Biomarker Detection of Neurological Disorders through Spectroscopy Analysis

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Abstract

Background: The accurate and efficient diagnosis at the early stages of neurological disorders is the key feature for effective treatment and productive research for finding out new ways to combat diseases. It is essentially true for neurological disorders where there is no effective cure, but only treatments are available for slowing down the procedure. Neurological disorders reveal only non-specific clinical symptoms of mental changes/decline starting from a few days to decades after initiation which goes very challenging to differentiate even at later stages when the disorder becomes way aggressive. Despite the fact of having great need, the current availability of diagnostic tests is unable to diagnose different forms of neurological disorders.

Aim: The aim of this review is to explore the application of Raman spectroscopy (RS) and mass spectrometry (MS) for the detection of changes in the biochemical composition of cerebrospinal fluid (CSF), blood serum, urine, and saliva. The approach will be based on probing biochemical composition of a biofluid totally using the spectroscopy analysis with advanced statistics. The power of high differentiation method will promote the translation of RS from mere a laboratory technique to clinically useful tool. Demonstration of biochemical information derived from RS from CSF, blood, saliva, and urine that will yield accurate and selective detection of neurological disorders. It will also provide diagnostic and prognostic indicators and will also play a significant role in the development of personalized medicine.

Conclusion: Combination of RS and other techniques such as MS and advanced molecular techniques will allow differentiating CSF, blood serum, saliva, and urine samples of common neurological disorders from normal control patients with sensitivity and specificity close to 95%.

Clinical Significance: The outcome of the research methods explained will demonstrate an accurate discriminative method which will be based on RS for the detection of neurological disorders. The findings of research in the review will furthermore confirm that if the biomedical application of the methods will either allow to distinguish and detect biomarkers of neurological disorders from biofluids and is it a viable clinical tool that can be used for an accurate diagnosis through a simple test.

Keywords: Biological fluids, biomarkers, diagnosis, neurological disorders, spectroscopy analysis

Introduction

Despite the considerable advances, neurological disorders remain the world’s leading cause of disability and hospitalization. Biomarkers, on the other hand, are multifaceted indicators of pathological disorders. Potential biomarker discovery from biological fluids has been widely applied to several disorders. Therefore, the study of neurological disorders can benefit by the use of biomarkers because of inherent disease heterogeneity. The application of associated techniques for the identification of biomarker signatures in neurological disorders through spectroscopy analysis is less well established. Hence, we aimed to standardize by describing together multiple experimental approaches for sample preparation, instrumentation, acquisition
parameters, and processing of data related to neurological disorders. Using the standardized approach, high-quality data for biological fluid analysis will be generated. Furthermore, by investigating the targeting biomarkers either exploratory or diagnostic, the desired analytical goals for classification for diagnosis, pattern finding, and biomarker investigation in neurological disorders will be accomplished. Furthermore, in this field by biomarker discovery and validation in neurological disorders, future development of this area is being explored.

Neurological disorders are diseases of the central and peripheral nervous system including the brain, spinal cord, peripheral nerves, cranial nerves, nerve roots, neuromuscular junction, autonomic nervous system, and muscles. Neurological diseases seem to be subtle in their clinical manifestations and are mostly prone to misinterpretations. They are becoming more prevalent, and burden is increasing as the world’s population ages.\(^1\)

Prevalence and incidence information of neurological diseases plays an imperative role in the assessment of burden and furthermore treatment planning. However, epidemiological studies associated with the diseases face challenges, first, because the diagnostic criteria are prone to misclassification. Second, the diagnosis is not only based on clinical phenotype but also dependent on sophisticated technologies such as magnetic resonance imaging or measuring biomarkers from cerebrospinal fluid (CSF) and serum that require specialists skills as well. Third, the variable long gaps between the occurrence of disease and manifestation of symptoms to get visible and observable among patients. In addition to it, in vivo pathological confirmations are very difficult. For this reason, there is a guideline which is being developed that consists of a checklist and recommendations known as “the standards of reporting of neurological disorders (STROND)” for the facilitation of improved reporting of prevalence studies of neurological diseases.\(^2\)

Mental and neurological illnesses together encompass disorders of the brain. According to the latest estimates, disorders of brain account for 13% of global disease exceeding cancer and cardiovascular diseases.\(^3\) Furthermore, according to the World Health Organization [WHO], 2008, mental illnesses burden the world more than any other chronic diseases. Many epidemiological studies suggested that about one-third of the adult population suffers from mental disorder every year.\(^4\)

More than 6 million people die due to stroke and more than 50 million people have epilepsy while 47.5 million people are suffering from dementia and 7.7 million cases are reported every year. While globally prevalence of migraine accounts for more than 10% of adult population in western countries.\(^5\) Approximately 45 million cases of neurological disorders were reported in the UK. The most prevalent was anxiety disorders, headache, sleep disorders, and somatoform disorders and the most costly disorders were psychotic disorders, dementia, mood disorders, addiction, and anxiety disorders.\(^6\) Due to burden of disease, the 1990 Global Burden of Disease (GBD) study, a time-based metric was used to assess the disease that measures disability weighted by severity (years of healthy life lost because of disability or years lost due to disease [YLD]) and premature mortality or years of life lost (YLL). Both of them together are called as disability-adjusted life years (DALYs).\(^7\) According to the GBD report, due to neurological and psychiatric disorders, 13% of patients lived with DALYs, where only 33% of years lived with disability.\(^8\) The need for updated projections on burden of disease and mortality by its cause and region is being provided by the WHO, and Table 1 shows the global DALYs neurological disorders for the years 2005, 2015, and 2030. The WHO uses the terms DALYs for the measurement of disease burden and YLDs and YLLs tell about the premature death due to disability. Neurological disorders such as epilepsy, Parkinson’s disease (PD), dementias, migraines, cerebrovascular disease, and multiple sclerosis contribute to 92 million DALYs in 2005 (percentages shown in Table 1) which is projected to increase up to 103 million in the year 2030. Moreover, Figure 1 indicates the global DALYs for neurological disorders for the years 2005, the regions which are included DALYs/100,000 population for neurological disorders by the WHO region, and mortality stratum for Africa which was 1536 DALY/100,000 population, America 1214, Southeast Asia 750, Europe 2920, East Mediterranean 1089, and Western Pacific 1543 DALY for neurological disorders.\(^9\)

Classification

Neurological disorders include epilepsy, neuroinfections (bacterial and viral), brain tumors, cerebrovascular diseases, Alzheimer disease and other dementias, migraine and other headache disorders, multiple sclerosis, PD, and traumatic disorders due to head trauma. Many viral (i.e., enteroviruses, human immunodeficiency virus, West Nile Virus, and Zika), bacterial (i.e., Neisseria meningitidis and Mycobacterium tuberculosis), fungal (i.e., Aspergillus and Cryptococcus), and parasitic (i.e., Malaria and Chagas) infections can affect the nervous system. Figure 2 shows the detailed general most common classification of neurological disorders.

Clinical manifestations and investigations

The main causes of neurological disorders for different disorders for instance, for multiple sclerosis it could be trauma, skull fracture, spinal cord injury, (Down’s syndrome) is caused by

Figure 1: The incidence and prevalence based on several global research\(^{9}\)
immunological factors, genetic disorders, stroke is caused by cerebrovascular accident, meningitis is caused by infection, neoplasia, diabetic neuropathy they are the metabolic disorders, heavy metal encephalopathies are caused by environmental factors. The chronic pain is frequent with many disorders. As the disease progresses, 1) altered sensation, i.e., hyperalgesia and allodynia, 2) altered emotion, i.e., reward deficit syndrome and depression anxiety, 3) altered cognition/integrative processing, i.e., memory, and 4) altered pain modulation, i.e., increased sensitivity, because of these symptoms it diminishes gradually the response to analgesics. Moreover, lifestyle factors such as tobacco use, less physical activity, obesity, alcohol, and certain diet such as Vitamin D have shown to develop MS, and these all contribute to the development of neurological diseases.\(^{(10-13)}\)

Specific tests for clinical neurological examination include mental status examination which includes Glasgow Coma Scale, cranial nerve examination, examination of motor system, deep tendon reflexes such as masseter, biceps, triceps, knee tendon, ankle jerk, and planter through reflex hammer, sensory system examination includes provoking sensations of fine pain, touch, and temperature, and finally, cerebellum testing is examined by finger to nose test, assessment of gait, nystagmus, and intention tremor, while neuroimaging (brain scans), angiography, biopsy, CSF analysis, computed tomography (CT) scans, discography, electroencephalography, electromyography, single-photon emission CT (SPECT), ultrasound imaging, and magnetic resonance imaging also help in coordination with clinical tests to diagnose the disease.

Neurological disorders are screened by neurological screening examination which is a basic procedure for neurological complaints assessment. It includes six areas:

- Mental status: By assessing the normal orientation to place, time, space, and speech.
- Motor: For checking drift, tone, and heel and toe walking.
- Sensory: For cold and vibration.
- Reflexes: To tap an instrument above a nerve to check a reflex which is emitted by nerve.
- Coordination: By observing patient’s walk and finger to nose testing.
- Cranial nerves: Checking the eyes by ophthalmoscope and assessment of facial muscles function and strength.

**Need of biomarkers**

Biological markers are defined as biochemical, cellular, or molecular alterations that can be measured in biological media, i.e., human cells, tissues, or fluids. Biomarkers include technologies and tools that help in the understanding of prediction, cause, diagnosis, regression, progression, and outcome of the treatment of disease.\(^{(14)}\) Figure 3 shows, according to Perera et al., classified biomarkers in four categories on the sequence of events on exposure to disease such as predictive, diagnostic, prognostic, and monitoring. Table 1:

<table>
<thead>
<tr>
<th>Neurological disorders</th>
<th>Number of DALYs 2005 (%)</th>
<th>Number of DALYs 2015 (%)</th>
<th>Number of DALYs 2030 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epilepsy</td>
<td>7308 (0.50)</td>
<td>7419 (0.50)</td>
<td>7442 (0.49)</td>
</tr>
<tr>
<td>PD</td>
<td>1617 (0.11)</td>
<td>1762 (0.12)</td>
<td>2015 (0.13)</td>
</tr>
<tr>
<td>Dementias</td>
<td>11078 (0.75)</td>
<td>13540 (0.91)</td>
<td>18394 ((1.20)</td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>1510 (0.10)</td>
<td>1586 (0.11)</td>
<td>1648 (0.11)</td>
</tr>
<tr>
<td>Migraine</td>
<td>7660 (0.50)</td>
<td>7736 (0.52)</td>
<td>7596 (0.50)</td>
</tr>
<tr>
<td>Cerebrovascular disease</td>
<td>50785 (3.46)</td>
<td>53815 (3.63)</td>
<td>60864 (3.99)</td>
</tr>
</tbody>
</table>

DALYs: Disability-adjusted life years, PD: Parkinson’s disease

**Figure 2: Classification of neurological disorders**

**Table 1:** Number of global DALYs for neurological disorders for the years 2005, 2015, and 2030\(^{(7)}\)
and monitoring of the pharmacodynamic response of drugs after the delivery of drugs. Studying these “biomarker” may shed light on the underlying pathological pathways involved and indicate a potential target for therapeutic treatment. It may also help to determine treatment response on the proposed target and whether the drug has altered the course of the disease.

**Biomarkers in neurological diseases**

Neurological disorders have a wide range of techniques to gain information which may involve biological media such as CSF, serum, urine, saliva, or measurements such as brain imaging.

Table 2 shows the list of various neurological disorders such as multiple sclerosis, Alzheimer’s disease (AD), PD, epilepsy, stroke, and brain tumors, already identified biomarkers associated with them, their methods of identification such as enzyme-linked immunosorbent assay (ELISA), isoelectric focusing combined with immuno blotting, indirect immunohistochemistry, western blotting, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), MS, microarray analysis, quantitative polymerase chain reaction, surfaced-enhanced laser desorption/ionization (SELDI)-TOF, Raman spectroscopy (RS), HC, nuclear magnetic resonance-based metabolomics, and many more. The basic function of identified biomarkers is in prediction, diagnosis, prognosis, and treatment.

**Methods of Collecting Samples**

**Sampling, transportation, and storage of biological fluids**

**Blood**

Blood sample will be collected by single venepuncture from patients arm complying with the standard procedure of the local hospital about 3 ml. An EDTA vacutainer tube will be collected at baseline and stored in −70°C freezer. During visit, blood will be collected and processed for serum separation within 1 h. Serum will be stored in single aliquots −70°C freezer. For Raman analysis, CSF droplet has to be dispended on to the calcium slide to allow it to dry for 30–45 min (can be varied depending on the room temperature). After the slide preparation and drying procedure, it has to be stored for further analysis.

**Urine**

Urine specimens will be collected in 50 ml sterile containers. Moreover, they will be stored in capped containers at room temperature between analyses.

**CSF**

For disinfection of the skin, chlorhexidine denatured ethanol 70% will be used. CSF samples will be taken through lumbar puncture. For sampling, 1–5 mL of CSF dripped through air into a 15 mL polypropylene Falcon tube. CSF will then be centrifuged at 4°C. After centrifugation, supernatant will be transferred to a 15 mL polypropylene falcon tube and divided into 0.5 mL aliquots, placed on dry ice within 30 min of sampling, and transferred to −80°C for storage within 60 min of sampling. For Raman analysis, CSF droplet has to be dispended on to the calcium slide to allow it to dry for 30–45 min (can be varied depending on the room temperature). After the slide preparation and drying procedure, it has to be stored for further analysis.

**Laboratory assessment**

Blood, saliva, CSF, and urine samples will be collected and in several aliquots according to the standardized procedure and protocols decided by the hospital. All samples after separation and storage will be evaluated in double-blind procedure in which individual assays will be performed at the time of study and at the same time to reduce variability. Figure 4 explains the various methods of protein analysis from biofluids such as high-performance liquid chromatography (LC), MALDI/SELDI, ELISA, two-dimensional (2D)-polyacrylamide gel electrophoresis, MS/MS, and isotope-coded affinity tags (ICAT)/isobaric tags for relative and absolute quantitation (iTRAQ). Apart from all these common techniques which are being used frequently, our study will focus on RS and MS to analyze the panel of candidates.

**Biomarkers Discovery**

**RS**

RS is a powerful analytical technique based on inelastic scattering of light by vibrating molecules that are able to provide chemical fingerprints and can measure chemical composition of biological fluids, biological samples cells, and tissues. It has the ability to detect biochemical changes at molecular level and therefore can be utilized for diagnostics, prognosis, or for development of new therapies. It has also been applied to diagnose many diseases,
### CSF, serum, saliva, and urine-based biomarkers identified with several techniques in neurological disorders

<table>
<thead>
<tr>
<th>Disease</th>
<th>Identified biomarkers</th>
<th>Methods of identification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Aβ42, t-tau, p-tau/p-tau/A β42</td>
<td>MS</td>
<td>[20,21]</td>
</tr>
<tr>
<td>PD</td>
<td>Alpha-synuclein, neurofilament light chain (NFL), oxidative stress markers, amyloid and tau-proteins</td>
<td>MS</td>
<td>[22]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>alpha-synuclein, NCAM-1, superoxide dismutase 1, Gelsolin, CD-133-enriched membrane particles</td>
<td>MS</td>
<td>[23]</td>
</tr>
<tr>
<td>Stroke</td>
<td>GFAP, MBP, FABPs, Tau protein, neurofilament light protein, visinin-like protein-1</td>
<td>MS, ELISA</td>
<td>[24]</td>
</tr>
<tr>
<td>Brain tumors</td>
<td>Cancer cells, hHCG and AFP, Apolipoprotein, CD27, AT III, chemotacttractant, CCL13, CCL12 and IL10, IgG, EGFR, VEGF, PGD2, CYFRA 21-1, miR-21 and miR-15b, NSE, GDF15</td>
<td>Proteomic analysis, microRNA analysis</td>
<td>[25]</td>
</tr>
<tr>
<td>Serum-based biomarkers and the techniques used for their identification in neurological disorders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Humoral factors adhesion molecule sVCAM-1, sICAM-1, sL-selectin. Cellulr factors intracellular cytokine: IFN-y CD4, IL-4, IL-13, TNF-a+CD4+cells, IL-17, IL-12, IL-23, Protein: SPP1, anti-EBNA, KFLC, BDNF, KIR4.1 (KCNJ10), Complement regulator factor H, MMP-9, mtHSP70. Metabolite: Nitric oxide (NO) metabolites. DNA: Glicipan 5 (GPC5), HLA Class II alleles</td>
<td>ELISA, PCR, 2DE, 2D-IB and Competition Assay, SECARS, LC-MS/MS,</td>
<td>[16,19,26,27]</td>
</tr>
<tr>
<td>AD</td>
<td>Complement factor H, a2-microglobulin, Abeta42</td>
<td>ELISA</td>
<td>[20,28,29]</td>
</tr>
<tr>
<td>PD</td>
<td>α-Synuclein, DJ-1, Uric Acid, ApoA1, Epidermal growth factor</td>
<td>Immoassays, 2-DGE, LC-based high-resolution tandem MS</td>
<td>[30]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>alpha-synuclein, fibronectin, HSP70, Bcl-2</td>
<td></td>
<td>[31]</td>
</tr>
<tr>
<td>Stroke</td>
<td>S100-β, N-acetylaspartate</td>
<td>MS</td>
<td>[32]</td>
</tr>
<tr>
<td>Brain tumors</td>
<td>S100A8, S100A9 and CXCL4, microvesicles</td>
<td>SELDI-ToF MS, ELISA</td>
<td>[32-34]</td>
</tr>
<tr>
<td>Serum-based biomarkers and the techniques used for their identification in neurological disorders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>sHLA</td>
<td>ELISA</td>
<td>[35-37]</td>
</tr>
<tr>
<td>AD</td>
<td>salivary p-tau and/or the p-tau/t-tau</td>
<td>ELISA</td>
<td>[38]</td>
</tr>
<tr>
<td>PD</td>
<td>TNF-a, asynuclein, CCL10, interleukins, Sicam1, Clusterin</td>
<td>ELISA</td>
<td>[39]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>Nesfatin-1, inflammatory biomarkers</td>
<td>Immunochemistry and ELISA</td>
<td>[23,40]</td>
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<tr>
<td>Stroke</td>
<td>NSE</td>
<td>NSE EIA kit</td>
<td>[41]</td>
</tr>
<tr>
<td>Brain tumors</td>
<td>FRAP, protein thiols, MBD3L2, KRAS, STIM2, DMX1L2, ACRV1, DMD, CABLES1, TK2, GLTSCR2, CDKL3, TPT1, DPM1</td>
<td>Spectrophotometric, qPCR,</td>
<td>[42]</td>
</tr>
<tr>
<td>Urine-based biomarkers and the techniques used for their identification in neurological disorders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple sclerosis</td>
<td>Neopterin, urinary kappa and lambda FLC, NO metabolites, MBPLP, higher urinary aluminum and iron (UCHL1)</td>
<td>ELISA, HPLC, MS</td>
<td>[17,35,43]</td>
</tr>
<tr>
<td>AD</td>
<td>AD7c-NTP, Urinary F2-isoprostanes</td>
<td>MS, ELISA</td>
<td>[44,45]</td>
</tr>
<tr>
<td>PD</td>
<td>Cortisol, 11-deoxycortisol, 21-deoxycortisol, histidine, urocanic acid, imidazole acetic acid, hydroxyphenylacetic acid</td>
<td>LC-MS-based metabolomics</td>
<td>[46]</td>
</tr>
<tr>
<td>Epilepsy</td>
<td>mTOR pathway</td>
<td>Western blotting</td>
<td></td>
</tr>
</tbody>
</table>

(Contd...)
Diagnosis of neurological disorders

Khan, et al.

Table 2: (Continued...)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Identified biomarkers</th>
<th>Methods of identification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke</td>
<td>Transferrin, hemopexin, albumin, transthyretin, a2-HS-glycoprotein, Gc-globulin, a1-antitrypsin, kallikrein-binding, alphalipocalin (NGAL), and VEGF</td>
<td>2DE, CE-MS, NMR-based metabolomics</td>
<td>[47]</td>
</tr>
<tr>
<td>Brain tumors</td>
<td>MMP-2, MMP-9, MMP-9/neutrophil gelatinase-associated lipocalin (NGAL), and VEGF</td>
<td>ELISA, HC</td>
<td>[48]</td>
</tr>
</tbody>
</table>


sVCAM-1: Soluble vascular cell adhesion molecule-1, sICAM-1: Soluble intercellular adhesion molecule-1, sL-selectin: Soluble L-selectin, SPP1: Osteopontin, anti-EBNA: Antibodies to Epstein–Barr virus nuclear antigens, 2DE: Two-dimensional electrophoresis, 2D-immunoblotting and competition assay, SECARS: Surface-enhanced coherent anti-stokes Raman scattering microscopy, LC-MS/MS: Liquid chromatography tandem–mass spectrometry, mHSP7: Antibody against mitochondrial heat shock protein 70

NSE: Neuron-specific enolase, evaluated FRAP: Ferric reducing antioxidant power, Shla: Saliva soluble HLA


such as diabetes, different types of cancer, atherosclerosis, Alzheimer’s, and PD.[49] It provides specific information on macromolecules, i.e., nucleic acids, proteins, and lipids.[50,51] Their structure, composition, and conformation which is unique to each macromolecule which therefore provides total biochemical state like finger printing of molecule.[52-54]

Sample preparation is mostly simple and reagent free, and material tested with a Raman can be low as or femtoliters or picograms. A Raman spectrum has several narrow bands which are a vibrational signature of the material being tested.[55,56] RS monitors the small changes in the overall biochemical composition of a sample biofluids, tissues, or cells.[57] It has the potential to be a powerful diagnostic tool for neurological disorders. It also reveals very little interference from water and this feature makes it a powerful tool for characterizing blood, its derivatives, and other body fluids.[58,59]

The basic setup requires traditional confocal light microscope with Raman spectrometer consisting of laser as a monochromatic light source that has an function of the separation of the elastically Rayleigh scattered light of the sample from inelastically scattered light, a spectrograph, and a detector. Raman spectra can be collected in pre-programmed point-mapping mode of Raman microscope. Spectroscopic mapping permits the resourceful collection of information about intrinsically heterogeneous samples. The laser beam is focused on the sample using the microscopic scanning technique, and hence, the scattered light that is carrying valuable spectroscopic information gets registered. After that, the inelastically scattered light from every point of sample is projected onto charge couple device.[57] When studying biological fluids, it is essential to use an optimal excitation wavelength for RS.[60] Thus, the unique technique and inherent specificity of RS allows the fingerprinting of each analyte and hence eliminates the comprehensive sample preparations which are attractive for identification and detection of different chemical and biological samples.

The mechanistic heterogeneous nature of neurological disorders creates difficulties in diagnosis, disease monitoring, and new therapy development. The discovery of biomarkers may address these issues. RS can extract differences between diseased and healthy biological samples and therefore may be a promising tool for identifying spectroscopic signatures acting as biomarkers in the CSF, serum, urine, and saliva of neurological patients. RS has the potential to be a powerful diagnostic tool. It has been reported that the composition of proteome in blood changes with AD progression. It gives strong basis that RS can be used for monitoring changes in the biochemical composition of blood. A diagnosis can then be made based on a comparison of the spectroscopic changes in blood in a library of Raman spectroscopic signatures. In the early stages, many types of dementia show only non-specific symptoms. For example, in PD patients who eventually develop dementia, the symptoms start as mild deficits in cognitive function and it progresses over time to the symptoms that are recognizable as mild cognitive impairment similar to AD. Still, there is no single biomarker or cognitive test that can conclusively distinguish between a person having PD dementia and AD. Thus, the development of an efficient and selective test to detect the presence of an AD biomarker signature in blood would have tremendous utility. Therefore, analyzing the panel of biomarkers allows for AD detection up to approximately 2 years before disease onset. Each single panel of biomarkers has a different sensitivity and specificity profile. A combined implementation of these panels can significantly improve the differentiation power of the diagnostic method. The emerging advances in vibrational spectroscopy and advanced statistics offer a real opportunity
for probing multiple biochemical markers of disease through their overall spectroscopic signature.\textsuperscript{[61,62]}

**Mass spectrometry (MS)-based method**

In MS-based analysis of proteins, MS is paired with 2DGE, and LC with either electrospray ionization (ESI) MS, and protein chips with one particular type of MALDI or MALDI which are together called as SELDI. MS-based quantitative proteomics uses chemical reactions for the introduction of isotopic tags at proteins called ICAT and iTRAQ. CSF proteome is being investigated by ICAT analysis in which it generates the mass signatures that have the ability to identify sample origin and gives accurate quantification, and hence, it compares two proteomes simultaneously. Moreover, LC-MS/MS can also be used actively for comparative proteomics. Zhang et al. used micro-LC-MS with ICAT labeling and identified more than 300 proteins in Alzheimer’s patients.\textsuperscript{[63]} Multiple reaction monitoring-MS along with isotope-labeled QconCAT is also being used for quantification of proteins.\textsuperscript{[64]}

**Biomarkers verification and validation**

Once the list of panel of candidates of prospective biomarkers will be identified, the validation tests should be followed for potential clinical diagnosis. Setting up supplementary analytical system for the validation of biomarkers is recommended because of the long experimental cycle of spectrometry-based methods. Immune-based assays such as western blotting, ELISA, and radioimmunoassay are considered ideal for validation of biomarkers.

ELISA is a biochemical technique used in immunology for the detection of antibody or antigen in a sample. It is used as a validation/diagnostic tool in medicine pathology. There are two variations for this method: First, ELISA can be used to detect the presence of antigens that are recognized by an antibody, and second, it can be used to test for antibodies that recognize an antigen. Western blotting has the principle of separation of a mixture of protein on polyacrylamide gels which are then transferred by electrophoresis onto NITROCELLULOSE sheets. The sheets are then probed with antibody specific for the protein of interest. The “blots” are then incubated with secondary antibody coupled to enzyme. The blots are developed with substrates that form insoluble colored complexes. This technique is used for immunogenetics, molecular biology disciplines, and validation of biomarkers. Furthermore, “omics” technologies are also used for identification, quantification, and in the diagnostic and prognostic approaches of biomarkers validation.

**Practical implications and conclusion**

The ultimate goal of this review was the development of biomarkers which allowed the identification of neurological disorders. The several advantages of RS of biofluids for disease detection includes, possibility to profile spectral disease-related changes of fluid composition, and the analysis methods that are suitable for automation. Yet, several challenges remain to be conquered, which includes the integration of these techniques into clinical practice. Moreover, close collaboration of clinicians with spectroscopy technicians will turn the use of spectroscopy to biofluid classification into a valuable diagnostic and screening tool in clinical practice. However, large multicentered randomized control studies with the gold standard of sample handling protocols and current diagnostic methods will help in the validations of biomarkers.

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