Proteomics Approaches in Neurodegeneration: Advances, Biomarkers and Challenges

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***Abstract*—Changes in protein structure and function, alterations in protein-protein interactions, and significant differences in protein concentration in the body may indicate abnormalities before clinical symptoms develop. They can serve as critical detection and diagnostic tools, commonly referred to as biomarkers. Neurodegenerative disorders, such as Parkinson's, Alzheimer's, Huntington's, prion diseases, and multiple sclerosis, are marked by neuronal deterioration, which leads to specific changes in neuronal proteins. To stop more harm, early diagnosis is essential. New targeted therapeutics and more precise diagnostic and prognostic disease indicators could be developed more quickly in pre-clinical and clinical stages with the help of a CNS proteome database of primary human CNS tissues. These biomarkers may be found in cerebrospinal fluid, blood, serum, plasma, saliva, or urine. Modified protein expression patterns may be associated with changes in protein isoforms brought about by post-translational modifications, such as phosphorylation from cell signalling events and distinct splice variants of receptors. The types of biomarkers, their origins, and the techniques employed to find biomarkers for the early identification of neurodegenerative illnesses are all covered in detail in this article.**

***Index Terms*—Neurodegeneration, Alzheimer's, Biomarkers, Mass spectrometry, Proteomics etc.**

I. INTRODUCTION

Proteomics is the branch of biomedical studies that focuses on analysing the entire protein content, their functions, and interactions within a cell or an entire organism. Although there are many ways to divide it into subgroups, the most commonly accepted division identifies three main subdivisions [1]:

1. Clinical proteomics: This field looks at how proteins can serve as potential biomarkers for specific diseases.
2. Structural proteomics: This area focuses on understanding the three-dimensional structure of a protein and how it relates to its function within the cell.
3. Functional proteomics: The primary aim in this area is to study how a specific protein interacts with other proteins or types of molecules in relation to its role in complex (Patho) physiological processes.

Proteomics tools allow for detailed protein profiling of tissue, plasma, serum, and CSF to discover biomarkers. Discovery proteomics technology that uses mass spectrometry, immunoassays, and aptamers can find thousands of different proteins that are expressed differently and their altered forms related to disease [(2)].

Post-mortem tissue proteomics is an important method for finding disease biomarkers in bulk tissues, tissue sections on slides, and single cells. Several workflows have been proposed for analysing bulk tissue, including suspension trapping filter-aided sample preparation (FASP), single-pot solid-phase enhanced sample preparation (SP3), and in-Stage Tip (iST) for disease biomarker analysis [(3)].

Neurologic diseases are the main cause of loss in disability-adjusted life-years. An increasing number of current diagnostic criteria exist, including those for Alzheimer's disease and multiple sclerosis [(4).]

Alzheimer's disease (AD) is the most common type of dementia, and its occurrence is rising quickly in aging populations. Patients with AD usually show memory loss and struggle with daily activities. This condition

involves the build-up of amyloid plaques and the formation of neurofibrillary tangles [5].

Amyotrophic lateral sclerosis (ALS) is a serious neurodegenerative disease marked by the gradual loss of upper and lower motor neuron sALS can sometimes be classified as sporadic ALS (sALS) due to point mutations, and familial ALS, which is associated with mutations in many different genes that appear to be unrelated [6].

Biomarkers are biological traits that can be measured directly to assess the normal or abnormal state of biological systems [7]. Efficient biomarkers should

1) show risk, presence, and status of a specific disease,
2) be used in clinical settings to predict, diagnose, and track disease progression, and
3) evaluate stage, response to treatment, or outlook.

Some of the best biomarkers for neurodegenerative diseases come from changes seen in neuroimaging techniques like magnetic resonance imaging (MRI) and positron emission tomography (PET) [7].

II. OVERVIEW OF NEURODEGENERATIVE DISEASES:

Due to their complexity and detrimental effects on cognitive and motor function, neurodegenerative disorders such as Huntington's disease (HD), Parkinson's disease (PD), Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) present a substantial scientific challenge [2]. In addition to their individual characteristics, all diseases have certain molecular mechanisms, such as protein misfolding and oxidative stress. For instance, misfolded proteins—alpha-synuclein in Parkinson's disease and amyloid-beta in Alzheimer's disease—accumulate in both conditions. There is a common pathogenic feature to these misfolded proteins, which cause toxicity and interfere with cellular functioning [8].

In Alzheimer's disease, a key factor is the amyloid-beta (Aβ1-42) peptide, which comes from the amyloid precursor protein (APP). When enzymes called beta and gamma secretases cut APP, they create Aβ fragments that tend to clump together outside cells. These clumps, known as plaques, have long been recognized as a major sign of the disease. However, recent research has shifted focus to the soluble forms of Aβ, suggesting that these smaller aggregates may actually be the main toxic agents. Soluble Aβ oligomers can directly interact with cell membranes, disrupt ion channels, and activate internal signaling pathways that affect synaptic function; this ultimately leads to neuronal death. Additionally, tau protein, which stabilizes the neuronal structure, undergoes hyperphosphorylation in Alzheimer's, transforming into tangled filaments within neurons [8].

In Parkinson's disease, the loss of dopamine-producing neurons in the substantia nigra region of the brain leads to common motor symptoms, like tremors and stiffness. This loss mainly happens because of alpha-synuclein, a protein that, under certain conditions, misfolds and accumulates inside neurons, creating structures known as Lewy bodies. In addition to protein misfolding, Parkinson's also includes serious mitochondrial issues. Key genes such as PINK1 and Parkin, which help maintain mitochondria, do not function correctly. This failure reduces the cells' ability to clear out damaged mitochondria. As a result, oxidative stress increases and further endangers the survival of dopamine-producing neurons [8].

One out of every 350 persons has ALS, a fatal neurological illness. Both upper and lower motor neurons degenerate as a result, resulting in gradual paralysis. About 10% of ALS patients have a strong family history of the disease, showing a significant genetic risk. So far, several genome-wide association studies (GWASs) have found up to six significant genetic locations linked to ALS, but these accounts for only a small part of the genetic risk. Some of the locations identified in these studies have rare variants with large effects that are also seen in family cases. For other locations, the role of rare variants remains unclear (9).

Huntington's disease has a unique molecular characteristic: an expansion of CAG repeats in the HTT gene. The mutant huntingtin (mHTT) misfolds and builds up in neurons, mostly in the striatum, which is crucial for movement and coordination. These clumps disrupt normal cellular processes by attaching to important transcription factors, causing broad changes in gene expression. Additionally, mHTT interferes with mitochondrial function, which reduces energy production and raises oxidative stress, worsening the damage to sensitive neurons (8).

The central nervous system is impacted by the chronic inflammatory condition known as multiple sclerosis (MS). It mainly impacts young adults between 20 and 45 years old, and it is more common in women, with a

ratio of three females to one male. MS is more prevalent among Caucasians, and its occurrence varies by geography. It is most common in Scandinavia and Canada, while it is nearly non-existent near the equator. MS is considered a rare disease, with about 2,800,000 patients worldwide [1].

III. PROTEOMICS TECHNIQUES

Mass spectroscopy

In the last few decades, the development and use of MS techniques have significantly contributed to proteomic studies. These studies focus on identifying proteins, characterizing their structures, and measuring their quantities. MS was mostly employed in large-scale biomarker research until recently, mostly using "shotgun" proteomic techniques that work from the bottom up. In these studies, proteins extracted from a sample are broken down into small peptides. To produce mass spectra, these peptides are then broken apart in the MS. The spectra are matched against a database to identify their original proteins. The reliability of protein and peptide identification and structural characterization is significantly increased by the fragmentation patterns from tandem mass spectrometry, which is commonly referred to as MS/MS but in this review as MS. MS-based proteomic analysis can also be quantitative, which is essential for spotting differences between biological samples in studies focused on disease states (7).

A wide range of technologies fall under the term proteomics. This includes traditional analytical methods like chromatography techniques and enzyme-linked immunosorbent assay (ELISA), as well as newer technologies like mass spectrometry and gel electrophoresis. It is most practical to summarize these technologies based on how they are commonly used in research (1).

Application of different proteomics technologies. ELISA, ICAT, SILAC, NMR spectroscopy (1).

A number of chromatography-based methods, including affinity chromatography, size exclusion chromatography, and ion exchange chromatography (IEC), can be used to purify biological materials [(10)]. Selective proteins are analysed using the Western blot and the enzyme-linked immunosorbent (ELISA). These technologies may identify a protein's presence in a sample at incredibly low concentrations, sometimes even detecting a single molecule [(11, 12)]. All of these techniques are helpful for analysing proteins qualitatively. Nonetheless, it is frequently necessary to separate and quantify complicated protein samples. Mass spectrometry, two-dimensional gel electrophoresis (2-DE), two-dimensional differential gel electrophoresis (2D-DIGE), or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used for both objectives [(13,14)].

Using their molecular size, mass, and charge, these methods determine which proteins are present in the sample. In the case of 2D-DIGE, a dyeing procedure allows for visualization of the proteins. For quantification, isotope-coded affinity tag labeling, stable isotope labeling with amino acids in cell culture, and isobaric tag for relative and absolute quantitation techniques can also be used[9,1].To analyse the three-dimensional structure of proteins, researchers use X-ray crystallography and nuclear magnetic resonance spectroscopy[(1)].Through well-maintained and expanding bioinformatics databases, scientists can explore the potential biological role and interaction of the assessed proteins.

IV. APPLICATIONS OF PROTEOMICS IN NEURODEGENERATIVE DISEASES RESEARCH

1. Biomarker discovery

Source materials used in MS genome and proteome investigations for biomarker development have varied greatly. These include post-mortem tissues from MS patients, cerebrospinal fluid (CSF), serum from patients, and tissue samples generated in mouse models like EAE [15]. In order to uncover molecular hallmarks and potential treatment targets, a number of studies have examined the genomes and proteomes of lesions using MS post-mortem tissue [16]. Despite the fact that it is typically impractical to sample CNS tissue from MS patients, employing CNS-derived materials such as CSF can yield valuable information on the disease causes.

Since pathogenesis occurs in the CNS, this research might eventually lead to blood-based biomarkers due to the increased permeability of the blood-brain barrier (BBB) in MS [15]. Using techniques like imaging mass spectrometry on CNS tissue may help distinguish proteins by brain regions [17].

2. Single-cell

Single-cell cytometry by time-of-flight mass spectrometry (CyTOF) represents another new area under the MS research umbrella. It involves labeling cells with antibody (Ab)-conjugated isotope reporters. Unlike fluorophores, whose detection is rather limited by spectral overlaps, isotope reporters allow the potential detection of dozens of cellular features by one individual cell and thereby permit ahigh-dimensional immunophenotyping of individual cells [19,20]. Thus, differences in signaling molecules and in the production of cytokines by myeloid populations when compared to naïve animals were found between EAE and naïve mouse CNS myeloid cells [20]. Moreover, CyTOF analysis of peripheral blood mononuclear cells and CSF cells in MS unveiled a T helper subset that expressed high levels of granulocyte-macrophage colony-stimulating Factor (GM-CSF) and C-X-Cmotif chemokine receptor 4 (CXCR4), which was reduced by disease-modifying therapy, thereby implicating these cells as markers for drug response (17).

3. Pathway & Network analysis

Pathway analysis refers to the use of lists of altered genes in biological pathways with an attempt to integrate multiple gene or protein alterations simultaneously to obtain lists of differentially altered pathway activities [19]. Pathways can be analysed either as networks or as gene sets. These pathway enrichment analyses may be alternative approaches. In a similar spirit, in MS, pathway analysis has been applied to SNP array data to broadly get a snapshot of pathway landscapes in MS, yielding, for instance, information on upregulations in JAK-STAT and TCR signaling pathways. Pathway analysis has been applied to studying specific processes too, such as transcriptional changes in oligodendrocytes during remyelination in the cuprizone model, revealing upregulation in cholesterol-synthesis pathways [15]. In contrast to RRMS, SPMS, or PPMS, others discovered that IFN-γ signaling was the most markedly dysregulated pathway in transcriptome datasets of healthy controls [21].

Network analysis groups proteins that have similar functions and show how molecules depend on each other, even if they are not part of a direct pathway [22]. For example, a network can consist of proteins that increase or decrease in a cell or tissue after treatment. It can also include molecules with broader connections, like a "neurological disorder" network that includes many genes and proteins affected in these diseases. Metabolic networks, transcriptional regulatory networks, and protein-protein interactions are examples of common network types (21).

4. Post-translational modifications (PTMs) profiling

High-throughput, in-depth mass spectrometry (MS) methods have mainly been used to study the dynamic changes of PTM-modified proteins in the brain, focusing on phosphorylation, ubiquitination, and glycosylation in Alzheimer's disease (AD). Profiling PTMs in brain tissue with these MS techniques can offer valuable insights into how abnormal PTM patterns contribute to human diseases and help identify markers that could be useful for diagnosis or treatment.

Going forward, more work should focus on identifying modified glycans and glycoproteins involved in neurodegenerative processes, especially in brain tissue and biofluids like cerebrospinal fluid (CSF) and serum. Understanding their roles could highlight important targets and eventually clarify how glycosylation affects disease mechanisms and progression (23).

V. PROTEOMICS IN SPECIFIC NEURODEGENERATIVE DISEASES

1. Proteomic studies in PD

Multiple research investigations examined differentially expressed proteins that develop in post-mortem tissue with Parkinson's Disease (PD). The proteomic analysis of post-mortem substantia nigra tissue from PD patients revealed changes in proteins that linked to mitochondrial dysfunction and oxidative stress and cytoskeleton impairment [24]. The study utilized isobaric tag analysis for sample evaluation followed by immunohistochemical and Western blot validation of specific markers.

The proteomic investigation revealing pathways linked to Lewy body pathology demonstrated that Arp2/3 together with synaptic function and hydrogen peroxide metabolism showed positive correlation with Lewy body pathology butpoly(A) RNA binding protein pathways like TDP43 and FUS showed negative correlation. The study comparing Lewy body pathology with neuronal loss demonstrated that CD59

levels increased while RGS6 and GANAB levels decreased [25].

2. Proteomic findings in ALS

So, here's the deal with ALS and proteomics: researchers dove into spinal cord tissue from people with and without sporadic ALS, using LC-MS/MS (fancy mass spec stuff), and guess what? They found ATP5D and calmodulin took a nosedive in ALS samples (22). Now, to sniff out biomarkers, they also used 2D-gel electrophoresis plus that same mass spec—think of it like sifting proteins by their quirks, but honestly, it's a pain. Super low throughput and you've gotta be a wizard to get the protein spots right every time. But here's where it gets interesting: When they checked out samples from folks carrying ALS mutations (C9orf72, SOD1, TARDBP), regular sporadic ALS, and controls, they spotted a whole bunch of proteins dialed up in ALS. Stuff like UCHL1, MAP2, CAPG, GPNMB, and a whole crew of others—some with names that sound like robot serial numbers (HIST1H4A, HIST1H2B, NEFL, NEFH, NEFM, CHIT1, CHI3L1). These weren't just in the spinal cord, but also floating around in the CSF. To make sure they weren't just seeing ghosts, they double-checked four of these proteins (UCHL1, MAP2, CAPG, GPNMB) in another group using this targeted method called MRM (22). They even looked at CSF from both symptomatic and asymptomatic mutation carriers, and the usual suspects—NEFL, NEFM, NEFH, CHIT1, CHI3L1—were up in the folks who actually showed symptoms (25,22).

3. Proteomic studies in AD

Level A protein biomarkers are the ones that doctors look at right now to help figure out if someone has Alzheimer's disease. These biomarkers are like little clues in your brain's spinal fluid that show if you've got the bad stuff associated with Alzheimer's, like too much of a protein called amyloid or messed-up tau proteins. The ATN criteria are like the rulebook they use to check for these clues. But they might swap out the total-tau biomarker for something called neurofilament light, because it's a better hint at what's really going on with the brain cells. These biomarkers can help them guess if someone with mild memory issues might go on to develop full-blown Alzheimer's. Now, not all of these biomarkers have been studied over a long time or in people who don't have any symptoms yet, which is why we're talking about a few that have more evidence behind them, like NfL and pNfH for ALS (a disease that messes with your muscles and nerves) and the Aβ42/40 ratio and phospho-Tau181 and 217 for Alzheimer's (27,28). Oh, and guess what? Some of these Alzheimer's-related proteins can be spotted in your spit too, so it's not all about poking and prodding. And lactoferrin? That's another cool thing they can check in your spit that might tell them if you're in the early stages of Alzheimer's (4).

VI. CHALLENGES AND LIMITATIONS

1. Sample complexity & Biological heterogeneity

In neurodegenerative disease research, scientists mainly use proteomics technology to analyse the brain and cerebrospinal fluid (CSF) from both humans and animals. The goal is to gather information about gene products involved in these disorders, including changes in protein levels and post-translational modifications (PTMs). Changes in protein levels may make them biomarkers or possible targets for drugs. These biomarkers can be helpful for diagnosing diseases if they appear in body fluids like blood and urine (29).

Researchers have identified more than 300 distinct proteins with altered levels or changes linked to neurodegeneration and psychiatric diseases since the early days of proteomics in neuroscience [30]. Signal transduction, detoxification, cytoskeleton formation, metabolic pathways, and transport are all associated with the majority of these proteins. Proteins found in these studies are mostly high-abundance proteins found in the brain or CSF. For instance, fructose-bisphosphate aldolase and peroxiredoxins 1 have shown altered levels in multiple disorders [30]. However, many of these changes have not been confirmed by other methods, and some are not reproducible, statistically insignificant, or even contradictory. These modifications can only be helpful in determining the associated illnesses following verification.

2. Post translational modifications (PTM's)

It is well known that many types of post-translational modifications (PTMs) can affect protein folding, influence real-time dynamics, and control the functions of most proteins. For instance, the addition

and removal of phosphate groups on proteins are crucial for cell signaling in the brain. Other significant PTMs, like glycosylation, methylation, acetylation, oxidation, nitrosylation, and ubiquitination, also impact the functions, targeting, and breakdown of proteins in the central nervous system (CNS). Therefore, beyond changes in protein levels, unusual PTM patterns in different proteins may be linked to the start and progression of several neurodegenerative diseases [29].

In summary, current mass spectrometry platforms and enrichment methods allow for either targeted or broad analysis of PTMs in Alzheimer's disease (AD) samples. Large datasets have been emerging, particularly in phosphoproteomics [31].

3. Sensitivity & Dynamic range

One method available for such characterization could involve top-down MS of proteins of interest-Aβ species and tau protein proteoforms [32]. Newer approaches to structural MS have appeared that allow one to analyse structural features of purified proteins, purified protein complexes, and sometimes even thousands of proteins [33,34,35,36] and will indeed be utilized to analyse global structural changes in AD. Subsequently, the combined use of bottom-up, top-down, and structural MS techniques would hence provide researchers with a more solid understanding of the proteotype (a state of the proteome associated with a certain phenotype) of AD patients [37]. Enhancing throughput, sensitivity, and affordability in proteomics must be done incessantly.

This means getting deeper and wider in the flavor of proteomes being detected and making it possible to work with large sample sizes that would eliminate limitations of protein dynamic range and clinical sample variation.

4. Translational Gap

An emphasis must be put to create a discrepancy existing between generation of proteomics data and discovery of disease drivers as, while proteome profiling shows disease-correlated components, correlation does not demonstrate causation. Heavy investments are made to set up causal relations with disease models and human patients [31]. It may, therefore, be difficult to directly define some regulators using gene/protein expression profiles. Alternatively, the driver activity may be inferred from the summary of expression alteration of its downstream target genes by systems biology approaches [38]. This network-activity based approach [39], along with other multi-omics network analyses [40,41], will reveal AD pathogenesis disease drivers. These new disease drivers will then be inspected through interactome profiling and genetics in laboratory cell (induced pluripotent stem cells) and animal models [42].

5. Data Analysis & Bioinformatics

Strong bioinformatics tools are required for data analysis and interpretation due to the massive volume of data produced by proteomic research. Protein identification, quantification, and PTM analysis are made easier by programs like MaxQuant, Proteome Discoverer, and Skyline. Furthermore, understanding the biological importance of proteomic data is aided by pathway analysis tools such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ingenuity Pathway Analysis (IPA) [43].

VII. FUTURE PERSPECTIVES

Single-Cell & Spatial Proteomics

Since proteins serve as mechanistic markers of function, knowledge of the dysregulated protein pathways seen in disease microglia may lead to the development of new therapeutic approaches [44]. Gaining a thorough grasp of the proteome diversity of microglia is essential, as single cell transcriptomics has transformed our comprehension of their diverse range of responses [45]. More advanced in-situ proteomics techniques, such as Nano String's new GeoMx protein panels, which enable the simultaneous, spatially resolved profiling of hundreds of protein targets, are presently being developed and implemented in addition to these established spatial proteomics approaches. Strong spatial multi-omics analysis may be possible when RNA and protein assays are used in the same tissue [45].

Artificial Intelligence (AI) and machine learning

Large and intricate proteomic datasets are available. AI systems are able to recognize trends, forecast the course of diseases, and propose new biomarkers. In recent research, for instance, the accuracy of protein identification has increased by 20% due to AI-driven analysis of mass spectrometry data [46].

Proteoform analysis

Protein detection is the main focus of current research, but identifying proteoforms—variants with unique modifications—offers more detailed information. This variety is being mapped using new methods, such as top-down mass spectrometry, which may reveal signs unique to a given disease [45]. Findings from the proteome map could be revolutionary. A complete database could identify elusive targets for rare disorders, where single protein abnormalities frequently predominate. Proteomic information may provide new avenues for treatment in common diseases like diabetes.

Clinical Translation & Multi-Omics Integration

These databases were combined in a 2023 colon cancer trial to improve survival estimates and find patients who would benefit from adjuvant chemotherapy. Breast cancer treatment is a real-world example. In order to minimize overtreatment and improve results, proteomic analysis of tumours separates hormone receptor-positive (tamoxifen-responsive) from triple-negative (aggressive chemotherapy) instances. The specificity of precision medicine is what makes it promising. Clinicians can reduce adverse effects and increase efficacy by matching treatments to proteomic profiles rather than using trial-and-error methods. Standardized procedures and a strong infrastructure are necessary for incorporating proteomic data into clinical workflows, but [45].

VIII. CONCLUSION

In conclusion, our study shows how proteomic applications can be used to find conserved sets of proteins participating in processes common to many neurodegenerative disorders as well as sets of disease-specific proteins associated to specific neurodegenerative diseases. The study of neurodegenerative proteinopathies has rapidly expanded from the proteome to proteoforms due to their variety, and the availability of sensitive and high-throughput proteomic tools has become essential. Several top-down and structural mass spectrometric approaches are being refined to improve our understanding of this field, even though certain traditional techniques, such as 2D-GE, are already useful for this purpose.

As we look to the future, we believe that our findings will assist guide important choices regarding sample preparation and data processing when working with TMT in CSF samples and comparable clinical research where diverse samples are involved. Furthermore, we are certain that the list of biomarker candidates provided in this study might serve as the foundation for further research on biomarker creation.

Disclosure statement

The authors declare no potential conflicts of interest.

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