to albumin.

4. Biomarkers of protein oxidation: from the brain to the periphery

Several studies highlight the involvement of OS in the pathogenesis of AD, by identifying specific proteins oxidatively modified in different brain regions [91]. A significant increase in protein carbonyls

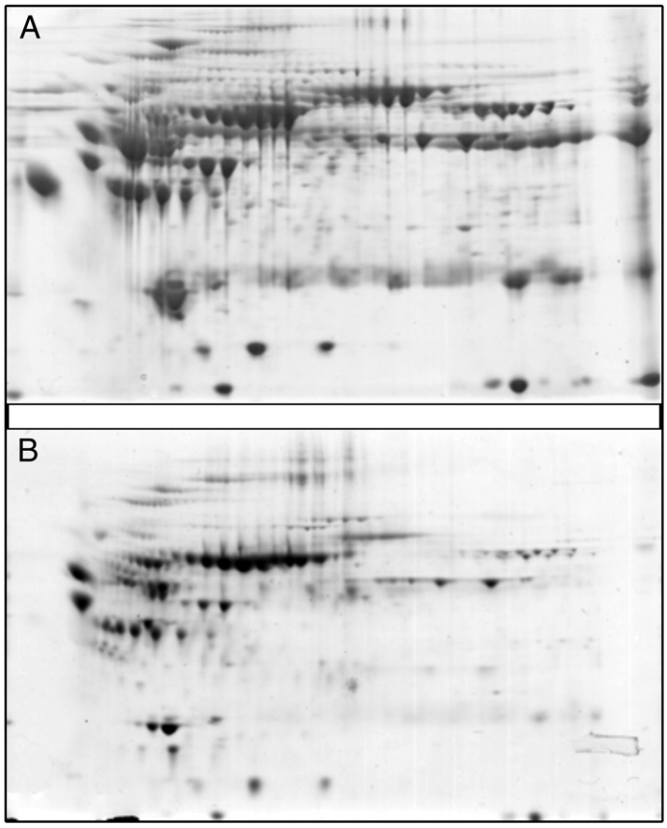


Fig. 1. Two-dimensional electrophoresis gel analysis of human plasma samples depleted with: A ProteoPrep blue Albumin & IgG depletion kit (Sigma-Aldrich); B Human 14 Multiple Affinity Removal System Spin Cartridge (Agilent).

in hippocampus (HP) and inferior parietal lobule (IPL) of AD subjects compared with age-matched controls was observed. Dityrosine and 3-NT total levels were reported to be elevated in the hippocampus, IPL, and neocortical regions of AD brain.

Alterations in brain phospholipids pattern, a more specific assessment of lipid peroxidation, have been reported for AD brain [92–94]. The levels of phosphatidylinositol (PI) and phosphatidylethanolamine (PE), rich in easily oxidizable PUFA, are decreased in AD brain. The levels of F(2)-isoprostanes [F(2)-IsoP], F(4)-neuroprostane [F(4)-NP], and isoprostane 8,12-iso-iPF2(α)-VI were also found to be increased in AD brain compared to controls [68,95]. An increase in free HNE has been demonstrated in amygdala, hippocampus, and parahippocampal gyrus of the AD brain compared with age-matched controls [35]. Several proteins mainly involved in energy metabolism pathways, pH regulation, and mitochondrial functions among others, were found carbonylated, HNE-bound or nitrated in AD brain [91]. Have been also reported that a number of proteins results modified by glutathionylation in AD IPL, [96,97].

AGEs accumulation in tissues has several toxic effects that comprises proteins modifications and direct damage to biological membranes and the endothelium. AGEs have been demonstrated to cause oxidative stress and cytotoxicity in neuronal cells [98]. Several evidences suggest that AGEs decrease mitochondrial activity and lead to energy depletion [99].

One of the major questions raised from both clinicians and researchers is: does body fluid reflect CNS pathology? Interpretation of changes of blood proteome is particularly complex since it could not necessarily reflect AD pathology only but also other systemic variations related to decreased cognitive function [79]. Thus, though the easy accessibility of peripheral body fluids, the degree to which peripheral fluids reflect biochemical changes in the brain is still under debate. Because the impossible direct access of brain tissue to blood for the presence of the blood brain barrier, CSF represents the closest approximation to analyze neurodegeneration process in living subjects. Therefore CSF can provide a biochemical window on brain status indicating neuronal cells alterations during onset and AD progression. Changes in Aβ40 and Aβ42 levels in CSF of AD patients were demonstrated to correlate well with amyloid burden in the brain, and the higher level of p-tau in CSF indicates more neurofibrillary tangles in the brain [100].

In recent years, growing studies have been focused to establish a direct link between tissue specific oxidation and systemic oxidative damage [11,79,101,102]. Correlations between total levels of oxidation markers in the brain and in the periphery have been shown. However none of the proteins found oxidatively modified in the brain, during AD or earlier stages of the disease, was also identified in any body fluid analyzed, raising doubts about the real suitability of circulating biomarker in the prediction of brain damage and neurodegenerative progression. Moreover, proteome profiles of neuronal cells are extremely different from protein composition of blood.

Thus, OS is a common feature of other degenerative and inflammatory diseases and the search for specific oxidative biomarkers in the periphery is challenging. Patients with AD often show other complications, such as weight loss and other chronic illness. In addition, other pathologies including diabetes, obesity and metabolic syndrome are characterized by oxidative stress conditions, which could lead to systemic oxidative stress. All these conditions could contribute to non-specific detection of oxidative biomarkers in the periphery.

This limitation that refers to lack of "specificity" is still the major issue in the field of biomarkers research. Genetic differences among population in addition to heterogeneity of disease clinical outcome, both tend to obscure what might otherwise be clear disease associations. To overcome this limit a combination of different approaches will be valuable.

1) Collect much more data from several pathologies, which will contribute to a have a more detailed and specific picture of a disease and make comparison among pathologies with similar features.
2) Use appropriate control population. For example, in the case of AD compare AD cases not only with healthy controls but also with other dementing disorders.
3) Analyze time-course variation of selected markers, that means to collect measures at different stages of the disease in order to understand when its variation is significant in clinical setting.
4) The most promising approach is offered by combining multiple markers which although not specific when considered alone may provide a more robust indication of the disease.
5) Large multicenter studies are needed to further define the added diagnostic value when multiple biomarker modalities are combined.
6) A single/panel biomarker/s need to be replicated in independent studies.

Many future studies should be performed toward these directions. Once such data will be available, reasonably novel putative markers could be discovered and validated.

5. Oxidatively modified proteins in biological fluids

As described above, protein carbonyls are the most often used markers of protein oxidation in tissue and blood. There are surprisingly few reports of plasma protein oxidation in vivo. The paucity of data on oxidized proteins in body fluids (CSF, plasma, serum and urine) is perhaps due to the difficulty of finding oxidized proteins in complex matrices, the multiplicity of possible oxidative modifications and low abundance [79]. In an effort to give insight into the mechanism of oxidative stress-induced neuronal loss, in recent years growing studies are focused on the search for circulating biomarkers of oxidative stress in patients with a clinical diagnosis of AD (Table 1). Indeed, post-mortem investigations cannot easily address the question of whether oxidative stress is an early component in the pathogenesis of the disease, or a common final step of the neurodegenerative process.

Table 1
Summary of available results on protein oxidation markers in CSF, plasma and serum from AD patients compared with controls.

Oxidative stress markers	Plasma AD vs CTR	Serum AD vs CTR	CSF AD vs CTR
Protein carbonyls	↑ (Conrad 2000) ≈ (Zafrilla 2006; Korolanein 2009)	≈ (Korolanein 2009; Sinem 2010)	↑ (Ahmed 2005) ≈ (Korolanein 2009)
Protein nitration		↑ (Sinem 2010)	↑ (Tohgi 1999; Ahmed 2005)
Lipid peroxidation	≈ F-2, F-4 isoprostanes (Montine 2002; Irizarry 2007; Mufson 2010)	↑ MDA (Padurariu 2010; Sinem 2011)	↑ F-2 isoprostanes (Pratico 1998; Montine 1998, 1999, 2001; Grossman 2005)
Oxidized LDL	↓ (Bergt 2006)	↑ (Sinem 2010)	
Redox Proteomics	Fibrinogen γ chain precursor and alpha 1 antitrypsin (Choi 2002) Hemopexin and transferrin (Yu 2003)		lambda chain precursor (Korolanein 2007)

↑ = increased oxidation; ↓ = decreased oxidation; ≈ = no difference.

5.1. Protein oxidation in CSF

Considering that AD is a "brain" disease, CSF is the most proximal to CNS and logical source to find any biomarker directly related to the pathology. Therefore, composition of CSF partially reflects cerebral metabolic changes and enables screening of ongoing pathophysiological process in brain [77]. The first report on protein oxidation in CSF samples was from Tohgi et al. [29] who demonstrated that 3-nitrotyrosine moderately but significantly increased with advancing age, and showed a remarkable increase in patients with AD. As the free tyrosine concentration did not decrease, the increase in 3-nitrotyrosine with age or associated with AD did not appear to be directly related to an increase in free-nitrated tyrosines. Rather, the increased 3-nitrotyrosine was likely due to an increase in nitrated tyrosines in proteins or increased degradation of 3-nitrotyrosine-containing proteins, which are highly vulnerable to degradation.

Subsequently, Ahmed et al. [101] measured in CSF the levels of protein glycation, oxidation and nitration. The authors found that the concentrations of 3-nitrotyrosine, N_{ε}-carboxymethyl-lysine, 3-deoxyglucosone-derived hydroimidazolone and *N*-formylkynurenine (as markers of protein glycation) were increased in subjects with AD. The Mini-Mental State Examination (MMSE) score correlated negatively with 3-nitrotyrosine residue concentration. These findings indicated that protein glycation, oxidation and nitration were increased in the CSF of subjects with AD. A combination of nitration and glycation adduct estimates of CSF may conceivably provide an indicator for the diagnosis of AD.

Previous studies on CSF nitrite and nitrate levels in patients with AD have provided contradictory results, with some showing decreased nitrate levels [103], others showing unaltered nitrite/nitrate levels [104], and still others increased nitrate levels [105]. However, a previous study from the same group showed that nitrite/nitrate levels in AD were stage-dependent, being elevated only in the early phase of AD and decreasing to control levels with disease progression [105]. This finding was interpreted to reflect progressive reduction of neurons. In contrast, free 3-nitrotyrosine levels increased significantly in parallel with the severity of AD, suggesting that protein degradation increases with disease progression, resulting in increased release of free 3-nitrotyrosine from tyrosine residues that have been nitrated. 3-nitrotyrosine and the 3-nitrotyrosine/tyrosine ratios in the CSF, both of which are believed to reflect degradation of nitrated tyrosine-containing proteins, increased significantly with age and were remarkably higher in patients with AD than in controls.

The most reliable CSF markers in AD are Aβ42 and tau. Low CSF Aβ 42 is associated with amyloid pathology in the brain and high Tau is linked with neurofibrillary pathology [106]. Most subjects with decreased CSF Aβ42 and high tau develop AD during the follow-up [107]. Therefore, these CSF markers may reflect brain pathology and identify preclinical AD. Interestingly, the levels of CSF Aβ42 showed a tendency to correlate positively with serum oxidative markers in the whole study population and with plasma nitrotyrosines in AD patients. Moreover, a negative correlation between CSF tau and serum nitrotyrosine levels was evidenced in controls [24]. The correlation between CSF AD markers and blood oxidative markers may suggest that oxidative metabolism is changed in AD. This hypothesis is further supported by the finding of decreased CSF protein carbonylation in APOE ε4 carriers, which is considered an important risk factor for developing AD [108] and correlates with redox proteomics studies that identified metabolic proteins as oxidatively modified and dysfunctional [109].

A few previous studies measuring total levels of protein carbonyls or nitrated proteins in CSF and plasma have yielded contradictory results [101,110–112]. Korolainen et al. [24] found that protein carbonyl levels did not differ in CSF between AD patients and controls. CSF represents a window to brain metabolism, therefore, alterations in oxidative stress in AD brain may not be detected as changes in total protein carbonylation in CSF. However, by using 2-D oxyblot, an increased degree of carbonylation for one single protein, lambda-chain, in CSF of AD patients as compared with controls [77] was showed. Further studies are needed to replicate this finding and also to add new information in this topic. Yet the analysis of CSF oxidized proteome present a number of challenges and it is likely that additional changes in protein carbonylation will be revealed by using more sensitive methods.

In late 90s several studies by Pratico et al. and Montine et al. showed increased levels of F-2 isoprostane in CSF of AD and probable AD subjects confirming increased oxidative stress indexed as increased lipid peroxidation in AD CSF [113–118].

5.2. Protein oxidation in serum/plasma

Overall, current data indicate that increased oxidation in AD patients is not restricted to the brain. The increase in oxidation may be due to increased ROS production, alteration of plasma protein composition or decreased antioxidant function.

Conrad et al. [119] were the first to measure the levels of oxidized proteins in plasma from AD patients compared with controls. The total oxidized proteins were determined by HPLC, while specific protein oxidation was assayed by western blot using anti DNP antibody. They found a statistically significant increase of total carbonyl groups in plasma proteins and specific oxidation of a 78 KDa protein appeared to be differentially oxidized in subjects with AD.

Yu et al. [120] investigated native and oxidized glycoproteins in plasma from AD patients. The plasma samples were fractionated by sequential affinity chromatography on heparin-agarose (HepA) and concanavalin A-agarose (ConA) columns followed by separation on one-dimensional and two-dimensional polyacrylamide gels. Carbonylation of proteins was monitored by in-strip derivatization with DNPH and anti-DNP immunoblotting. They showed increased levels of carbonylation for glycosylated hemopexin and transferrin in the AD subjects as compared to controls suggesting systemic derangements in heme/iron/redox homeostasis and activation of the acute phase response in sporadic AD [120].

A study by Choi et al. [72] identified uniquely oxidized proteins in AD plasma. These authors applied 2DE coupled with immunological staining of protein carbonyl and the oxidized proteins observed in the plasma of both AD subjects and non-AD controls were determined. However, the level of oxidation of these protein spots was markedly higher in the AD samples. They also found that the increased oxidation was not a generalized phenomenon. In the total protein stain profile, more than 300 spots were detected, but less than 20 spots were positive by immunostaining with anti-DNP antibody. Furthermore, of the seven proteins that were most intensively oxidized, their relative levels of oxidation differed. These scientists found that fibrinogen gamma chain precursor and alpha 1 antitrypsin precursor showed increased levels of carbonyl groups in AD compared with controls. Fibrinogen oxidation may be relevant to the mechanism since all six chains of fibrinogen (a2; b2; c2) must be intact for normal fibrinogen and oxidative modification of the c-chain may result in increased activation of plasminogen thereby contributing to fibrinolysis and proteolysis in areas of inflammation [121]. Similarly, a-1-antitrypsin oxidation may be particularly relevant to the disease. a-1-Antitrypsin, one of the major serine proteinase inhibitors (serpins) in human plasma, functions to inhibit overexpressed proteinases during inflammation [122]. These inhibitors tightly regulate proteinase activity, under normal physiological conditions. However, under some pathological conditions, proteinase activity may exceed the capacity of such proteinase inhibitors as a-1-antitrypsin. This could be caused by oxidation inactivation of the inhibitor [123].

Recently, plasma carbonyl content was found to be unchanged in AD patients as compared with age-matched controls [112]. In the same study, total antioxidant activity in plasma and the activity of

endogenous antioxidant enzymes such as glutathione peroxidase, glutathione reductase and superoxide dismutase were also evaluated. The total antioxidant plasmatic status of the patients with AD both in light-moderate phase and in advanced phase was lower than in the controls. No significant differences of protein oxidation levels were observed between the different stages of the disease. Peroxidation was higher in patients in the advanced stage of the disease than in the control group. However, no significant differences were observed between different stages. In this preliminary study, it was observed that AD patients in the light-moderate stage already present oxidative stress levels above those of the control group. The most recent study is the one published by Korolanein et al. 2009, who measured total levels of protein oxidation (carbonyl and 3-NT) in CSF, plasma and serum in AD. The result of protein carbonyls not differing in CSF between AD patients and controls corroborates their previous findings [77]. Accordingly, the levels of serum protein carbonyls were decreased in AD subjects when compared with controls whereas no differences were found in plasma. It must be noted that the reliability of these results is diminished by the fact that most values measured in blood samples were under the detection limit. Nevertheless, these data agree with the study by Zafrilla and collaborators [112] of similar plasma carbonyl concentrations among AD patients and controls. It seems that the total protein carbonylation may vary in different compartments; or alternatively, the separation of serum and plasma from whole blood may result in differences in measurable carbonyls. Reasons for the tendency of decreased levels of serum protein carbonyls remain unclear. Decreased serum carbonyls may thus be an epiphenomenon and not linked to AD per se. On the other hand, also previous studies have reported decreased levels of oxidative stress markers such as nitric oxide and oxidized high-density lipoprotein in blood from AD patients [124,125]. Bergt et al. [125] found significantly lower oxHDL levels in plasma of AD patients compared to age-matched, cognitive healthy individuals. The decrease was statistically significant in males (32% reduction) and it followed the same tendency in females (34% reduction), however, without reaching statistical significance. However, they did not observe a marked decrease in apo A-I plasma levels as reported by others [126]. These results are in contrast with the findings of Sinem et al. [127] and Dildar et al. [128] that demonstrated that serum NO and oxLDL levels in patients with AD were significantly higher than in both controls and patients with vascular dementia (VaD). However, no significant differences in plasma NO and ox-LDL levels were found between VaD and controls. On the other hand, they did not find any significant difference in serum 3-NT values of both AD and VaD patients when compared with controls. Although it has been suggested that nitrotyrosine levels were increased in the brain of AD patients, several factors might be responsible for these results, for example 3-NT is not stable in serum or protein nitration occurs at undetectable levels. Some previous studies [29,101] reported evidence of increased CSF nitrotyrosine concentrations in AD patients as compared with controls, whereas another study revealed no differences [110]. Overall, these results point to the fact that the concentrations of nitrotyrosines in individuals may vary greatly. However, further studies with more sensitive methods are needed to assess nitrotyrosine levels in AD plasma or serum.

As well as in the CSF F-2 and F-4 isoprostane levels were also investigated in plasma of patients with AD and subjects with MCI. The results showed were somehow controversial, however, in most of the cases no differences in F-2 and F-4 isoprostane, between AD and control subjects, were showed [129–132].

Studies from Padurario et al. demonstrated a progressive increase of the peripheral levels of MDA in patients with MCI and AD [133]. The end-products of lipid peroxidation, so called lipofuscin-like pigments (LFP), were also found increased in erythrocytes of AD patients compared to controls. The specific fraction of LFP in AD patients, which was isolated, might represent a disease-specific product in the blood [134].

A reduction of plasma 24S-hydroxycholesterol that correlated with the severity of dementia and degree of brain atrophy was also found. However different results have been shown by other groups and might be explained by differences in the severity of the disease [135].

Recently, markers of oxidative damage were found to be elevated in mitochondria isolated from lymphocytes from AD patients compared with their age-matched controls [136]. Indeed, mitochondrial dysfunction has been widely implicated in the etiology of AD. This is the first report to show mitochondrial alteration in peripheral lymphocytes thus suggesting the oxidative stress indices could potentially be used as putative biomarkers for AD.

6. Concluding remarks

Although some of the reported results in AD are controversial, most of them support the presence of peripheral oxidative damage and of a characteristic panel of systemic oxidation that correlates with the occurrence of the disease.

Studies investigating oxidative stress outside of the CNS, particularly in blood, while prove the occurrence of oxidative reactions, are not fully elucidating the complex cascade of events. Thus, one hypothesis is that oxidative stress first develops in the periphery as a result of different causes, and then it will contribute to perturb neuronal homeostasis, either by increasing the production of ROS or by depleting antioxidant defense, which will eventually lead to oxidative damage of the brain and final neurodegeneration. On the other hand, it is also possible to imagine that oxidative stress starts in the CNS where several different metabolic end-products are formed and released into the blood stream. In this context, an important issue is to perform further studies in order to investigate the timing of appearance of oxidative damage signatures at systemic level during the onset of AD early stages and the progression to late stages.

There are several different reasons to support the development of more sensitive method to detect a biochemical marker in AD: to increase diagnostic accuracy; to identify MCI subjects who will progress to clinical AD; to monitor pharmacological and biological effects of drugs. It is clear from the present review that there is an urgent need to add further peripheral markers of oxidative stress as useful diagnostic biomarker. Recently, important steps have been accomplished but there is still a lot of work to be directed towards the discovery, testing and validation of a panel of novel and old assays that could serve all the requirements for an ideal biomarker. However, the emerging trend which results from the collection of multiple data (as shown in Table 1) is the wide variability among different studies that led to contrasting results. Thus, there is an urgent need to standardize protocols for replicate experiments on large population, which may allow to better understand the effect of systemic oxidative damage in the pathogenesis and progression of AD. Indeed, this is also evident by the lack of redox proteomics studies applied to biological fluids. This approach has the “power” to search for specific protein oxidative modification thus allowing the identification of altered protein in complex matrices such as body fluids, which may discriminate a pathological vs healthy condition. Once proteins have been identified, the results would need to be further validated by testing in large population studies with commercially available kits.

The essential goal in biomarker discovery studies is the identification of preclinical marker, which facilitates disease diagnosis at early stages, monitors disease progression and assesses the response to treatments by the time that disease-modifying treatments become available in clinical practice.

Currently, novel approaches derive from the combination of putative peripheral biomarkers and structural (MRI) and/or functional (PET) brain imaging that should provide increased diagnostic accuracy compared with circulating biomarkers or one type of imaging used in isolation. So far, only a few studies have directly examined this

possibility. CSF biomarkers combined with MRI measurements of medial temporal lobe atrophy have been reported to increase the accuracy of AD diagnosis [11]. In fact only a combination of different markers could, most likely, offer a certain diagnosis and be able to capture all aspects of the disease.

Our laboratory is presently performing the analysis of both CSF and plasma samples from MCI and AD living patients, by proteomics and redox proteomics approach coupled with the use of prefractionation methods, in the effort to find potential markers of peripheral damage, which could contribute to a more accurate diagnosis and prognosis.

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