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The Importance of Proteomics in Saliva and Tears as Potential Non-invasive Methods for Identifying Biomarkers in the Prognosis and Diagnosis of Multiple Sclerosis Patients

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Abstract

Over the past two decades, the advancement of analytical examinations like proteomics for investigating neuronal-related biomarkers has emerged as one of the most important and effective tools for clinical evaluation and prognosis. However, there remains an unmet need in this area. With advances in quantifying methods and detection in omics research, including proteomics and metabolomics, the close association of the salivary glands and tears with the nervous system has become increasingly evident. As noninvasive specimens, saliva and tears serve as attractive substitutes for neuronal biomarkers, containing numerous proteins and metabolites that represent various ailments in neurodegenerative illnesses like multiple sclerosis (MS). These noninvasive biomarkers might potentially correlate with susceptibility, severity, and pathogenesis of neurological disorders and could be utilized in early diagnosis and prognosis. Therefore, tear and salivary proteomics present novel insights into understanding disease progression and offer personalized treatment options with greater sensitivity. This approach helps highlight the most relevant experimental outcomes related to tear and salivary biomarkers for multiple sclerosis.

Keywords: Proteomics, Metabolomics, Multiple Sclerosis, Tears, Saliva

1. Introduction

Over recent decades, proteomics has achieved enormous success in identifying protein biomarkers. Recent advancements in equipment have facilitated the investigation of the human biological fluids proteome (1), serving as a primary source for disease-related biomarker discovery (2). Among human specimens, blood, urine, cerebrospinal fluid (CSF), saliva, and tears are commonly used in proteomic analysis to establish protein biomarker profiles for human diseases, particularly central nervous system (CNS) disorders (3). Among human biological fluids, serum is the most commonly analyzed because of its noninvasive sampling and reliable information content on human disorders. Additionally, cerebrospinal fluid (CSF) is routinely used for diagnosing specific neuronal pathologies (1), which is critical for identifying and detecting potential and sensitive biomarkers in CNS disorders like multiple sclerosis (MS) (4). However, the identification of protein biomarkers in CSF is limited due to the invasive nature of the lumbar puncture procedure, the risks associated with repeated lumbar punctures, and the potential for blood contamination (5). Therefore, CSF is not recommended for routine proteomic assessment. The use of biomarkers collected

through non-invasive means serves as an attractive alternative for detecting neuronal disorders like MS (6). Multiple sclerosis is a recurrent and progressive multifactorial chronic inflammatory-autoimmune and neurodegenerative disorder of the CNS (7), causing demyelination (8) due to complex interactions among genetic and environmental risk factors (9). MS presents with neurological deficits, including sensory and motor function loss, attributed to demyelination and subsequent axonal damage (10, 11). Currently, due to the lack of specific diagnostic methods, an inadequate understanding of the disease's causes, and the absence of specific biomarkers for MS, there are numerous challenges in diagnosing, treating, and monitoring MS patients. This leads to difficulties in the diagnostic process, disease management, and a high rate of misdiagnosis (5, 8). At present, the diagnosis of MS is based on the McDonald diagnostic criteria, which include clinical examinations, such as (a) clinical evaluations, (b) magnetic resonance imaging (MRI), and (c) detection of oligoclonal bands (IgG) in CSF (12). These criteria currently allow for the diagnosis of MS (9, 13). However, the low specificity of MRI and other diagnostic tests, as well as the unpredictable course and prognosis of MS, means that none of the present diagnostic criteria can definitively recognize MS. Despite many studies, there are still many unknown aspects of MS that need further evaluation to reflect the probable pathological processes involved in the development of the disease (13, 14). Hence, these findings indicate a fundamental need to identify biomarkers involved in the prognosis and diagnosis of MS. They emphasize the urgent requirement for the development of an authentic diagnostic solution, like proteomics, with high specificity and sensitivity that would be accessible to both medical staff and patients (8). A hallmark of these research efforts is the identification of viable molecular biomarkers in biological fluids, which could aid in the differential diagnosis, prognosis, and therapy of various MS phenotypes (13). Previous studies suggest that protein components are integral to cellular functions, with diverse cellular signaling activities. Using advanced proteomics technologies, such as biosensors for MS biomarker identification, offers significant advantages (8), and has led to the promising investigation of protein biomarkers in human body fluids (2). Proteomics is a key technique for investigating the human proteome, which includes the complete set of cell-expressed or secreted proteins in human body fluids. It involves characterizing protein structures and specific functions in physiological and pathological processes (15). As a rapidly advancing field in molecular biology, proteomics has the potential to meet the unmet need for discovering molecular biomarkers (13) to detect human pathological disorders and their processes (16). Among human specimens, tears (17, 18) and saliva (19) are recognized as suitable noninvasive specimens that contain proteins which might serve as potential biomarkers for MS. However, the development of practical and noninvasive detection methods for neuronal biomarkers, as crucial tools for clinical use and prognosis evaluation, remains an unmet need (20). Most critically, the absence of verified clinical biomarkers is hindering the optimal diagnosis and treatment of MS (5).

The investigation of novel specific biomarkers through advancements in proteomics techniques offers the potential for targeted and personalized therapy, allowing a proactive approach to managing neuronal diseases (5). Proteomic analysis of clinical specimens to assess the diversity and abundance of proteins is a powerful tool for identifying potential biomarkers associated with the susceptibility, severity, and pathogenesis of MS (21). Current research efforts are focused on investigating sensitive disease biomarkers to provide accurate diagnosis, prognostic information, and effective monitoring of disease severity. Therefore, the proteomic evaluation of saliva, salivary glands, and tear samples in CNS abnormalities (including MS) can identify novel biomarker candidates in neuronal disorders (22). These samples should be used regularly instead of CSF or CNS tissue for proteome-based biomarker discovery (23, 24). These biomarkers can be used as predictors of axonal damage in diagnosis or as indicators of recurrence of attacks, nerve damage, and disease progression. The scope of this current review is to investigate and reconcile the gathered information on the importance of protein profiles in saliva and tear fluid proteomics assessments as powerful tools in the diagnosis of MS. The aim is to encourage further experiments to identify and validate more authentic biomarkers (biological parameters) for MS diagnosis. The research selection was conducted by focusing on related experimental papers that provided findings on the use of salivary and tear diagnostic proteomic biomarkers for the initial diagnosis of MS, using search engines like Web of Science, PubMed, and Google Scholar. The newest findings from each research paper were carefully examined to highlight the prime role these biomarkers play in salivary and tear diagnostics in MS disease.

1.1. Saliva

Currently, proteomics experiments identify body fluids as rich sources of pathological biomarkers (4).

Saliva is a unique specimen with specific proteins and metabolic compositions that have clinical applications for disease diagnosis. It is secreted from the submandibular, sublingual, and parotid glands, which are under the direct innervation of the parasympathetic nervous system (25, 26). Previous research findings support the notion of the conservation of the salivary proteome among human subjects. In earlier studies, evaluating a small number of salivary discriminating proteins (< 10%) out of over 900 proteins involved in tissue metabolism, immunity, and regeneration enabled the distinction among participants (27). Several sensitive attributes of saliva have verified its value as an efficient substitute sample in neurological ailments. Previous research has reported the role of salivary protein profiles in many aspects, such as prognosis and diagnosis evaluation, and even for potential age and gender determination in forensic contexts, including race/ethnicity (28). Additionally, a large variety of diseases can modulate the salivary proteome composition, which may also be detected for forensic purposes (29, 30). On the other hand, saliva sampling is a simple, painless, and non-invasive procedure with no undesirable side effects, requiring less sampling process without expert training (31, 32) Figure 1.

Moreover, the proteome content of saliva has a high overlap with blood sample proteins (26, 33). Furthermore, as a beneficial source of evidence for detecting immune-mediated inflammatory disorders, saliva is an incredible specimen for reliable assessment and storage (29, 34). Thus, saliva can be considered a reliable noninvasive alternative sample in some neuroproteomics research and offers a novel, naturally accessible physiological fluid that can be assessed by different analytical assays (35). However, some blood or CNS protein biomarkers do not present in saliva, and disease-related biomarker candidates may not be secreted into it (36). Nevertheless, with progress in omics detection and quantification techniques such as genomics, proteomics, and metabolomics, saliva has been verified as a good source of neuronal biomarkers (31). Salivary proteome examination has progressively expanded into various biomedical areas, including medicine, molecular biology, and genetics (37, 38). Following the advent of omics experiments, research focused on saliva analysis has significantly increased, although the human salivary proteome has only been partially characterized so far (34). The unique properties of saliva, such as its non-invasive nature and ease of replication on a large scale, make it an excellent choice for large-scale clinical trials (39). Additionally, the cost-effectiveness of manufacturing evaluations related to the development of most biosensors makes them attractive for low-resource settings (39). Consequently, the direct identification of saliva biomarkers may enable the early diagnosis of abnormalities, leading to timely treatments (39).

The use of these protocols could lead to simpler, earlier, and non-invasive diagnoses, potentially improving the lives of many patients and minimizing the social and economic burdens of these disorders (40, 41). More broadly, salivary biomarkers have various promising applications: They could be increasingly utilized in research settings, such as clinical laboratories, where they may help identify the characteristics of specific treatments (39). However, their current application in clinical practice is limited due to the lack of standardized methods for salivary sample collection and evaluation, as well as the absence of specific clinical parameters that can distinguish patients from controls based on the assessment of a single biomarker (39). Previous experiments suggest that the primary goal of proteomic assessment is to differentiate between pathological and physiological states (34). However, a unique proteomic platform to assay the entire saliva proteome is not currently feasible. Furthermore, metabolomics research indicates that the end products of interactions between environmental factors, proteins, and genes are concentrated in various abnormality approaches. Salivary metabolomics is an extremely unique and sensitive procedure for recognizing different conditions, making it an effective alternative to routine serum and tissue-based analyses (39, 42). These metabolomics experiments combine multiple analytical techniques to detect various contents that might serve as novel biomarkers. Therefore, we reviewed the current status of salivary proteomics and metabolomics, their future role in monitoring and detecting various systemic illnesses, their prognosis and diagnostic efficiency, and the related technologies (42). As mentioned, the collection of saliva offers several benefits, such as being stress-free, noninvasive, and repeatable. Moreover, the direct identification of salivary biomarkers could provide reliable protocols that allow for the early diagnosis of neuronal disorders, potentially leading to timely treatments (39). Salivary analysis can identify numerous biomarkers of aging, including a variety of metabolites, proteins, and modifications to DNA and miRNA (43). The direct relationship between the salivary glands and the neuronal system results in these glands' secretions having a high protein overlap with the nervous system. This verifies that these noninvasive body fluids are a useful source of biomarkers that reflect the pathological physiologies of nervous system disorders (44). Although

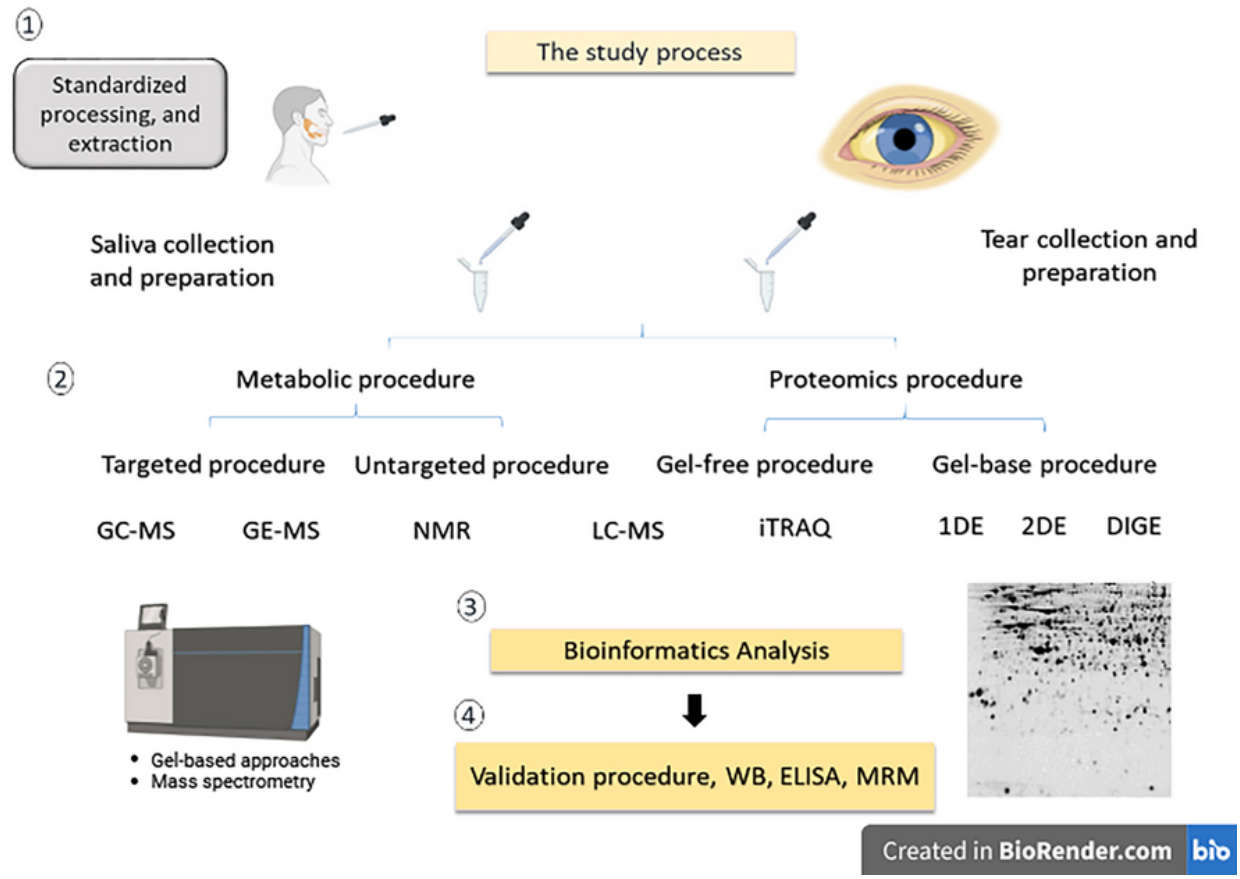

Figure 1. The proteomics procedure of saliva and tears as the noninvasive human sample

many salivary biomarkers for nervous system diseases are still being investigated (45), saliva proteome analysis has already been applied to examine its modifications in immune-mediated inflammatory systemic disorders. These include cystic fibrosis (46), diabetes mellitus (47), oral leukoplakia, chronic graft-versus-host disease, oral squamous cell carcinoma, and Sjögren's syndrome (48-51), as well as demyelinating and neurodegenerative disorders related to aging (39), such as MS (29), Parkinson's disease (PD) (52, 53), Alzheimer's disease (the most frequent form of dementia) (43), amyotrophic lateral sclerosis, and Huntington's disease. These conditions present significant salivary protein biomarkers that are commonly associated with them (39), demonstrating the promising potential of proteomics in biomarker detection and offering novel insights into the molecular processes underlying several systemic ailments. For instance, previous studies suggest that human saliva assessment could provide valuable information for the prognosis and diagnosis of various inflammatory immune-mediated skin illnesses in the near future (34). Moreover, the application of salivary biomarkers offers a novel diagnostic procedure that might be useful for distinguishing demyelinating syndromes (54), predicting disease status, and monitoring response to treatment. This approach allows for the selection of the most applicable and personalized pharmacological treatments. As a result, salivary analysis is gaining interest as an innovative and desirable area of research for multiple disorders. For example, research has demonstrated lower serum levels of VPS4B, S100-A16, and ARP2/3 in Parkinson's disease (PD) patients compared to healthy controls (HC), confirming a different salivary protein composition in PD patients. Additionally, lower concentrations of inflammatory proteins and those involved in exosome

formation were found in PD patients (55). In the case of saliva, previous research has recommended that noninvasive specimens be more deeply analyzed in MS experiments to discern beneficial proteomic biomarkers (5). The neuronal damage-associated proteins in MS patient samples indicated mechanistic concordance with previously reported CNS disease models and in vivo knockdown, verifying their probable value as specific therapeutic targets in MS treatments (56). Besides being a non-invasive diagnostic tool, the salivary protein content includes blood proteins transported from the blood through intra- and extra-cellular pathways. Therefore, the application of saliva biomarkers provides a valuable procedure for evaluating treatment response and monitoring disease activity in MS (57).

For instance, in MS, human leukocyte antigens (sHLA) II and sHLA I (58), immunoglobulin free light chains (FLC) (59), thiobarbituric acid-reacting substances (TBARS), advanced glycation end products (AGEs), advanced oxidation protein products (AOPP), Ferric Ion Reducing Ability of saliva and plasma (FRAS/P), and total antioxidant capacity (TAC) (60) were identified in select patient salivary specimens. The analyzed results represented the turnover impairment and differential expression of immune response, inflammatory, and antioxidant mechanism-related proteins like the cystatin protein superfamily in MS patients compared to healthy controls (57). Additionally, Manconi et al. (29) detected 119 salivary proteins from 49 MS patients and 54 healthy controls, using top-down proteomics. Among these, 23 proteins of the salivary proteomic profiles showed significant differences between MS patients and healthy controls (61).

They revealed that lower levels of identified proteins, such as mono-phosphorylated statherin, cystatin S1, and mono- and di-oxidized cystatin SN, were present in MS cases compared to healthy individuals. Additionally, they illustrated elevated levels of several protein variants, peptides, and fragments. For instance, fifteen proteins showed overexpression, including the cystatin SN P11 → L variant, P-C peptide (Fr.1 - 14, Fr. 26 - 44, antileukoproteinase, and Fr. 36 - 44), cystatin SN Des1-4, SV1 fragment of statherin, two proteoforms of Prolactin-Inducible Protein, and cystatin A T96 → M variant. These identified proteins in patients correlated with inflammation and immune response, which are known aspects of MS pathology (61).

Furthermore, in another study, myelin basic protein (MBP), a critical factor in the myelin cover (10), was found to be secreted at lower levels in MS patients with stimulated saliva compared to healthy controls. This finding had a remarkable equivalence with its level in triggered saliva. Hence, MBP represents crucial diagnostic potential for distinguishing MS patients from healthy controls and could be considered a promising biomarker for MS (10). In other MS proteomic research focusing on saliva, the soluble form of human leukocyte antigen (sHLA) has been evaluated in MS patients and healthy controls. It was found that sHLA class II was overexpressed in patients with Relapsing Remitting MS (RR-MS), reaching values similar to those found in CSF (58, 62). Additionally, increased sHLA class II levels after interferon β1a treatment indicated a promising response to the drug (62). In contrast, sHLA class I was undetectable due to low salivary expression levels (58, 62).

In another valuable study, Kaplan et al. reported the promising value of immunoglobulin-free light chains (FLC) in discriminating MS patients in the relapse phase of the disease. Higher levels of FLC in saliva were observed both compared to controls and to MS patients in remission, using semi-quantitative western blot analysis (59, 63, 64). Furthermore, Karlik et al. evaluated quantified salivary oxidative stress biomarkers, such as AGEs and TBARS, which were overexpressed in the salivary samples of MS patients compared to healthy controls. However, AOPP levels remained unchanged in saliva, possibly influenced by circadian rhythm and oral pathologies (60). Additionally, the authors reported lower salivary levels of Total Antioxidant Capacity (TAC) and FRAS/P in MS patients. However, FRAS/P showed a significant difference compared to healthy controls (60). Therefore, further experiments are needed to examine the importance of saliva-detected proteins and metabolomics in MS treatment response and pathology. Besides these findings, salivary metabolic profiling has emerged as a significant means of evaluation, with metabolic markers aiding in the early diagnosis of several systemic illnesses (42).

However, it should be acknowledged that current MS diagnostic criteria are sufficiently accurate to distinguish between MS patients and healthy controls (HC), even at early stages of the disease. Instead, the achievement of biomarkers as an alternative potential procedure for differential diagnosis among demyelinating disorders would be more applicable in a clinical platform. Therefore, future research in the area of saliva biomarkers should focus on the whole spectrum of demyelinating disorders. Additionally, salivary proteomics research in the dermatological field is still in the early stages. As potential biomarkers for MS, further research is required to assess the role of

these identified proteins in MS pathology and treatment response.

1.2. Tears

Biological methods are considered the most significant aspect for the authentic diagnosis of human disorders. However, advancements in proteomic diagnostic methods have allowed the identification of innovative biomarkers in human specimens (65). Tears, as a thin, moist layer covering the ocular surface, interface with the external environment and are rich in various components such as lipids, cellular debris, peptides, proteins, electrolytes, and mucins, which help preserve the normal status of the ocular surface (66). Like saliva, tears are easily available, repeatable for sampling (17), and noninvasive to collect without difficulties, making them a valuable source of information related to human disease states (66) (Figure 1).

Researchers have introduced tear fluid as a potential source of ailment-specific protein-based biomarkers for human neurological diseases due to its close relation to other body fluid components and its reflection of CNS status (67-70). Although proteomic identification has been challenged by the small volume of tears (65), limitations such as low amounts (5 - 10 μL) (17), the presence of high-abundance proteins, high interpersonal variability in its composition, and sensitivity to physiological and pathological conditions (71-73) exist. Despite these limitations, tears are an optional sample for proteomics in neurological experiments on a limited scale. Tear examination, as a less obtrusive method, may help patients avoid lumbar punctures (1).

Tears provide an interesting resource for biomarker research due to their close relationship with the CNS (57). In another notable study, Ornek et al. (74) evaluated the sensitivity of tear function for neurological disorders. The findings reported a possible correlation between neurodegenerative disorders such as MS, Alzheimer's disease, Parkinson's disease, epilepsy (EP), and Friedreich's ataxia (FA) (74). Moreover, Belviranli et al. (75) identified a relationship between MS and the quality/quantity of tears. Previous studies demonstrated that as a safe and noninvasive sample, alterations in the chemical barrier composition, total protein concentration, and tear flow rate in Alzheimer's disease (AD) could be considered a significant biomarker, with 77% specificity and 81% sensitivity (6). Furthermore, in another study, the application of selected reaction monitoring (SRM) as a novel targeted proteomics method led to the discovery of four tear proteins—dermicidin, lipocalin-1, lactritin, and lysozyme C—that had the same 81% sensitivity and 77% specificity for Alzheimer's disease (AD) (18). In addition to Alzheimer's findings, MS, known as a destructive chronic neuronal disorder of the CNS with significant heterogeneity (76, 77), presents beneficial biomarkers in other specimens like tears (78-80).

The results of two associated researches reported that the specificity and sensitivity of oligoclonal IgG bands in the tears of MS patients are comparable to those in CSF, while the collection procedure is less invasive (80, 81). This supports the notion that tears should be considered a reliable biological fluid for MS biomarker identification (80). Currently, the detection of CSF oligoclonal bands (OCBs) is a primary indicator for predicting and diagnosing MS as a subclinical inflammatory disease of the CNS (82, 83). OCBs are also detectable in the tears of MS patients (82); however, previous research indicates they are not considered definitive MS biomarkers.

In an interesting experiment, Bachhuber (84) reported that OCB detection in tear fluid could not be related to clinical parameters and therefore cannot replace CSF OCB detection in MS patients. He measured OCBs in CSF, tear fluid, and serum samples from 22 diagnosed MS patients, finding that tear fluid OCBs were not specific to MS or other inflammatory diseases (84). Additionally, two independent proteomic studies conducted by Salvisberg et al. and Brown et al. evaluated the tears of MS patients and healthy controls (HC) (17, 85). They reported that among forty-two differential proteins, only alpha-1 antichymotrypsin was significantly overexpressed in all experiments ($P < 0.05$) (86). Based on these findings and the results of other research, the authors confirmed that tear proteomics reflect biological oddities such as abnormal CNS protein modifications, which correlate with related neuronal inflammatory states and MS (17, 85). Therefore, the significant increase in tear alpha-1 antichymotrypsin production emerges as an invaluable MS biomarker, potentially replacing traditional lumbar punctures (87).

Based on available reports detailing the molecular crosstalk between tears and CSF, tears are positioned as a valuable source for exploring specific and sensitive neurological biomarkers (57). In another research, Salvisberg et al. illustrated that among the 42 different proteins, alpha-1 antichymotrypsin was the only protein significantly expanded in all experiments ($P < 0.05$) between the tears of MS patients and HC, making it an auspicious biomarker for MS that could substitute classic lumbar punctures (17).

Furthermore, several studies investigated the alteration levels of immunoglobulins (e.g., alpha, gamma, kappa, and lambda) in tears (17) and found increased levels of innate immune response-regulating proteins like calcium-binding cytosolic protein S100, which plays a key role in modulating macrophage-mediated inflammation (88). Comprehensive top-down research consistently found an enhancement in the abundance of antichymotrypsin in tears and CSF (17, 89, 90). The raised exuberance of heat shock protein, which functions in response to traumatic stimuli and is significant for protein folding dynamics (91), has been illustrated in tears from MS cases (17, 92). Likewise, a proteomics evaluation of secreted extracellular vesicles (EVs) in the tears and CSF of MS patients was carried out by Pieragostino et al. (93). An interesting finding they illustrated was the transportation of the same protein from the CNS to CSF and the tears, supporting the role of EVs in tears as an important diagnostic tool that can be collected in a noninvasive way (93).

Moreover, another study showed a considerable increase in microglial and neuronal-derived EVs in the collected tears of MS patients (94). Therefore, based on these studies, tear proteomics of MS patients can guarantee the high precision and sensitivity requisite for single-tear proteomics examination and biomarker discovery. The most important issue in the field of tear proteomics is the limited sample amounts, which hinder the depth analysis experiments on single-tear samples. This limitation may contribute to the identification of low-invasiveness, sustained-accessibility biomarkers and open novel avenues for the advancement of personalized diagnosis and therapy using tear quantitative proteomics (95).

However, additional clinical experiments are needed to discover and confirm unique and reliable biomarkers from body fluids and possibly CNS tissue in MS sufferers (17). Furthermore, beyond proteomics findings, a great number of metabolic proteins such as mitochondrial proteins, apolipoproteins, lipids containing choline, acylcarnitines, free carnitine, and some amino acids reflect the pathological states of the CNS, demonstrating their valuable role as potential biomarkers for MS (96, 97). Another interesting result from tear lipidomics suggests significant modulation of 30 phospholipids and downregulation of many sphingomyelins in MS (2). The investigation of the tear Metabo-lipidome may provide diagnostic and prognostic biomarkers, improving our understanding of neuronal disease pathogenesis (65). For instance, the overexpression of apolipoproteins (A, C, D, and E) was displayed in MS patient tear samples (17). Specifically, the increase in apolipoproteins AI and AII and the downregulation of apolipoprotein D have been illustrated in tear specimens (17). Moreover, complement proteins (complements B, C3, and I) were also identified in tears (17), demonstrating that tears could be a viable alternative to sampling CSF. Thus, tear proteomics and metabolomics are expected to play a strategic role soon, not only by supporting the three pillars of individualized medicine but also through valid molecular platforms, noninvasive samples, and endotype characterization by identifying innovative, low-invasiveness biomarkers (95). In parallel with previous research, our gathered information from several studies also confirms that tears may be a practical and valuable source of protein and metabolite biomarker profiles for neuronal dysfunctions, specifically MS. The identification and validation of MS biomarkers may allow for the development of a cost-effective and non-invasive diagnostic screening test. However, more clinical experiments are needed to evaluate and identify verified biomarkers in MS patients.

2. Conclusions

This review presents valuable biomarkers, enabling molecular diagnostics to encourage future experiments in this direction and pave the way for their clinical usage. These noninvasive discovered biomarkers may be potentially associated with susceptibility, severity, and pathogenesis of neuronal disorders, and might assist in early diagnosis, prognosis, and a better understanding of disease progression. Furthermore, salivary and tear proteomics research is still in its early stages. However, as potential biomarkers for MS, further research is required to assess the role of these identified proteins in MS pathology and treatment response. The application of salivary and tear proteomics platforms may have distinct advantages, as they can be self-collected with non-invasive procedures, leading to the advancement and verification of new health biomarkers.

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Footnotes

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scientific assessment; Azita Asadi, Vahid Ghafari and Mehdi Rostami: Data collection and drafting the article.

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