REVIEW ARTICLE

# Role of Proteomics in the Discovery of Autism Biomarkers

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## ABSTRACT

The epidemiology of autism is continuously increasing all over the world with social, behavioural and economical burdens. Autism is considered as a multi-factorial disorder, influenced by genetic, neurological, environmental and immunological aspects. Autism is still believed to be incurable disorder with little information about the role of proteins patterns in the diagnosis of the disease. Knowing the applications of proteomic tools, it is possible to identify quantitative and qualitative protein patterns in a wide variety of tissues and body fluids such as blood, urine, saliva and cerebrospinal fluid in order to establish specific diagnostic and prognostic biomarkers. The aim of this review is to provide an overview of the various protocols available for proteomics by using mass spectrometry analysis, discuss reports in which these techniques have been previously applied in biomarker discovery for the diagnosis of autism, and consider the future development of this area of research.

**Key words:** *Autism. Proteomics. Biomarkers. Spectrometry.*

## INTRODUCTION

Autism spectrum is a lifelong developmental disorder with psychological conditions characterized by impaired social interaction and communication along with restricted and repetitive behaviours defined by classification system such as DSM-IV.[1] These signs all begin within the first three years of life. Synaptic synthesis and organization is affected by autism in the brain but its exact mechanism is poorly understood.[2]

**Epidemiology:** An increase in the prevalence of all the autism spectrum disorder (ASD) is being reported worldwide. The number of reported cases of autism increased significantly in 1990s and early 2000s. This increase can possibly be due to greater public and professional awareness,[3] changes in diagnostic practices, referral patterns, availability of services and perhaps unidentified environmental risk factors may also be implicated.[4] The boy to girl's ratio is average 4.3: 1 and is highly affected by cognitive impairment. it may be close to 2:1 with mental retardation and more than 5.5:1 in the absence of mental retardation.[5] The aged fathers cause a greater risk than older mothers possibly due to increase in mutation in older sperm.[6] It is also believed that race, ethnicity, and socioeconomic conditions do not affect the occurrence of autism.[7] It has also been reported that in twenty first century the rate of autism cases per 1000 children grew significantly in the US. Two recent studies conducted in United States found that the incidence of ASD was about 1/91 children age 3 to 17 years and 1/110 children age 8 years.[8,9]

Epidemiological research on the prevalence of ASD in Asia has also been conducted.[10] The average prevalence of ASD before 1980 was around 1.9/10,000 while it is recorded 14.8/10,000 from 1980 to the new millennium. Prevalence of ASD was found higher in children of 2-6 years old and boys had a higher prevalence than girls.[11] Estimates of prevalence were also higher in urban than rural areas. Some studies also revealed an association between an increase in prevalence with younger children, lower parental educational level, native residents and urban areas. These studies revealed that ASD is probably more common in Asia.[10] Since autism is increasing worldwide, this area needs urgent attention particularly in the Middle East including Saudi Arabia where autism had increased dramatically.[12,13]

**Etiology:** Autism is a varied neurodevelopmental disorder. Many causes have been anticipated, yet its exact pathophysiology is unknown.[14] Autism is primarily inherited, although its genetics due to complexity remain unclear[15] whether ASD is explained more by rare mutations, or by rare combinations of common genetic variants.[16] In rare cases, autism is strongly associated with agents that cause birth defects.[17]

Association between autism and environmental exposure cannot be ruled out.[5] Environmental factors that have been claimed to be the cause of autism, or may be important in future research, such as certain foods, infectious disease, heavy metals, solvents, diesel exhaust, phthalates and phenols used in plastic products, pesticides, brominated flame retardants, alcohol, smoking, illicit drugs, vaccines,[5] and prenatal stress.[18] The evidence for environmental causes is

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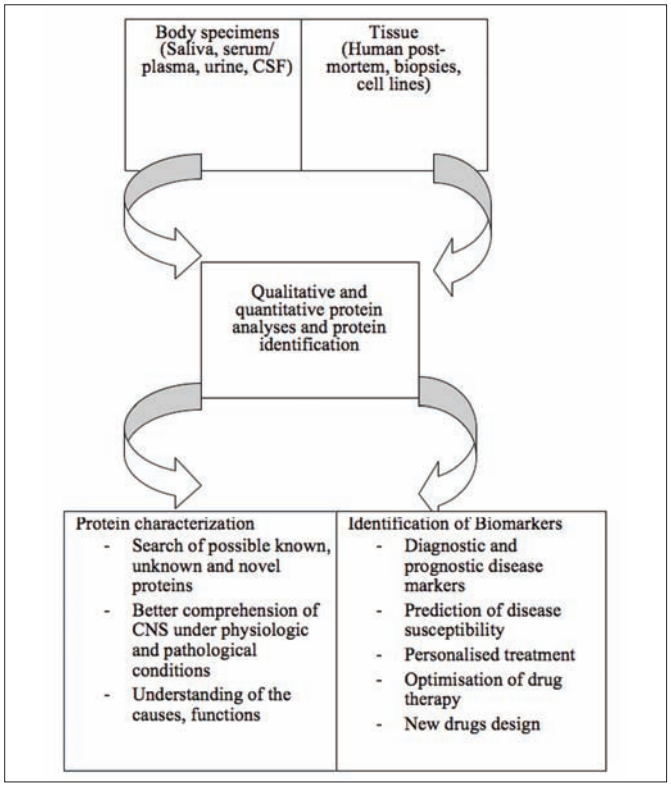


**Figure 1:** Possible proteomic studies with impact on neuropsychiatric diseases.[27]

unreliable and has not been confirmed by available studies. Although there is no known cure for autism, many treatments are available to improve core and associated symptoms.

**Proteomics:** By using proteomic techniques, it is likely to identify quantitatively and qualitatively protein patterns in a wide variety of tissues, in order to isolate particular diagnostic and prognostic biomarkers.

To understand the proteome, as shown in Figure 1, the complexities of protein-protein interactions, the structure and function of each protein will be vital for developing the most effective diagnostic tools and disease treatments. An interesting use of proteomics is using specific protein biomarkers to diagnose disease. A number of techniques including western blot,[19] immunohistochemical staining,[20] enzyme linked immunosorbent assay (ELISA)[21] or mass spectrometry[22,23] have been used to test for proteins produced during a particular disease, which helps to diagnose the disease quickly.

Quantitative proteomic profiling is becoming a widely used approach in systems biology and biomarker discovery.[23] In the past few years, reliable identification of proteins in complex biological samples has become routine practice in many laboratories across the world. Such qualitative information provides researchers with insights into the intricate structures of biological systems. However, many applications, such as disease biomarker discovery, also require the measurement of relative abundance of proteins. Modern advances in analytical instrumentation and bioinformatics now enable the relative quantitation of experiment.[24,25] Thus, it is possible to obtain both qualitative and quantitative information providing deeper and deeper insights into the origin and structure of biological systems as well as allowing global proteomic profiling for identification of disease specific biomarkers.

Proteomic-based approaches, which examine expressed proteins in tissues or cells, have great potential in the explanation of biological defects in heterogeneous neurodevelopmental disorders such as autism.[26] The proteomic approach requires limited number of tissue samples and the study can be completed in a relatively short time. Currently, these methods are available for relatively abundant proteins. The genetic defect results in either total loss of proteins or changes in molecular weight and/or isoelectric point will be detectable by the proteomic method.

The new research approach of proteomics, based on mass spectrometry, has recently been applied to psychiatric research.[22] Proteomic tools allow for an automated, technology-driven large-scale mode of examination that provides the chance to determine the whole proteome in a given tissue without prior assumptions about candidate molecules.[27] The rapid development of proteomics witnessed in recent times is due to the increasing sophistication of biological MS, improvements in bioinformatics and the magnitude of data resulting from genomic sequencing of different organisms. Indeed, proteomics is complemented by functional genomics approaches that provide complete genomic sequences, allowing for protein identification by correlation of MS measurements with sequence databases.

The assessment of biomarkers in easily accessible tissues such as saliva, blood, urine or cerebrospinal fluid (CSF) provide promising opportunities in diagnostic and prognostic processes.[20,27-29] Similar phenotypes with distinct nosological entities could be compared by assessing their differential protein patterns.

Proteomics also provides the opportunity to observe the effect of different psychotropic drugs on protein expression in post-mortem tissue, *in vivo* in patients and animal models, and *in vitro* in cell lines. Findings from proteomic research can hopefully point to new drug targets for psychiatric diseases and optimize current treatment strategies (Figure 1).

The most commonly used separation techniques[27] for proteins are two-dimensional gel electrophoresis (2D-PAGE) and high-pressure liquid chromatography (HPLC), as well as the usage of pre-coated protein chips, centrifugal filters or magnetic beads. The details and relative merits of sample preparation and protein fractionation techniques have been discussed elsewhere.[27,30,31]

**Mass spectrometry:** Mass spectrometry (MS) has been used as powerful tool for the structural characterization of proteins and peptides since many years[32] and now being widely used in autism.[27]

There are four basic types of mass analyzers used for protein studies: the ion trap (IT), time of flight (TOF), quadrupole (Q), and Fourier transform ion cyclotron (FT-ICR) devices. These are very different in design and performance, each with its own strengths and weaknesses. Two analyzers can be placed in tandem to perform two-stage mass spectrometry (tandem MS, commonly referred to as MS/MS). In MS/MS, with diverse mechanisms in different mass spectrometers, peptide ions are isolated and fragmented and the mass-to-charge ratio of the fragments is measured.[33] The most common tandem mass analyzers, able to perform peptide fragmentation, are quadrupole ion trap (IT), triple quadrupole (QqQ), quadrupole-TOF (Q-TOF) and TOF-TOF. The performance of different tandem mass spectrometers varies in terms of mass accuracy, mass resolution, robustness, and ease of operation.

In particular, two different mass spectrometer configurations have been widely used for protein and peptide analysis, either ion traps coupled to ESI sources (ESI-IT) or TOFs coupled to MALDI sources (MALDI-TOF). Both of which are 'soft' ionization techniques that enable the transfer of intact proteins and nucleic acids into the gas phase without fragmentation;[34,35] simple peptide samples may be examined with MALDI-TOF MS, while more complex mixtures may require separation using an analytical HPLC column coupled to the ESI MS systems (LC-ESI MS).

A variation of the MALDI technique, SELDI MS, has been developed. This approach is based on the retention of proteins of a sample via protein chip systems with pre-activated surfaces (e.g. with antibodies, receptors, ionic or hydrophobic material). The retained proteins are then ionized and detected by MS similar to the process using MALDI-TOF MS.[35,36] Details of MS technique and the uses of instruments are already described in many previous studies.[22,27]

After protein identification and quantification by MS, high-throughput collection of consistently high-quality data, therefore, remains a challenge in proteomics. The analysis and interpretation of the enormous volumes of proteomic data remains an unsolved challenge, particularly for gel-free approaches. Expert manual analysis is incompatible with the tens of thousands of spectra collected in a single experiment and is inconsistent. Therefore, the development of transparent tools for the analysis of proteomic data, using statistical principles, is a key challenge.[37,38]

**Peripheral biomarkers of autism:** To-date, the diagnosis of autism is solely based on the patient's history and the observation of behavioural abnormalities. No disease markers for the diagnosis of autism have been validated. A reliable protein biomarker, however, could significantly contribute to an early and more exact ASD diagnosis, a crucial pre-requisite for an early behaviour-modifying therapeutic intervention. Further-more, a diagnosis at an early stage could contribute to developing better coping strategies within families confronted with classical ASD features of a child's behaviour. It has been suggested that molecules involved in serotonin metabolism[39,40] and several cytokines and chemokines are associated with ASD.[41,42] Altered blood levels of neurotrophic factors have also been reported.[43]

Several review articles on autism spectrum disorder associated biomarkers have also appeared which examines recognized clinically available biomarkers as testing for the evaluation and treatment of ASD.[44-47] They attempted to explain the clinical significance of the findings and, where possible, explored potential treatment options.

Various screening and diagnostic tools were used to identify autism. Proteomics is likely to play an increasing role in identifying biomarkers that may assist in early diagnosis and in monitoring progression and, most importantly, response to therapy. After a successful identification of disease biomarkers proteomic technologies will hopefully overcome the obstacle of designing clinically useful and easily applicable laboratory or bedside tests.

**Proteomics studies in autism:** Protein biomarker discovery from biological fluids, such as serum, has been widely applied to disorders such as cancer,[48,49] and has more recently also been utilized in neuropsychiatric disorders with relatively clear biological causes.[50] The application of the associated technologies for the identification of protein biomarker signatures in neurodevelopmental disorders, such as autism spectrum disorder, is comparatively less well established.[51]

Several studies have demonstrated the power of proteomics in understanding disease processes and elucidating the molecular defects associated with various conditions.[52,53] However, in past years very limited studies were reported on proteomics based research on autism (Table I).

Junaid *et al*. used a proteomic approach to identify protein abnormalities due to unusual gene expression in the grey matter of eight ASD autopsied autism brains.[19] In four of eight autism brains, they have found an increase in polarity (more acidic) of glyoxalase I (Glo1) by two-dimensional gel electrophoresis. Sequencing of this more acidic protein revealed a single nucleotide polymorphism causing an AlaGlu exchange. A possible role of this gene for the aetiology of autism was confirmed by the finding of a reduced glyoxalase 1 activity in the brain lysates and a further population association study.

**Table I:** Findings for the comparison of proteomic studies for autism.

| Approach | Tissue, conditions | Proteins | Method | Ref |
|---|---|---|---|---|
| Postmortem | Total brain grey matter | Glyoxalase | 2D-PAGE, MS/MS | 19 |
| Peripheral biomarkers | Serum | ApoB100 prec complement factor H-related protein prec; complement, C1q chain prec; fibronection 1 isoform 1 preportion, cold-insoluble globulin | Spin filters, LC-ESI, MS/MS | 54 |
| Peripheral biomarkers | Serum | Peak with the m/z ratio of approx 10.38 kDa which differentiated significantly between the ASD patient and control group. | Magnetic beads, MALDI | 55 |

Corbett *et al*. analysed the serum proteome by first MS based study on peripheral markers. Group of children with autism (n = 69) were compared to typically developing children (n = 35) with similar age and gender distributions.[54] A total of five peptide components corresponding to four known proteins [Apolipoprotein (apo) B-100, Complement Factor H Related Protein (FHR1), Complement C1q and Fibronectin 1 (FN1)] were found greater for autism compared to controls. In addition, apo B-100 and apo A-IV were also higher in children with high compared to low functioning autism. Study summarized that identified proteins that might be differentially expressed in autism.

Apos are involved in the transport of lipids, cholesterol and vitamin E. The complement system is involved in the lysis and removal of infectious organisms in blood, and may be involved in cellular apoptosis in brain. It suggested that peripheral differences of immune molecules including complement that could impact indirectly on the developing brain in autism. On the other hand, the same or similar molecules within the brain might be abnormal and contribute directly to abnormal brain development and autism.

Recently, Taurines and co-workers conducted proteomic studies, using serum samples in order to primarily determine a protein pattern that can be used as biomarker, rather than to identify specific differentially expressed proteins.[55] In contrast to Corbett *et al*.[54] they analyzed whole proteins not peptides after tryptic digest-using serum protein pre-fractionation with C8-magnetic beads and protein profiling by matrix-assisted laser desorption/ionization time of flight-mass spectrometry (MALDI-ToF-MS) to identify possible differences in protein profiles in patients and controls. Data revealed three potential biomarker peaks all showed m/z ratios of approximately 4.40, 5.15 and 10.38 kDa that significantly differentiated the ASD sample from the control group. Results suggested that altered protein levels in peripheral blood of patients with ASD might represent useful biomarkers for this devastating psychiatric disorder.

## DISCUSSION

Autism is considered as severe and disabling disease like other psychiatric disorders such as schizophrenia, Alzheimer's disease and mood and anxiety disorders. The new research approach of proteomics, a systematic analysis of all expressed proteins, based on MS, has recently been applied to psychiatric research.[27] Currently available data reflects new developments and opportunities in modern autism research. Proteomic analysis of brain tissue, peripheral tissues and body fluids (blood, saliva and urine) is now a promising tool to better understand the complexity of neuropsychiatric disorders and drug effects as well as explore corresponding early disease markers.[51,56]

Despite the encouraging progress in proteomic technologies this approach is still in a developmental stage with drawback and hurdles to overcome. In general, in a given tissue, a proteome of millions of proteins could be expected, but so far only a small fraction has been detected with current proteomic methods. One reason for this is that certain characteristics of brain molecules complicate their analysis. Proteins of interest in CNS are often transmembrane and membrane-associated proteins, including ion channels G proteins and receptors.

Consideration to insoluble nature of protein molecules, proteomic methods in neuropsychiatric research have to be specially adapted. The widely used 2D-PAGE imposes clear limitations in representation and detection of these proteins, as well as for the detection of low abundance gene products, proteins of small size, hydrophobic, acidic and basic proteins, all of which often escape 2D-PAGE analyses. Furthermore, neuropeptides and proteins only exist in very low concentrations, in addition, the amount of available brain and CSF tissue is limited.

High concentration of few proteins is dominated in every type of tissue and causes hindrance in the detection of low abundance proteins by mass spectrometry. To deal with these difficulties, different fractionation and advanced procedures are adopted before mass spectrometric analysis. A sophisticated and efficient sample refractionation and the recovery of more homogenous sample fractions/sub proteomes prior to MS will finally provide valuable information data. Except adapting 2D-PAGE methods, non-gel-based techniques such as LC-ESI MS, Pre-coated protein chips, Magnetic beads are gaining in importance as they seem more suitable for the analysis of interesting protein populations in blood and brain research. These progressive methods make possible these systems to allow and detect very small protein or peptide amounts on nano scale.

Despite the difficulties and challenges, proteomic technologies provide rapid progress and immense benefits and now already create important biological data in neuropsychiatric research. Proteomic approaches allow a large-scale high-throughput qualitative and quantitative protein analysis complementing other traditional methods used in molecular genetics. Proteomics has the advantage of being relatively unbiased without a prior assumptions about differences between sample groups. It is a powerful research field that can reveal the function of so far uncharacterized proteins and generate new hypotheses to improve the understanding of the basic physiology of CNS under normal and disease conditions. In contrast to molecular genetic studies, proteomics has the great advantage of analyzing processes at the protein level, thereby possibly being closer to the pathophysiological processes underlying the clinical phenomenology of specific psychiatric conditions.

As already mentioned for several psychiatric disorders, proteomic studies present the vast opportunity to identify surrogate biomarkers in easily accessible tissue for early disease detection, perhaps disease prevention, and the differentiation of stages and similar phenotypes of different nosological entities. Furthermore, biomarkers are suitable for a personal drug-monitoring scheme. The assessment of the patients' individual protein profile before and in the course of medication might in the future allow a prediction of drug response and an adequate treatment modification. Ideally the marker recovery should be simple and non-invasive enough to perform in easily accessible tissue whose gene expression profile is similar to more inaccessible CNS tissues. In a recent study, the gene expression patterns in blood and brain were compared and significant expression similarities in whole blood and multiple CNS tissues were found.[57] Results concluded that surrogate marker search in blood is a useful tool when it has been determined that the relevant genes are expressed in both tissues.

## CONCLUSION

Proteomics research in autism is still incomplete, but important advances have been made. Protein profiling is a promising tool for the discovery and subsequent identification of proteins and peptides associated with various diseases including autism. However, knowledge is still limited regarding the sensitivity and reproducibility of various techniques for analyzing the wide dynamic range of peptides and proteins in blood.

In spite of many challenges, the impact of proteomics on clinical and biological research is growing rapidly. It seems that beyond its great current contribution to cell biology, proteomics may have a huge influence on clinical diagnosis. MS-based proteomics seems capable of detecting patterns of differentially expressed proteins in easily accessible clinical samples such as blood plasma or serum.

These types of analyses have the potential to diagnose the presence and stage of many neurological diseases; including autism. Clinical diagnosis will be further advanced with the advent of mass spectrometers with higher mass accuracy, dynamic range and resolution, and with the ability to identify specific sequences of diagnostic analytes and the use of accurate quantification procedures.

In proteomic biomarker research it is certainly necessary to verify preliminary mass spectrometric findings in independent, sufficiently large sample sets and to validate results with different methods such as Western blot or ELISA to confirm relevant proteomic findings. Furthermore, pilot studies may be used to calculate false-positive rates, allowing for better confidence in protein identification.

Proteomics based MS approach provides the opportunity to analyze and identify the complexity and dynamics of pathophysiological processes in neuro-psychiatric disorders at the protein level. Understanding the molecular mechanisms of synaptic transmission, protein interactions and signaling cascades will provide crucial insights into brain diseases, allowing the search for diagnostic and prognostic biomarkers as well as new therapeutic targets.

In conclusion, proteomics is still a new investigational approach that can be used to identify biomarkers for neuropsychiatric disorders including autism. Future studies will help to determine the role of this approach in the diagnosis and differential diagnosis of neuropsychiatric disorders.

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