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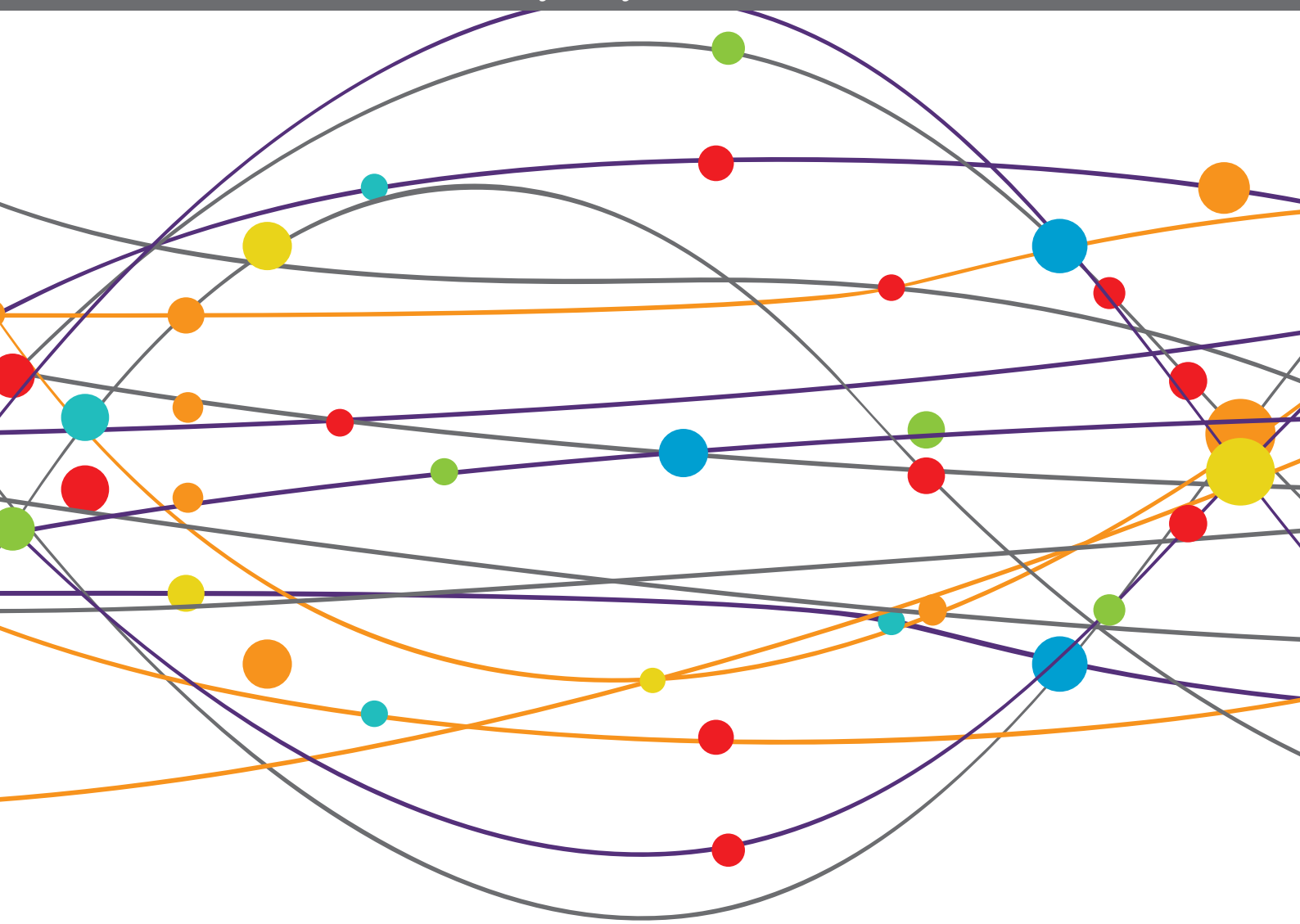

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BIOMARKERS IN NEUROLOGY

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EDITORIAL
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Editorial: Biomarkers in Neurology

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Keywords: biomarkers, neurological disorders & brain damage, diagnostic test, prognosis, neurodegeneration, brain Injury - Traumatic

Editorial on the Research Topic

Biomarkers in Neurology

Neurological disorders constitute a major health and socioeconomic problem. They represent the second cause of death and the leading cause of disability throughout the world. Despite the implementation of strategies and intervention programs to reduce the burden, over the past 25 years, the incidence, prevalence, mortality, and disability rates of neurological disorders are rising globally, mainly due to population aging and growth (1). This has placed heavy pressure on health-care systems pointing out the urgent need to identify new strategies to improve patient outcomes and reduce health costs by enabling more effective drug development and establishing a more personalized medicine approach.

Rapid scientific and technical advances have enabled reliable and affordable measurement of novel biomarkers—biological indicators that objectively measure and evaluate physiological or pathophysiological processes or pharmacological responses to a therapeutic intervention (2)—which have been suggested to help assessment and management of patients with neurological disorders beyond current practice standards (3–5). Evidence suggests a potential variety of clinical applications, including enhancing diagnostic and prognostic accuracy, improving the existing decision criteria for early diagnosis and risk stratification, as well as assisting in disease monitoring, and acting as surrogate endpoints in experimental studies and clinical trials (6–10). In addition, biomarkers may reliably capture the different aspects of disease heterogeneity and pathogenesis, helping characterize patients, and thereby informing targeted tailored treatments and predicting response outcomes to interventions (11–18). However, despite large numbers of candidate biomarkers have been proposed and extensively evaluated, very few are currently integrated into routine clinical practice and the quest for novel brain injury markers in still ongoing (19).

This book aimed at providing an overview of the biomarker landscape in neurological disorders. The diverse authors discuss established and emerging biomarkers as well as innovative strategies for identifying novel candidates offering new and unique perspectives. Several articles in this volume have been focused on Alzheimer's disease and other neurodegenerative disorders, exploring potentially relevant genetic signature (Chen et al.) and the pathogenetic and prognostic role of circulating cytokines (Kim et al.). Importantly, using a methodologically novel approach that

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combines computational prediction and experimental validation, Yao et al., for the first time, identified VLDLR, an apolipoprotein E receptor involved in synaptic plasticity, as a circulating signature for Alzheimer's disease. Accordingly, several lines of evidence are pointing toward the added and complementary value of markers of synaptic function owing to their close link with cognitive deterioration (20).

Contemporary investigations on microRNAs (miRNAs) (Di Pietro et al.) and high mobility group box protein 1 (HMGB1) (Paudel et al.) are also presented, highlighting the fact that these markers may be risk factors themselves and therefore potential targets of therapy (21). Diverse contributions recognize the urgent need for reliable diagnostic and prognostic biomarkers in peripheral demyelinating diseases (Kamil et al.) and spinal cord injury (Albayar et al.), with an emphasis on recent advances in medical knowledge and practice; while other work provides an opportunity to study established markers, such as neurofilament light chain, in neonatal neuronal injury (Depoorter et al.), and to demonstrate the theragnostic potential—capability to identify and monitor the drug effect on the molecular pathology—of PAS-positive vacuolated lymphocytes in late-onset Pompe disease patients treated with ERT (Parisi et al.). Finally, the role of lipidomic analysis (Sabogal-Guáqueta et al.) and Fourier-transform infrared imaging spectroscopy and Laser ablation LA-ICPMS techniques (Ali et al.) in biomarker discovery is outlined.

Overall, this volume offers a unique opportunity to foster knowledge and innovation in the arena of biomarkers for neurological disorders, while stimulating testable hypotheses and the development of a strategic research agenda to accelerate their incorporation into routine clinical practice.

AUTHOR CONTRIBUTIONS

SM wrote the original draft, assembled and incorporated comments from the co-authors and crafted the final draft. All of the other co-authors contributed to manuscript review and revision.

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ORIGINAL RESEARCH
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Non-Motor Symptoms as Predictors of Quality of Life in Egyptian Patients With Parkinson's Disease: A Cross-Sectional Study Using a Culturally Adapted 39-Item Parkinson's Disease Questionnaire

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Objective: The prevalence of non-motor symptoms (NMSs) and their impact on health-related quality of life (HRQoL) in Parkinson's disease (PD) has been reported inconsistently among different populations. In this study, we aimed to investigate the NMSs and HRQoL profiles and their correlation in Egyptian PD patients, using a culturally adapted Arabic version of the 39-item Parkinson's disease questionnaire (PDQ-39).

Methods: Ninety-seven PD patients were rated using the unified Parkinson's disease rating scale (UPDRS), the non-motor symptoms scales (NMSS), Beck depression inventory (BDI), and the Arabic version of PDQ-39. We used the Spearman's rank correlation and multiple linear regression analyses to evaluate the relationship between NMSs domains and HRQoL dimensions.

Results: Fatigue/sleep (91.3%) and mood/cognitive disturbances (87%) were the most frequently and severely affected NMSS domains. Other common NMSs included urinary (75.9%), memory/attention (72.4%), gastrointestinal (67.8%), and cardiovascular problems (64.8%). The total NMSS scores were positively correlated with UPDRS I, II, and III scores. Depression was prevalent in 76.7% of PD patients. Moreover, all enrolled PD patients reported impairment in different HRQoL dimensions, especially mobility (98.9%), activities of daily living (97.8%), and emotional well-being (95.5%). The summary index of PDQ-39 was correlated to the total NMSS, UPDRS-I, UPDRS-II Off, UPDRS-III (Off and On states), and BDI scores.

Abbreviations: ADL, activities of daily living; BDI, Beck depression inventory; HRQoL, health-related quality of life; H&Y, Hoehn and Yahr scale; NMSs, non-motor symptoms; NMSS, non-motor symptoms scale; PD, Parkinson's disease; PDQ-SI, Parkinson's disease questionnaire-summary index; S&E, Schwab and England scale; UPDRS, unified Parkinson's disease rating scale.

Conclusion: This study showed the high prevalence of NMSs and the value of NMSS and BDI scores as predictors of HRQoL in Egyptian PD patients. Therefore, characterizing the NMSs profile is essential for tailoring management strategies for PD patients.

Keywords: 39-item Parkinson's disease questionnaire, Arabic, Egypt, non-motor, Parkinson's disease, quality of life

INTRODUCTION

Parkinson's disease (PD) is the second most common neurodegenerative disorder worldwide, caused by degeneration of dopaminergic neurons in the substantia nigra, and affects approximately 1–3% of the elderly population (≥60 years) (1). Health-related quality of life (HRQoL) is a multidimensional, self-reported measure of the disease impact on the patients' lives. Improving the HRQoL is the aim of care in chronic diseases, especially PD in which HRQoL is determined by motor, non-motor symptoms (NMSs), and other social factors (2).

The NMSs consist of autonomic dysfunction, sensory symptoms, neuropsychiatric disturbances, sleep problems, fatigue, and gastrointestinal (GIT) disorders. Their impact on HRQoL was reported to be greater than the motor symptoms of PD (3). However, the prevalence of NMSs and their influence on HRQoL in PD patients had been shown to vary among different countries and cultures (4–8). For example, constipation and cognitive deficit were highly prevalent in Asian (especially Chinese) patients (8), compared with Western countries. Moreover, another study showed that bodily discomfort and stigma were the most impaired HRQoL domains among Chinese PD patients, in contrast to other populations (9). These differences could be attributed to several demographic, genetic, and clinical variations in PD patients (10). Moreover, different NMS profiles were identified, resulting in the proposal of NMS subtypes of PD (11). Therefore, identifying the prevalent NMS subtype in various settings will help to personalize the management of PD patients (12).

Despite the high prevalence and genetic variability of PD in Egyptian and Arab populations (13–15), only few studies investigated the NMSs profile in Egyptian PD patients and none—to the best of our knowledge—explored their value as predictors of HRQoL (16, 17). The prevalence of PD among the Egyptian population is the highest compared with other Arabic countries, which may be due to genetic or environmental factors (14, 18). Providing a culturally adapted Arabic version of the 39-item Parkinson's disease questionnaire (PDQ-39) would facilitate epidemiological studies and improve patients' care.

Therefore, this study aimed to explore the NMS and HRQoL profiles and their correlations in Egyptian PD patients. Furthermore, it provides a culturally adapted Arabic version of the PDQ-39 scale.

MATERIALS AND METHODS

Patients

Ninety-seven patients diagnosed with idiopathic PD were recruited from the movement disorders outpatient clinic at Ain Shams University Hospitals (Cairo, Egypt) between 2013 and 2017. The recruited patients were diagnosed according to the British Parkinson's disease Society Brain Bank criteria (19). Patients with atypical or secondary Parkinsonism and those with other comorbid chronic diseases that could affect PD NMSs and/or HRQoL were excluded. Moreover, PD patients who refused or could not complete questionnaires, e.g., due to severe cognitive impairment or being on antidepressants were excluded. The Ethical Committee at the Faculty of Medicine, Ain Shams University approved the study protocol. All subjects gave written informed consent according to the Declaration of Helsinki (1975, as revised in 2008).

Outcome Measurement

All subjects were evaluated using the unified Parkinson's disease rating scale (UPDRS), Hoehn and Yahr scale (H&Y), and Schwab and England scale (S&E) in medication "off" and "on" states by a movement disorders expert. Different UPDRS subscales were estimated, including cognition (UPDRS-I), activities of daily living (ADL, UPDRS-II), motor (UPDRS-III), and total UPDRS scores. Moreover, depression was assessed using the Beck depression inventory (BDI) (20).

The NMSs were assessed in all patients, using the non-motor symptoms scale (NMSS) (21), which consists of 30 items, grouped in nine domains [cardiovascular (CVS), sleep/fatigue, mood/cognition, perception/hallucinations, memory/attention, GIT, urinary, sexual, and miscellaneous symptoms]. The frequency of each NMS (item) was calculated (item score ≥ 1), and the summary index for each domain was estimated (the sum of included items divided by the maximum possible score then multiplied by 100) to allow crude comparisons between the severity of different domains.

The HRQoL of PD patients was measured using a culturally adapted Arabic version of the Parkinson's disease questionnaire (PDQ-39) (22), which is formed of 39 items (ranged from 0 = never to 4 = always) that are grouped into eight dimensions. These dimensions include mobility (1–10, 10 items), ADL (11–16, 6 items), emotional well-being (17–22, 6 items), stigma (23–26, 4 items), social support (27–29, 3 items), cognition (30–33, 4 items), communication (34–36, 3 items), and bodily discomfort (37–39, 3 items). Following answering the questionnaire, a summary index for each dimension (subscales) was calculated by dividing the sum of included items by the maximum possible score then multiplying by 100. Then, the total score [PDQ-39 summary index (PDQ-39 SI)] was calculated by summation of the eight dimensions' scores divided by 8. Consequently, lower scores reflect better HRQoL (23). The frequency of impairment of each dimension to any degree (score > 0) was also estimated.

PDQ-39 Cultural (Same Language) Adaptation

After a written agreement from the scale provider (Isis outcomes), the Arabic version of PDQ-39 (Tunisia) was culturally adapted and changed to an Egyptian version to avoid some words that were not familiar with the Egyptian culture. According to the standard methodology (24), two Egyptian native speakers reviewed the Tunisian Arabic version and identified terms that were unfamiliar separately. The comments of the two reviewers were revised by a third Egyptian individual to confirm their acceptability and cultural relevance. Finally, back-translation of revised items from the Egyptian Arabic version to English was performed to ensure accuracy, followed by a cognitive debriefing with 10 Egyptian patients to confirm that the new version is well understood.

Statistical Analyses

Statistical analyses were performed using the SPSS (version 23 for windows). Categorical data, such as the frequency of NMSs, were described using frequencies and percentages, while continuous data were expressed as mean and standard deviation (SD) values. The comparison between two independent variables was made using the independent *t*-test, while the Spearman's rank correlation coefficient was used to evaluate the association between PDQ-39 dimensions, NMSS domains, and other variables. Moreover, we used the multiple linear regression analysis to determine the predictors of PDQ-SI. The level of statistical significance (*p*-value) was set at 0.05.

RESULTS

Clinical and Demographic Characteristics of PD Patients

Ninety-seven PD [58 males (59.8%) and 39 females (40.2%)] patients were enrolled in this cross-sectional study (mean age at enrollment: 55.3 ± 10.8 years and mean age of disease onset: 50.1 ± 11.2 years). The mean disease staging in the Off state (H&Y Off scale) was 2.8 ± 1.1 (stage 1: 6.4%; 1.5: 12.8%; 2: 13.8%, 2.5: 11.7; 3: 34%; 4: 12.8%; 5: 8.5%), while the mean motor severity in the Off state (UPDRS III Off) was 38.04 ± 19.37. The prevalence of depression was 76.7% in the enrolled cohort (**Table 1**). Females had younger age at study enrollment ($p = 0.007$) and at disease onset ($p = 0.004$), as well as worse S&E Off scores ($p = 0.01$); however, males and females had comparable disease duration, staging, and UPDRS scores.

TABLE 1 | Demographic and clinical motor characteristics of PD patients.

	Mean (SD)	Range
Number	97	
Gender		
Male, *n* (%)	58 (59.8)	
Female, *n* (%)	39 (40.2)	
Age (years)	55.3 (10.8)	23–77
Duration of illness (years)	5.3 (4.1)	0.3–20
Age of onset (years)	50.1 (11.2)	20–70
H&Y off	2.78 (1.1)	1–5
H&Y On	1.00 (0.8)	0–3
S&E Off	56.0 (22)	10–90
S&E On	86.38 (13.9)	40–100
UPDRS I	3.83 (2.87)	0–12
UPDRS II off	17.88 (10.8)	0–44
UPDRS II on	7.38 (8.1)	0–41
UPDRS III off	38.04 (19.4)	4–86
UPDRS III on	15.93 (14.0)	0–92
BDI	17.5 (8.7)	

Data presented are the mean ± SD and range unless otherwise indicated.
BDI, Beck depression inventory; H&Y, Hoehn and Yahr scale; S&E, Schwab and England scale; UPDRS, unified Parkinson's disease rating scale; PD, Parkinson's disease.

NMSs Prevalence and Correlations

All patients (100%) suffered from one or more NMSs. The most common and severely affected domains were sleep/fatigue (91.3%), mood/cognition (87%), miscellaneous symptoms (78.2%), urinary symptoms (75.9%), and memory/attention impairment (72.4%) (**Table 2**). Fatigue was the most frequent NMS (81.8%), followed by mood symptoms (sadness 75%, nervousness 69.6%, and lack of motivation 68.5%), forgetfulness (65.5%), and urinary symptoms (nocturia and urgency 58.6%) (**Figure 1**). No gender differences were detected except that females had worse miscellaneous domain scores ($p = 0.018$, females experienced more pain and sweating) and higher BDI scores ($p = 0.012$).

The total NMSS score was significantly correlated with S&E Off, UPDRS I, UPDRS II (Off & On), UPDRS III (Off & On), and BDI scores, but not with age, duration of the disease, or H&Y staging ($r = 0.204$, $p = 0.06$). Most of NMSs domains (except sexual and urinary domains) were correlated with UPDRS-I and II Off scores (Table A in Supplementary Material).

TABLE 2 | Non-motor and HRQoL characteristics of enrolled PD patients.

	Mean (SD)	Frequency (%)
Non-motor		
NMSS total	61 (42.9)	100
Cardiovascular	2.7 (3.9)	64.8
Sleep/fatigue	10.98 (8.7)	91.3
Mood	18.2 (16.2)	87
Perceptual problems	1.4 (3)	37
Attention/memory	6.1 (6.8)	72.4
Gastrointestinal	5.4 (6.98)	67.8
Urinary	7.3 (9.5)	75.9
Sexual function	3.4 (5.96)	43.5
Miscellaneous	6.7 (6.7)	78.2
HRQoL		
PDQ-39 SI	37.5 (18.6)	100
Mobility	46.8 (29.92)	98.9
ADL	44.4 (28.1)	97.8
Emotional well-being	45.1 (30.1)	95.5
Stigma	50 (31.7)	86.4
Social support	18.3 (24.6)	56.8
Cognition	27.05 (20.94)	88.6
Communication	26.6 (25.2)	73.9
Bodily discomfort	40.15 (24.9)	94.3

Data presented are mean ± SD.
HRQoL, health-related quality of life; NMSS, non-motor symptoms scale; PDQ-39 SI, Parkinson's disease questionnaire-summary index; ADL, activities of daily living; PD, Parkinson's disease.

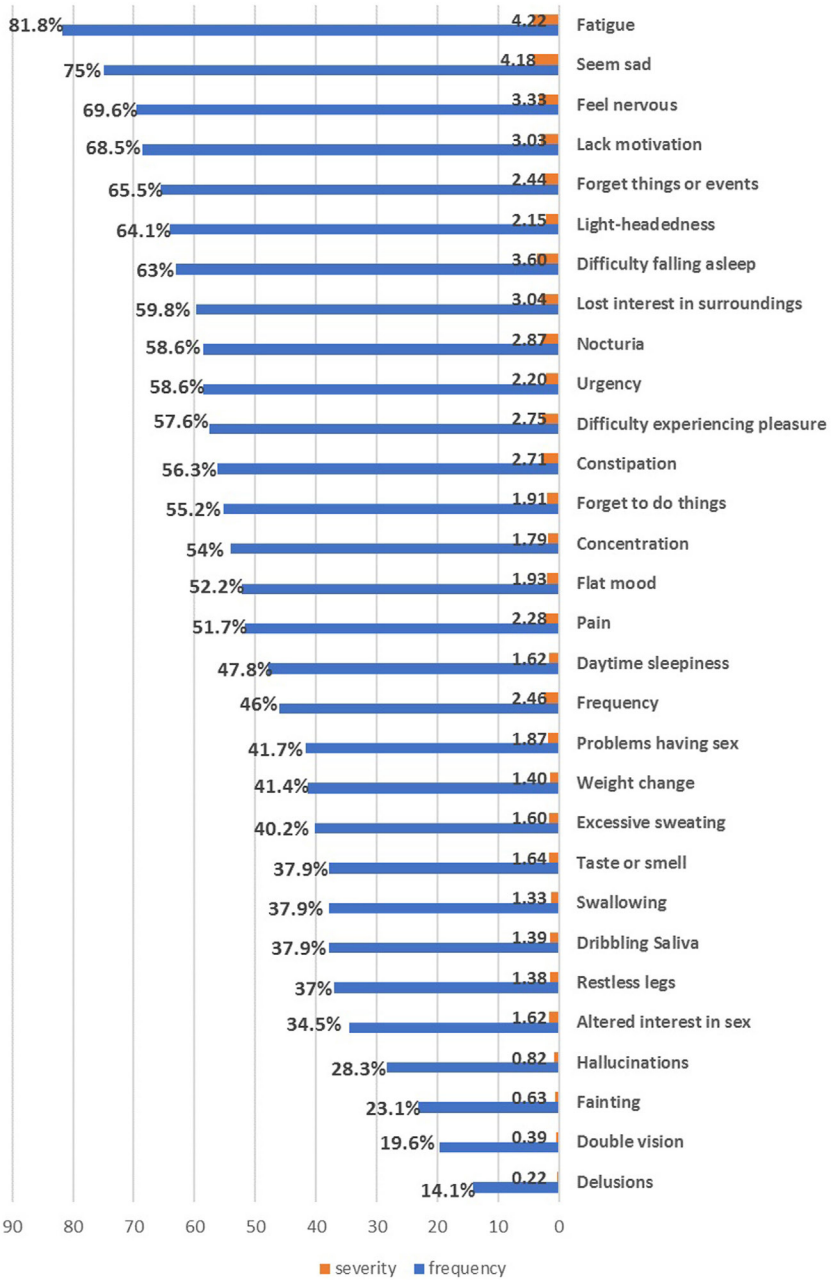

FIGURE 1 | The frequency and severity of individual non-motor symptoms in the enrolled Parkinson's disease patients.

Quality of Life State and Correlations

All enrolled PD patients had impaired HRQoL with comparable values (scores between 40 and 50) and frequency (88.6–98.9%) in most dimensions, except for social support, cognition, and communication (had lower scores, indicating better HRQoL aspects) (**Table 2**). About 86% of PD patients felt stigmatized to some degree, while 56.8% of the cohort reported incomplete social support. Females showed significantly more impaired (higher values) PDQ-SI, mobility, ADL, and bodily discomfort scores ($p = 0.016$, 0.001, 0.003, and 0.047, respectively).

The PDQ-SI score was inversely correlated with the patients' age at enrollment ($r = -0.220$, $p = 0.04$), age of disease onset ($r = -0.272$, $p = 0.01$) and S&E daily activity scores (Off & On scores) ($r = -0.483$ and -0.361, $p < 0.01$, and 0.01, respectively) (Table B in Supplementary Material). This means that younger age at enrollment or disease onset and less independence in daily activity were associated with more impaired HRQoL. In addition, the PDQ-SI was positively correlated with female gender, H&Y (Off and On) disease stage ($r = 0.318$ and 0.298, $p = 0.003$ and 0.006, respectively), UPDRS-I and UPDRS-II Off ($p < 0.001$),

UPDRS-III (Off and On) scores (**Table 3**). Thus, HRQoL impairment was associated with more impaired cognition, daily activity, and "Off and On" motor features. The disease duration did not correlate with PDQ-SI; however, it was correlated with ADL and Stigma dimensions ($r = 0.226, 0.229$; $p = 0.030, 0.036$, respectively).

Moreover, PDQ-SI was strongly correlated to the total and most of NMS subscales' scores (except CVS and sexual domains) and BDI score ($p < 0.001$). The total NMSS score was strongly correlated to PDQ-39 dimensions ($p < 0.001$), except social support. Likewise, most of the PDQ-39 dimensions had a significant correlation with most of NMSS domains, except social support (**Table 4**). In summary, the more severe and frequent NMSs are, the more impaired HRQoL becomes in PD patients.

Multiple linear regression analysis was performed to determine the predictors of the PDQ-SI from different variables including age at enrollment and disease onset, gender, disease duration, UPDDRS I, II, III, BDI, H&Y, S&E, and total NMSS scores. Depression (BDI) and the total NMSS score explained 50.5% of the PDQ-SI. Depression was the primary predictor

TABLE 3 | Correlations between quality of life domains and disease stage, patient disability and UPDRS subscores.

		PDQ-39 SI	Mobility	ADL	Emotional well-being	Stigma	Social support	Cognition	Bodily discomfort	Communication
H&Y off	*r*	0.318	0.281	0.438	0.153	0.168	0.051	0.151	0.118	0.449
	p	0.003	0.008	<0.001	0.160	0.121	0.638	0.165	0.279	<0.001
H&Y On	*r*	0.298	0.291	0.340	0.230	0.096	−0.138	0.249	0.098	0.380
	p	0.006	0.006	0.001	0.034	0.383	0.209	0.022	0.372	<0.001
S&E On	*r*	−0.361	−0.414	−0.402	−0.269	−0.167	−0.001	−0.208	−0.130	−0.305
	p	0.001	<0.001	<0.001	0.013	0.127	0.992	0.057	0.237	0.005
S&E Off	*r*	−0.438	−0.433	−0.463	−0.294	−0.285	−0.099	−0.252	−0.214	−0.375
	p	<0.001	<0.001	<0.001	0.006	0.007	0.364	0.018	0.047	<0.001
UPDRS I	*r*	0.599	0.514	0.495	0.612	0.338	0.234	0.459	0.377	0.452
	p	<0.001	<0.001	<0.001	<0.001	0.002	0.032	<0.001	<0.001	<0.001
UPDRS II off	*r*	0.548	0.539	0.552	0.467	0.335	0.122	0.270	0.410	0.390
	p	<0.001	<0.001	<0.001	<0.001	0.003	0.282	0.016	<0.001	<0.001
UPDRS II on	*r*	0.217	0.209	0.178	0.189	0.085	0.048	0.180	0.108	0.302
	p	0.057	0.061	0.112	0.098	0.457	0.675	0.115	0.348	0.007
UPDRS III off	*r*	0.384	0.415	0.426	0.280	0.208	0.004	0.169	0.217	0.375
	p	<0.001	<0.001	<0.001	0.011	0.061	0.971	0.129	0.051	0.001
UPDRS III on	*r*	0.283	0.276	0.275	0.202	0.076	0.042	0.276	0.079	0.364
	p	0.010	0.010	0.010	0.067	0.492	0.703	0.012	0.478	0.001

Data presented are p values and correlation coefficients (r).

H&Y, Hoehn and Yahr scale; S&E, Schwab and England scale; UPDRS, unified Parkinson's disease rating scale; ADL, activities of daily living.

TABLE 4 | Correlations between NMSs and quality of life domains.

		Mobility	ADL	Emotional well-being	Stigma	Social support	Cognition	Communication	Bodily discomfort	PDQ-39 SI
CVS	*r*	0.221	0.150	0.237	0.079	0.027	0.242	0.197	0.154	0.198
	p	0.039	0.163	0.030	0.475	0.807	0.026	0.072	0.161	0.071
Sleep and fatigue	*r*	0.643	0.548	0.563	0.389	0.148	0.508	0.559	0.415	0.659
	p	<0.001	<0.001	<0.001	<0.001	0.176	<0.001	<0.001	<0.001	<0.001
Mood and cognition	*r*	0.518	0.444	0.742	0.423	0.182	0.501	0.471	0.531	0.681
	p	<0.001	<0.001	<0.001	<0.001	0.096	<0.001	<0.001	<0.001	<0.001
Perception	*r*	0.202	0.164	0.348	0.013	0.007	0.382	0.234	0.412	0.263
	p	0.058	0.125	0.001	0.905	0.953	<0.001	0.031	<0.001	0.015
Memory	*r*	0.289	0.303	0.378	0.137	0.242	0.669	0.404	0.174	0.421
	p	0.008	0.005	0.001	0.223	0.029	<0.001	<0.001	0.120	<0.001
GIT	*r*	0.455	0.343	0.433	0.148	0.049	0.474	0.415	0.466	0.452
	p	<0.001	0.001	<0.001	0.188	0.666	<0.001	<0.001	<0.001	<0.001
Urinary	*r*	0.333	0.359	0.383	0.182	0.099	0.308	0.335	0.340	0.414
	p	0.002	0.001	<0.001	0.104	0.380	0.005	0.002	0.002	<0.001
Sexual	*r*	0.060	0.164	0.217	0.035	0.154	0.261	0.201	0.053	0.151
	p	0.593	0.140	0.054	0.760	0.176	0.020	0.076	0.643	0.184
Total	*r*	0.625	0.573	0.697	0.400	0.160	0.613	0.594	0.526	0.723
	p	<0.001	<0.001	<0.001	<0.001	0.155	<0.001	<0.001	<0.001	<0.001
BDI	*r*	0.671	0.578	0.745	0.524	0.309	0.569	0.574	0.387	0.765
	p	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001	<0.001	<0.001

Data presented are p values and correlation coefficients (r).

ADL, activities of daily living; BDI, Beck depression inventory; CVS, cardiovascular; GIT, gastrointestinal; PDQ-SI, Parkinson's disease questionnaire-summary index; NMSs, non-motor symptoms.

(adjusted R^2 0.497, $p < 0.0001$). On removing BDI score from the model, the total NMSS score and UPDRS-I (cognition) were the independent predictors of HRQoL and explained 53.7% of patients' HRQoL. The total NMSS score was the primary predictor (adjusted R^2: 0.480, $p < 0.0001$).

DISCUSSION

This study explored the non-motor and HRQoL profiles of PD patients in an African country with a high prevalence of PD. It confirmed the marked impact of NMSs and depression on HRQoL impairment. Moreover, it provides a culturally adapted Arabic version of the PDQ-39 to promote further research and care of Arabic PD patients. To the best of our knowledge, this is the first study to investigate the HRQoL and its association with NMSs in Egyptian PD patients.

Our study showed a high prevalence of NMSs in Egyptian PD patients (100%). Fatigue, mood disturbances (sadness, nervousness, and lack of motivation), and memory impairment were the most frequent NMSs. Likewise, the most severe and frequently affected domains were sleep/fatigue and mood/cognition domains. Gender differences were minimal and restricted to miscellaneous symptoms. A similar high prevalence of NMSs and frequency of NMS domains were reported in Upper Egypt by Khedr and colleagues (16). This NMS profile in the Egyptian patients is more consistent with the depression/anxiety PD subtype (11).

Previous studies have identified differences in NMS profile between different countries and races. Reports from different Asian countries identified GIT symptoms, especially constipation as the most frequent NMS (8). Nocturia was the most frequent NMS, followed by fatigue and dribbling of saliva in a multicenter European study (3). Cognition, sleep followed by urinary symptoms were the most common NMS in an Estonian cohort (25), while fatigue followed by urinary symptoms were more common in the Spanish population (26). These differences could be attributed to several demographic, genetic, and clinical variations in enrolled patients (age, the age of onset, disease duration, and severity), as well as different assessment questionnaires (10, 12).

The different dimensions of HRQoL were impaired in all patients and markedly correlated to NMSS total score and depression severity. Moreover, it was associated with motor severity, disease staging, and impaired daily activity in Off and On states. However, regression analysis confirmed the independent predicting effect of NMSs for HRQoL. This is consistent with the findings of former studies in different populations in which the total NMSS score was the main predictor of QoL. Mood/cognition and sleep/fatigue had the strongest correlation with PDQ-SI in accordance with prior studies (3, 7, 27).

Neuropsychiatric symptoms, especially depression, were reported as independent determinants of HRQoL (28). In consistence with previous studies (25, 26, 29), depression was recognized as the main independent predictor of HRQoL. This could be explained by the high prevalence of depression (76.7%) and the strong correlation between depression and all HRQoL dimensions, several NMS domains and total NMSS scores. Consequently, depression affects HRQoL directly and indirectly through its impact on the NMSs, especially emotional well-being and cognition (28). In agreement with several previous studies, depression was recognized as the main predictor of poor HRQoL in PD patients (29, 30). Antonini et al. reported improvement in mobility and ADL after depression treatment, indicating that mood problems affect how PD patients perceive their motor functions (31).

Intriguingly, 86.4% of PD patients felt stigmatized, while 56.8% of the cohort reported incomplete social support. Stigma was associated with younger age of enrollment, younger age of disease onset, disease severity, impaired daily activities and cognition, as well as depression. This is consistent with prior studies (32, 33). Furthermore, it was correlated with fatigue/memory, mood/cognition domains, and total NMSS scores. Stigma is an important determinant of HRQoL, related to cultural and social factors (32, 34). Consequently, the identification and management of this feature are crucial for the improvement of HRQoL of PD patients.

Although this study provides a comprehensive assessment of the impact of different NMS domains on HRQoL for the first time in Egyptian patients, it has some limitations. The relatively small sample size and the fact that the majority of enrolled patients came from a low socioeconomic class restrict the generalizability of our findings. Moreover, as a clinic-based study [plus the relatively short disease duration (5.3 years)], patients with advanced stages and cognitive impairment were under-represented. Furthermore, some NMSs are prevalent in the elderly population without PD; therefore, a case–control study would elucidate whether the investigated NMSs are PD related.

In conclusion, this study demonstrated the high prevalence of NMSs in Egyptian PD patients and the significant impact of NMSs and depression on HRQoL. Thus, the assessment and management of NMSs are as important as the motor aspects of PD. Future larger studies can use our culturally adapted questionnaire to evaluate the correlation between NMSs domains and HRQoL dimensions in patients with advanced PD.

ETHICS STATEMENT

The ethical committee of the faculty of medicine, Ain Shams University has approved this study. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008. All subjects gave written informed consent according to the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions, revised the manuscript, and approved the final version. AS, HE, SA, and ME: conception and design of the study. AS, AB, MH, MK, and EH: data collection and analysis. AS, AB, AA, and HE: first draft writing. All authors: revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00357/full#supplementary-material.

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REVIEW
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MicroRNA Signature of Traumatic Brain Injury: From the Biomarker Discovery to the Point-of-Care

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Traumatic brain injury (TBI) is a serious problem that causes high morbidity and mortality around the world. Currently, no reliable biomarkers are used to assess the severity and predict the recovery. Many protein biomarkers were extensively studied for diagnosis and prognosis of different TBI severities such as S-100β, glial fibrillary acidic protein (GFAP), neuron-specific enolase (NSE), neurofilament light chain (NFL), cleaved tau protein (C-tau), and ubiquitin C-terminal hydrolase-L1 (UCH-L1). However, none of these candidates is currently used in the clinical practice, due to relatively low sensitivity, for the diagnosis of mild TBI (mTBI) or mild to moderate TBI (MMTBI) patients who are clinically well and do not have a detectable intracranial pathology on the scans. MicroRNAs (miRNAs or miRs) are a class of small endogenous molecular regulators, which showed to be altered in different pathologies, including TBI and for this reason, their potential role in diagnosis, prognosis and therapeutic applications, is explored. Promising miRNAs such as miR-21, miR-16 or let-7i were identified as suitable candidate biomarkers for TBI and can differentiate mild from severe TBI. Also, they might represent new potential therapeutic targets. Identification of miRNA signature in tissue or biofluids, for several pathological conditions, is now possible thanks to the introduction of new high-throughput technologies such as microarray platform, Nanostring technologies or Next Generation Sequencing. This review has the aim to describe the role of microRNA in TBI and to explore the most commonly used techniques to identify microRNA profile. Understanding the strengths and limitations of the different methods can aid in the practical use of miRNA profiling for diverse clinical applications, including the development of a point-of-care device.

Keywords: traumatic brain injury, biomarkers, microRNA, diagnosis, prognosis, therapy, high-throughput technology, point-of-care

MICRORNA SIGNATURE IN TRAUMATIC BRAIN INJURY

Traumatic Brain Injury

Head injuries are a significant cause of disability and mortality worldwide and one of the most common reasons of emergency department visits especially among young males (1), creating a severe physical, psychological and socioeconomic burden on the patients, their families and the community (1, 2).

In particular, traumatic brain injury (TBI) is a complex pathological alteration in the neural homeostasis which is triggered by an external mechanical force resulting in a broad spectrum of temporary or permanent injuries and outcomes (3). Annual TBI incidents are estimated to be more than 10 million patients worldwide (4–6). The most frequent causes of TBI are falls, road traffic accidents, sport and recreation activities, military injuries and assault or abuse. TBI pathology can be classified as primary and secondary brain damage (7). The primary injury occurs immediately after receiving the mechanical impact which disrupts the integrity of neuronal, glial, endothelial cells and dysregulates the cerebral blood flow (CBF), whereas the secondary brain injury is due to a range of biochemical and cellular changes that causes neuronal apoptosis and death, blood-brain barrier (BBB) disruption, etc. (8–15). Controlling the development of secondary injury is the only strategy that can be beneficial to improve the outcome of the primary injury that cannot be managed medically. The heterogeneity of the disease makes an accurate assessment of the severity of trauma and the prediction of patient outcome, challenging. Clinically, head injuries are diagnosed as mild, moderate, or severe according to the Glasgow Coma Scale (GCS) score, which uses a motor, eye and verbal responses to assess the conscious level of the patient. However, this score might underestimate mild TBI (mTBI) cases (16). Computed tomography (CT) or magnetic resonance imaging (MRI) scans are also used to assess TBI according the current guidelines (17). Although these techniques show limited diagnostic ability for the detection of mild brain tissue insult with concerns for radiation risks from CT scans and the escalating costs of diagnostic imaging techniques (18, 19) in the future, imaging has the potential to complement molecular diagnostics (20).

For this reason, mTBI detection remains one of the most difficult clinical diagnoses, it accounts for 75–90% of the TBI cases in the United States (21) and 10–20% of the patients remain symptomatic and complain of post-concussive syndrome (PCS) symptoms (22). In addition, people such as military, sportive and children are at risk of repeated concussions and may develop depression (23) and neurodegenerative conditions in later life, e.g., Parkinson's disease, motor neuron disease, and chronic traumatic encephalopathy (CTE) (24, 25).

Biomarkers of Traumatic Brain Injury

Currently, no TBI biomarkers were identified that could reliably be used in the clinical practice for diagnosis and prognosis.

Recently, the U.S. Food and Drug Administration reviewed and authorized for marketing the Banyan Brain Trauma Indicators which are ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP), to evaluate mTBI in adults. These two proteins are released from the injured tissue into the blood and can be quantified within 12 h of the brain injury and can help to predict the patients with detectable intracranial lesions on the CT scan with 97.5% of accuracy. However, a biomarker able to accurately diagnose mTBI is still needed.

In the last decades, many molecules were proposed as promising TBI biomarkers, but the complicated anatomy of the brain and the disparate pathology of the TBI make it challenging to apply into the clinical practice (26).

Biofluid biomarkers would be preferable as they present various advantages such as cost-effective and minimally invasive sample collection.

Among the most extensively studied biomarkers in the serum and cerebrospinal fluid (CSF), there are S-100β and GFAP. S-100β is an extracellular protein with a short half-life of <30 min (27). However, because of its size, it does not cross an intact BBB. Besides, S-100β is not a brain-specific protein and can also be released by other organs in case of polytrauma (28–31). In 2013, the S-100β serum level was used to reduce the unnecessary CT scans in the adult mTBI patients among the Scandinavian population. However, it remains challenging to find the appropriate cut-off value of S-100β that correlates with the injury primarily because of the lower sensitivity in polytrauma patients (32, 33).

On the contrary, GFAP is a structural protein exclusively expressed in the astroglial cells and plays a pivotal role in the astrocyte's cytoskeleton as a component of the intermediate filament (IF) network (34). GFAP was found to be slightly elevated in mild TBI and when added to the clinical data, it improved the power of outcome prediction (35). Animal studies also showed GFAP to be a promising biomarker, since its cellular release is correlated to all grades of injury severities (36). The only limitation in the use of GFAP as a biomarker is related to the release into the bloodstream or CSF, which is, indeed, strictly BBB-damage dependent (35, 37).

Neuron-specific enolase (NSE), neurofilament light polypeptide (NFL), cleaved tau protein (C-tau) and UCH-L1 were also considered promising biomarkers. However, the biological significance of these biomarkers cannot be confidently declared, due to the lack of studies with adequate sample size and low sensitivity for mTBI in individuals without detectable structural brain abnormalities. A summary of papers showing the area under the curve (AUC) of representative TBI biomarkers is presented in **Table 1**.

Enolases are glycolytic enzymes composed by three different subunits (α, β, γ). The two most stable isoforms are γγ and αγ, which are referred to as NSE, are particularly abundant in the neuron cytoplasm, however NSE proteins can also be found in erythrocytes and platelets making the process of haemolysis a significant extracranial source when measured in trauma (46, 47). In the context of mild TBI, NSE can predict the early prognosis of patients when measured in combination with S-100β (48). However, its slow

TABLE 1 | Area under the curve (AUC) of representative TBI biomarkers.

Biomarkers	AUC	Cohort	Condition	N	Controls	Reference	Timing	Comment
S100B	0.87	TBI all severity	TBI vs. non-TBI	50	50	(38)	Within 6 h	Non-specific
S100B	0.68	mTBI	Ice hockey vs. pre-season	28	28	(39)	Within 1 h	Poor performance
NSE	0.82	TBI all severity	TBI vs. non-TBI	50	50	(38)	Within 6 h	Non-specific
NSE	0.54	mTBI		28	28	(39)	Within 1 h	Poor performance
NSE	0.64	mTBI	Clinically important injury	25	82	(40)	Day 1	Non-specific
Myelin-basic protein	0.66	TBI all severity	TBI vs. non-TBI	50	50	(38)	Within 6 h	Poor performance
Cleaved Tau	0.74	mTBI	Injury vs. pre-season	28	28	(41)	At 36 h	Late
Total Tau	0.8	mTBI	Ice hockey vs. pre-season	28	28	(39)	Within 1 h	Promising
GFAP	0.84	mild-moderate TBI	Positive CT	209	188	(42)	At 4 h	Limited sensitivity
UCH-L1	0.87	mTBI	GCS 15 vs. controls	86	199	(43)	Within 1 h	Promising
UCH-L1	0.73	TBI	positive CT	N/A	199	(43)	Within 1 h	Promising
Amyloid-β	N/A	sTBI	TBI vs. controls	12	20	(44)	Day 1	poor sensitivity
All-Spectrin break-down	0.76	mTBI	Injury vs. pre-season	25	N/A	(41)	At 36 h	Late
CTS5	N/A	TBI all severity	sTBI vs. orthopedic injury	30	30	(45)	Within 1 h	Promising

elimination from plasma, leads to difficulties in distinguishing between primary and secondary insults to the brain (49, 50).

One of the most recently identified biomarkers is NFL. It was suggested as a potential, sensitive and specific marker in detecting axonal injury in mTBI (51). One of the advantages of its clinical use is the relatively long half-life which was estimated to be ∼3 weeks (52). NFL also plays a vital role in the neuro-axonal cytoskeleton (53). Therefore, increased NFL levels were found in the CSF and serum of individuals with a wide range of neurodegenerative and neuroinflammatory diseases (54, 55). Another proposed serum marker is C-tau, which is a microtubule-associated protein (MAP) primarily found in the neuronal axons and dendrites (49, 56). After an axonal injury, tau protein can be detected in the extracellular space and diffuses into the CSF after N- and C- terminals cleavage (56). In 2006, a study demonstrated that higher levels of post-traumatic CSF C-tau were associated with a poorer clinical outcome following severe TBI (sTBI) (57). However, there was no significant correlation between the levels of C-tau and the outcome following mTBI (58).

UCH-L1 was identified as highly specific to the human brain (59) and the increased levels were correlated with the TBI severity and a worse outcome. Its diagnostic value was found to be beyond the first 24 h of injury (60–63). It could also distinguish between the patients with TBI and the uninjured patients with altered GCS secondary to drugs and alcohol intoxication (50).

Recently, a new protein Cystatin D (CTS5), which inhibits lysosomal and secreted cysteine proteases, was also identified as a potential biomarker to assess the severity of TBI and its expression at very early time points, makes CTS5, an ideal biomarker for a point-of-care (PoC) device (45). To the best of our knowledge, none of the previous protein biomarkers has been successfully used in the clinical setting for diagnosis and prognosis of TBI patients.

MicroRNAs as Emerging Biomarkers in TBI

MicroRNAs (miRNAs or miRs) are a class of molecular regulators discovered for the first time in *Caenorhabditis elegans* in 1993 (64). Then, dozens of miRNAs were identified in worms, flies and human suggesting that miRNAs represent a previously unknown group of molecules (65).

miRs are short (∼22 nucleotides) non-coding, single-stranded RNAs that play key roles in the regulation of several biological processes such as cell proliferation and differentiation, survival, and motility via negative feedback mechanism at the post-transcriptional level by binding to the 3′-untranslated region (UTR) of the target miRs and leading to either suppression of the translation process, mRNAs degradation or both. A single miRNA can regulate multiple mRNAs and vice-versa because they do not always require a perfect complementarity for target recognition. Therefore, they can briefly interchange between the cellular programs (66).

The synthesis of miRs begins in the nucleus with transcription by the RNA polymerase II or III producing long primary miRNA transcripts (pri-miRNA) that contain functional secondary structures, termed stem-loops and carrying mature miRNA sequences. Maturation of the pri-miRNA transcripts includes several steps which are initiated by RNase III endonuclease Drosha and produces the precursor-miR (pre-miR) (67, 68). Following Drosha processing, a complex of proteins, exportin-5 (EXP5) with GTP-binding nuclear protein Ran-GTP, transports pre-miR from the nucleus into the cytoplasm where it is cleaved by Dicer and TAR RNA-binding protein (TRBP) (69, 70). This produces a double-stranded RNA molecule composed of 20–24 nucleotide miR and a complementary miR* of the same length (71). It has been found that not only the mature miR strand is biologically active, but also the miR* strand is functional and not just degraded as was previously hypothesized (72). Then, mature miRNAs bind to mRNA molecules through a process facilitated by the RNA-induced silencing complex (RISC), which consists of RNase Dicer, TRBP, PACT (protein activator of PKR) and the Argonaute proteins (68).

The resulting RISC-miRNA complex binds the complementary regions of the target mRNAs by partial or total base-pairing at the 3′ UTR. This interaction, controlled by nucleotides 2–8 at the 5′ end of the miR and known as "seed sequence" (73), reduces protein production by translation inhibition and mRNA degradation (74). However, miRNAs do not target all mRNAs because there are only binding sites in one-third of the mRNAs (75).

Currently, in the human genome, over 2,000 miRNAs were identified and numerous studies were mainly focused on the miRNA profiling in various tissues and biofluids that can aid the diagnosis of a wide range of diseases, including cancer, cardiovascular, nervous system disorders and many other disorders (76, 77, 147). Since miRs are relatively abundant and stable in the human biofluids, they are considered to be better than protein biomarkers and therefore are now being investigated as the new class of markers for numerous pathologies including but not limited to neurodegenerative diseases. However, a better understanding of the biological mechanisms of miRNAs in these diseases is required to improve their application as biomarkers (78).

With the discovery of miRNAs and its critical role as regulators in various diseases, it is now possible to investigate their role as biomarkers and emerging therapeutic targets. Based on the antisense technology, very potent oligonucleotides targeted against miRNA known as anti-miR were developed (79, 80).

TBI research associated with the changes in miRNA expression is only at the beginning to be understood. Few studies showed the miRNA profile in serum plasma and CSF after different TBI severities and at different time points (81–85).

Redell et al. found a downregulation of miR-16 and miR-92a in severe TBI patients and an upregulation of miR-765 in mild and severe TBI, within the first 24 h and by using a microarray approach (81). Bhomia and collaborators analyzed microRNA profile in serum and CSF of patients grouped in three different categories, mild-moderate TBI (MM-TBI), severe TBI and orthopedic injury patients with samples collected within 48 h from injury and compared to healthy volunteers. Eighteen and 20 miRNAs were observed in MMTBI and sTBI groups respectively and among these, 10 miRNAs were present at both TBI severities. Finally, four of these 10 miRNAs were also found in CSF (85). Di Pietro et al. screened 754 microRNAs using TaqMan Array Human MicroRNA A+B cards in mTBI+EC (extra-cranial injury), sTBI+EC, EC only groups and compared the results to healthy volunteers at different time points. Particularly interesting were the results obtained within the first hour from injury, in serum of mTBI+EC. These data reported two microRNAs, miR-425-5p and miR-502, having high diagnostic accuracy (AUC $>$ 0.9) in differentiating mTBI from sTBI (84).

Recently, saliva was also explored as potential source of biomarkers for TBI. Salivary microRNA changes were found to be associated with prolonged concussion symptoms in pediatrics (86). Five miRs (miR-320c-1, miR-133a-5p, miR-769-5p, miR-1307-3p and let-7a-3p) were detected in the patients with prolonged post-concussive symptoms, and three of them; miR-320c-1, miR-629, and let-7b-5p were associated with memory problems, headache and fatigue that were developed 4 weeks after head injury. The same group has also matched miRNA changes in saliva and CSF, identifying six miRs (miR-182-5p, 221-3p, 26b-5p, 320c, 29c-3p, and 30e-5p) with similar changes in both biofluids (87).

A completed list of microRNA detected in different biofluids in TBI patients can be found in **Table 2**. Results presented, were not always consistent. However, it is not always possible to compare these studies, since sample collection timing or the different biofluid analyzed, play a relevant role to uniform the biomarker discovery.

Many microRNAs were also described in the brain of injured animals by using different models of TBI. Some of these studies have also investigated the potential pathobiology of the microRNAs differentially expressed in tissue.

Human miR-21 is one of the most studied miRs in TBI. It is a polycistronic miR (chromosome 17q23.2), and it overlaps

TABLE 2 | MicroRNA differentially expressed according the severity of TBI and the different human biofluid.

Sample	microRNAs	TBI patients	References
Plasma	miR-765, miR-16, miR-92a	Mild and severe	(81)
Plasma	miR-142-3p, miR-423-3p	Mild, moderate, and severe	(83)
Plasma	miR-23b	Severe	(88)
Serum	miR-1255b, miR-151-5p, miR-194, miR-195, miR-199a-3p, miR-20a, miR-27a, miR-27b, miR-30d, miR-328, miR-362-3p, miR-381, miR-486, miR-505*, miR-625*, miR-638, miR-92a, miR-451, miR-1291, miR-130b, miR-19a, miR-20a, miR-296, miR-29c, miR-339-3p, miR-579, miR-601, miR-660, miR-9*	Mild, moderate, and severe	(85)
Serum	miR-425-5p, miR-502, miR-21, miR-335	Mild and severe	(84)
Serum	miR-93, miR-191, miR-499	Severe	(82)
CSF	mir-9	Severe	(89)
CSF	miR-451, miR-328, miR-362-3p, miR-486	Severe	(85)
CSF	miR-141, miR-257, miR-181*, miR-27b*, miR-483-5p, miR-30b, miR-1289, miR-431*, miR-193b*, miR-499-3p, miR-1297, miR-33b, miR-933, miR-449b	Severe	(82)
CSF	miR-182-5p, miR-221-3p, miR-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p	Severe pediatric TBI	(87)
Saliva	miR-182-5p, miR-221-3p, miR-26b-5p, miR-320c, miR-29c-3p, miR-30e-5p	Severe pediatric TBI	(87)
Saliva	miR-320c-1, miR-133a-5p, miR769-5p, miR1307-3p, let-7a-3p, miR629, let-7b-5p	Children with Post-concussion symptoms (PCS)	(86)

miRNA = The RNA strand of the miRNA duplex that is complementary to the mature miRNA is shown with a star symbol (miRNA*).*

with the Vacuole Membrane Protein 1 (VMP1) coding gene, also known as Transmembrane Protein 49 (TMEM49) (90).

Recent studies have demonstrated high miR-21 expression levels after TBI. Also, it has been found to improve the neurological outcome through inhibiting apoptosis and targeting angiogenesis molecules. In particular, the upregulation of miR-21 was found to reduce brain oedema derived by BBB-leakage. Hence, ago-miR-21 treatment was proposed as a potential therapy to decrease BBB damage (91) by inhibiting the loss of occludin and claudin-5 among other tight junction proteins. It also increases the levels of Angiopoietin-1 and its Tie-2 receptor, which maintain the normal BBB condition. MiR-21 was also found to improve experimental TBI mice cognition after the running wheel exercise (92, 103). The therapeutic role of miR-21 might also be due to inhibiting apoptotic cell loss by targeting the phosphatase and tensin homolog (PTEN)-Akt pathway (91). In an interesting study, extracellular vesicles (EV) were isolated from the brain of injured mice and controls, and the expression of miR-21 was found significantly increased with the injury. Concomitantly, an increase of miR-21 in neurons was observed, suggesting miR-21 secretion from neurons by EV cargo (92). Further support via the upregulation of miR-21 was also found in the serum of sTBI patients but not of mTBI, at very early time points and up to 15 days from injury. Also, no increase was found in the musculoskeletal injured patients, and for this reason, miR-21 was considered as a potential new TBI biomarker and a future therapeutic target for TBI (84).

Another exciting miR associated with TBI is miR-16, involved in the regulation of several biological processes activated after TBI; such as being involved in apoptosis by targeting BCL-2 (93) and in the cell cycle by targeting CDK6 (cyclin-dependent kinase 6), CDC27 (cell division cycle 27) and CARD10 (caspase recruitment domain 10) (94, 95, 148). Also, miR-16 was significantly increased within the first 24 h in the mild TBI patients and significantly decreased in the severe TBI patients compared to the healthy volunteers (81). MiR-107 was found to be underexpressed in cortex and hippocampus of a rat model of severe controlled cortical impact (CCI) (96). MiR-107 can regulate granulin (GRN) mRNA, suggesting a role in inflammatory process, energy metabolism and neuron regeneration (104). MiR-27a and miR-23a were downregulated in mouse cortex in a moderate model of CCI and was found to regulate pro-apoptotic Bcl-2 family members (97). Furthermore, miR-711, was upregulated in hippocampus after severe CCI, (96) and was found to reduce the neuronal cell death and lesion volume via Akt-pathway.Let-7i is another exciting biomarker with potential implications in TBI. It was upregulated in the serum and CSF of the rodent model of mild to moderate blast overpressure wave. It might be a potential regulator of many proteins and inflammatory cytokines, including S-100β and UCH-L1 (98). A detailed list of the pathobiology for miRNA differentially regulated in animal models of TBI can be found in **Table 3**.

TABLE 3 | Pathobiology of the main differentially expressed microRNAs in brain of different animal injury models.

microRNAs	Tissue	Animal injury model	Pathobiology	References
miR-21	Cortex\ hippocampus	FPI/CCI	Apoptosis, dendritic spine morphogenesis, Angiogenesis, alleviating BBB leakage, cognition	(90–92, 99–103)
miR-107	Hippocampus	CCI	Neuron regeneration, inflammation	(96, 104)
miR-16			apoptosis, cell cycle	(93–95, 148)
miR-9	Cortex	FPI	Damaging the cytoskeleton and cellular integrity	(105)
mir-27b	Cortex	FPI	Disrupting amino acid and nucleic acid metabolic processes, hindering macromolecule complex assembly	(105)
miR290, miR-497	Cortex	FPI	Intracellular transport	(105)
mir-451	Cortex	FPI	Inflammation	(106)
miR-874	Cortex	FPI	Intracellular transport, apoptosis, inflammation	(105, 106)
miR-34a	Cortex\ hippocampus	FPI	Inflammation, apoptosis,	(106)
mir-144	Hippocampus	CCI	Synaptic function, cognition	(107)
miR-153	Hippocampus	CCI	Cognition	(107)
miR-23a, miR-27a	Cortex	CCI	Apoptosis	(97)
mir-155, miR-223	Hippocampus	CCI	Mitochondria associated miRs, inflammation	(108)
miR-711	Hippocampus\ cortex	CCI	Apoptosis	(96, 109)
let-7i	CSF\ serum	Blast	Regulator of inflammatory cytokines	(98)
miR-92a, miR-674, miR-138, miR-124, let-7c	Cortex	CCI	Behavior	(103)
miR-142-3p, miR-221	Hippocampus	CCI	Cell proliferation and angiogenesis of PDGF signaling pathways	(110)
miR-23b	Plasma\ hippocampus\ cortex	WDI	Reduce lesion volume of contused hemisphere and brain oedema, cognition	(88, 97)

BIOMARKER DISCOVERY: MICRORNA PROFILING

Numerous studies investigated the global profiling of miRNAs in human diseases with the aim to identify a variety of biomarkers when compared the normal and affected tissues, which can further be correlated with the prognosis or the therapeutic response. MicroRNA can be extracted from a variety of sources, including cell lines, fresh tissues, formailin-fixed paraffin embedded (FFPE) tissues and also biofluids such as plasma, serum, urine, saliva and CSF.

Many are the techniques used to analyze microRNAs. Generally, qPCR is suitable to investigate one or two miRNAs, whereas for larger studies examining multiple miRNAs at once, platforms such as TaqMan™Array Microfluidic Cards, miScript miRNA PCR Array or nCounter® microRNA panels, are more suitable. Finally, to discover new miRNA variants,

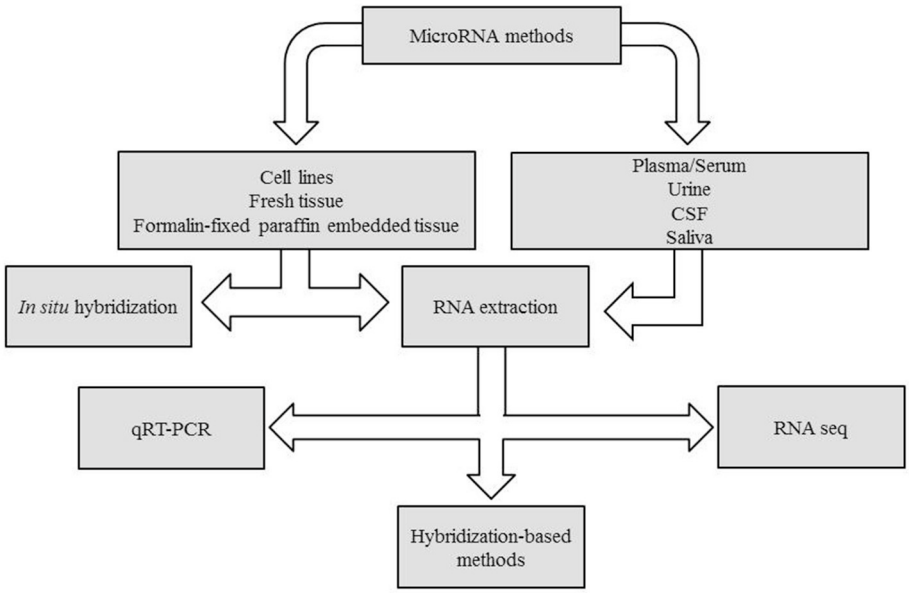

FIGURE 1 | Decision making chart. MiRNAs can be extracted from different sample types, such as tissues and body fluids. The experimental design determines the methodology chosen for miRNA detection.

the Next Generation Sequence (NGS) solution results are more appropriate. In **Figure 1** a decision making chart is represented.

Sampling

Sample processing and storage is the first step to perform miRNA profiling. This step is particularly crucial in order to obtain high-quality microRNA, especially for the determination of miRNA expression in biofluids. MiRNAs are stable in biofluids because of their molecular size and because they are protected within protein complexes or contained within EVs (microvescicles or exosomes). However, an immediate separation of cells is required to prevent lysis of cells and to avoid RNA contamination.

In addition, caution must be taken when collecting plasma or serum. Heparin-plasma for example, is a potent PCR inhibitor (111). Differentially, plasma-EDTA does not affect PCR and can overcome clotting due to platelet activation. In addition, plasma content of miRNA is higher than serum which is confirmed by slightly lower Ct value in the plasma (112).

RNA Extraction

Different kits are commercially available for miRNA extraction from different tissues or biofluids such as miRNeasy (Qiagen), mirVana TM (Ambion) or PureLink TM (Invitorgen) miRNA. The most commonly used kits are based on two main steps. The first one, is a chemical extraction with guanidine thiocynate (e.g., Trizol and QIAzol reagents); the second one, is an extraction procedure based on silica columns. New phenol-free kits were also recently developed such as ISOLATE II miRNA (Bioline) or ReliaPrep^{TM}miRNA (Promega).

Alternative strategies apply magnetic bead–based technology to purify samples, such as TaqMan^{TM}miRNA ABC Purification Kit (Thermo Fisher). All these kits differ, compared to those used for total RNA extraction, for additional steps to enrich the smallRNA fraction (113). Extraction from biofluid samples are particularly challenging, compared to extraction from tissues or cells, because of the lower RNA content, the possibility of hemolysis or platelet contamination and presence of serum proteins (such as RNases and PCR inhibitors). In addition the lack of well-established reference genes makes it difficult to analyze and interpret the data (114).

Nevertheless, several strategies can be used to maximize RNA yield. Among these, RNase-free glycogen, which acts as nucleic acid carrier can be added during the extraction (112). Similarly, other RNA carriers, such as the bacteriophage MS2 RNA, can also help to maximize RNA recovery (115). Therefore, monitoring the efficiency of the RNA extraction by addition of a known amount of synthetic miRNA spike-in is recommended (116). Alternatively, isolation of exosomes from biological fluid can help to increase the amount of retrieved RNA.

Exosomes are vesicles with diameter between 30 and 100 nm, originated from multivesiculated body (MVBs) and released into the extracellular space. The exosomes are able to carry different molecules as mRNAs, miRNAs, lipids and proteins and to transfer their contents to recipient cells, therefore influencing different physiologic and pathologic processes (117). The current techniques to separate exosomes from biological fluids include methods based on exosome size differences, as ultracentrifugation or size exclusion chromatography, or on identification of specific surface markers as immunoaffinity capture-based techniques (118). In ultracentrifugation procedure, the force used ranges from ∼100,000 to 120,000 × g. After the centrifugation step, the exosome pellet is dissolved in phosphate buffered saline (PBS) and subjected to subsequent

centrifugation runs with increasing force. Finally, isolated exosomes can be stored at −80°C until further analysis or in Trizol for RNA extraction. Based on their size, exosomes can also be purified by using membrane filters with 0.2 μm of diameter. This method, although widely used, can result in samples contaminated by others EVs and a large sample volume is requested. Other techniques were also developed to isolate exosomes. The presence of tetraspanins as exosomal surface markers, for example, is used for immunoaffinity reactions and different companies have already developed specific kit, based on affinity spin columns for exosome purification (Invitrogen, Qiagen).

Quantification and Quality Control

The measurement of RNA concentration by using conventional spectrophotometers, such as nanodrop, is not possible for miRNA quantification and quality control (119). However, RNA integrity can be checked by spectrophotometry and automated capillary electrophoresis instruments such as the Bioanalyzer 2100 (Agilent) and Experion (Bio-Rad). In particular, the Bioanalyzer 2100, can also estimate miRNA concentration as the result of the ratio of 15-40nt RNA fragments and the total RNA, (120) providing that RNA integrity is very high. For this reason; it is a common practice to perform the analysis using established volume and not concentration of RNA extracted from the same volume of biofluid or tissue. However, accurate strategies to relatively quantify the sample are still necessary.

miRNA Profiling

The most widely and well established approaches used to determine microRNA profile can be divided into three main categories: quantitative real time PCR (qRT-PCR), hybridisation-based methods (i.e., Microarrays, Nanostring) and high-throughput next generation sequencing. The main advantages or disadvantages in using the above techniques are reported in **Table 4**.

qRT-PCR

qRT-PCR is the most popular technique to accurately assess miRNAs.

The single assay is primarily used to efficiently validate the results of large screening studies or for relatively small experiments.

The technique is relatively expensive and can be divided in two main steps: the conversion of miRNA into cDNA and quantitative polymerase chain reaction.

Because of the length of miRNAs and the lack of a common sequence such as a poly(A) tail that can be used for reverse transcription, cDNA synthesis presents its own challenges.

Two main strategies are used to generate cDNA:

1) The use of a stem–loop RT primer which first hybridizes with the miRNA strand, followed by reverse transcription using MultiScribe reverse transcriptase. cDNA products are then amplified using conventional TaqMan PCR.
2) The addition of a poly(a) tail using *E. coli* poly(A) polymerase (assay). An oligo-dt primer is then used to pair the miRNA tailed and allows the retro-transcription of the resulting cDNA, which is further amplified using specific primers and detected by the use of a fluorescent dye such as SYBER green.

However, large experiments using qRT-PCR can become quite laborious to perform. In order to overcome this problem, reactions can also be carried out in high-throughput form.

Pre-plated PCR primers, for example, are commercially available and distributed typically across multiwall dishes, or alternatively microfluidic cards containing nanoliter-scale wells. However, performing highly parallel qRT-PCR might present some challenge due to differences in primer annealing temperatures. However, it is still possible to solve this issue by using the locked nucleic acids (LNAs) into primers and allowing the optimal hybridisation conditions for several PCR assays to be run simultaneously (114).

qRT-PCR allows both absolute and relative quantification. In the first one, a standard curve from serial dilutions of known concentrations of synthetic miRNA is generated and used to calculate the number of copies of a specific miRNA. In the second case, before setting up the microRNA expression analysis, an endogenous normalizer (reference gene) has to be chosen, among several control candidates tested. These candidates offer stable expression over the whole range of samples, and are selected based on the literature or pre-existing data.

Hsa-miR-16-5p is widely used in the literature as an endogenous miR, despite the lack of a panel of endogenous miRNA consensus (121). Hsa-miR-223 (116) hsa-let-7d-5p (122) hsa-miR-484 (123), hsa-miR-191-5p (124), and hsa-miR-423 (125) were also described as relatively invariant reference genes in plasma/serum. MiR-331 and miR-223 were identified as the most stables in traumatic brain injury patients (84). MiR-202 was also used as normalizer gene in CSF of TBI patients (85).

In addition, to identifying the appropriate endogenous controls, it is also possible to use some software as geNorm Algorithm (https://genorm.cmgg.be/) and DataAssist v.3 software (Applied Biosystems). GeNorm is used to normalize the data from a large and unbiased set of miRNAs. DataAssist is useful to quantify gene expression in samples when using the comparative CT (ΔΔCT) method (126, 127). However, it is always preferable to add a spike-in control during the RNA extraction and to normalize the microRNA using an exogenous control (e.g., cell-miR-39).

Hybridization-Based Methods

Several hybridization-based methods exist to identify microRNA abundance. *In situ* hybridization (ISH) is the most used method to localize DNA or RNA using labeled complementary nucleic acid probes in tissue section or fixed cells (128). However, this technique is not suitable for miRNA detection because of their length, but the introduction of LNA showed a significant improvement in the sensitivity and specificity of this technique applied to miRNAs detection (129). Microarray-based technique is another powerful high-throughput method extensively used for microRNA profiling, because of their ability to screen large number of miRs simultaneously in large variety of samples (from tissue to biofluid). MiRNA microarray is a nucleic

TABLE 4 | Advantages and disadvantages of the main microRNA profiling methods.

Profiling methods	Time	Sample input	when to use it	Advantages	Disadvantages
qPCR/Microfluidics	≤6 h	500/10 ng	small scale experiments large experiments also possible (multiwell dishes or microfluidic cards)	established protocol, high sensitivity and specificity, absolute and relative quantification, used for validation of large scale experiments.	intensive labor, requires quality miRNAs, relatively expensive, cannot identify novel miRNA.
Microarray	∼2 days	100 ng−1 μg	large studies	established protocols, easy and fast, inexpensive.	less sensitive than qPCR, hard distinguishing similar sequences, no absolute quantification.
NextGen Sequencing	1–2 weeks	500 ng−5 μg	discovery phase	whole content analysis, single base resolution, not depending on any prior sequence knowledge, can detect low abundance transcripts, can detect new miRNAs.	equipment costs, bioinformatics support, less sensitive than qPCR, no absolute quantification.

acid hybridization technique which uses amino-modified 5' termininal complementary probes immobilized onto glass slides through covalent crosslinking between the amino-groups and the self-assembling monolayer (130). After RNA purification, miRNAs are tagged with fluorophore-labeled nucleotides at their 3′ end. LNAs can also be incorporated into capture probes to increase specificity and sensitivity (131). The main advantages of using micrarray are the low costs and the parallel measurements. Typically microarray involves a comparison between two or more groups and cannot be used to determine absolute quantification. Because of limited specificity, data obtained are typically validated by a qRT-PCR.

A new technology, the Nanostring nCounter Analysis System, was recently developed to allow the quantification of more than 800 RNA molecules in 12 samples, in a single assay. The nCounter Analysis System is a very new technology which uses digital color-coded barcode for precise multiplexed measurement of the gene expression (<1 copy per cell). This system is more sensitive than microarrays and as sensitive and accurate as qRT-PCR. The combination of color-coded barcode attached to a single target-specific probe corresponding to a gene of interest and the single molecule imaging, allows detecting and counting hundreds of unique transcripts in a single reaction. Each color-coded barcode represents a single target molecule. No amplification is required (132).

Finally, to identify the significant differentially expressed miRNAs in a large genomic data, such as the microarray data but also the microfluidic card and the RNA sequencing data, the most frequently used method is the Significance Analysis of Microarrays (SAM) computed by Multi Experiment Viewer (MEV) v4.8.1 (http://www.tm4.org).

RNA Sequencing

The introduction of the next generation sequencing has become increasingly popular in biomedical research, overcoming the limitations of the microarray analysis (133). While it cannot quantify miRNA levels with the same resolution of qPCR, it still has the advantage to detect all known or unknown miRNAs present in a sample and to precisely distinguish all isoforms in the absence of background and cross-hybridization problems. IsomiRNAs, indeed are miRNA containing sequence variations, typically by shortening or lengthening of the 3′ end. Over 3,300 miRNA variants were identified and reported at the following website http://galas.systemsbiology.net/cgi-bin/isomir/find.pl. However, one or two isomers contribute to >90% of the signal detected, while the remaining variants are not abundant enough to be revealed.

The procedure consists in a small-RNA cDNA library preparation followed by "massive parallel" sequencing on a single run. First of all, miRNA fragments are extracted from total RNA. Running the sample on an agarose gel and cutting out the band corresponding to the miRNA size is the second step. Then, the selected RNA fragments are ligated to sequencing adapters and transcribed into cDNA by ∼12–15 RT-PCR cycles of amplification and using a reverse transcription primer which hybridizes to the 3′ adapter.

At this point, another run on agarose gel of the obtained cDNA library is performed and the band with size corresponding to the length of adapter sequences plus the miRNA insert of ∼20–30 bases (for a total length of 120 bp) is cut out and ready for sequencing. The gel size selection is particularly crucial because of the potential presence of adapter dimer side products created during the ligation step as well as higher molecular weight products generated from ligation of other RNA fragments, such as tRNA and snoRNA, containing 5′ phosphate groups.

Significant computational resources and bioinformatics expertise are required for data interpretation not only for known miRs but also for the newly discovered miRs. Initially all generated reads are aligned to the reference genome of the sequenced organisms. Short read aligner tools are available to process the reads such as maq (http://maq.sourceforge.net/maq-man.shtml), sop (http://soap.genomics.org.com) or bwa (http://bio-bwa.souceforge.net/). In addition, it is also important to filter

out reads that align against other non-coding small RNA species and RNA degradation products which sequences are available on the University of Santa Cruz (UCSC) Genome Browser.

Another bioinformatics challenge is the relative quantification. Expression levels are analyzed on the base of the read counts for each sequenced sample. The number of reads of each individual molecule is normalized against the total number of reads produced in the same sample (134).

Different tools are also available to predict novel miRNAs from generated data. One of the commonly used is mirDeep (https://www.mdc-berlin.de/8551903/en/) (135).

Although the NGS is one of the most advanced techniques currently used, other challenges, beside the bioinformatic support, need to be faced. One of these is the cost required for equipment, software and consumables. In addition a high quality of purified RNA and a large amount of RNA, usually 5 μg, are required for the analysis. Validation is another important aspect to address in order to use this technology for diagnosis and prognosis of diseases.

MicroRNA Database and Target Prediction

Since miRNAs control the regulation of several genes and they are linked to many disorders, it is also possible to reliably predict potential miRNA targets which can be involved in these pathologies. The prediction of the mRNA targets is based on the partial complementary sequences between the mature miR and the mRNA candidate target. This search is generated by miRNA target prediction algorithms able to seek for putative binding sites in the 3′UTRs of the candidate mRNAs (i.e., PicTar, TargetScan, DIANA-microT, miRanda, rna22). High complementarity between the miRNA and the target binding region results in the degradation of the target, whereas the presence of mismatches represses the translational process. However, results of their applications are often not consistent and must be experimentally validated. Many lab-based techniques can be used to overcome the challenge of target validation such as the inverse correlation between the expression of miRNA and its target, the effect on protein expression /function or a direct validation by using the luciferase assay or their functional effects (proliferation, differentiation, apoptosis) on a cell culture system.

In addition, databases such as mirTarBase or miRewcords collect both predicted and experimentally confirmed miRNA targets.

Finally, functional analysis of miRNAs or miRNA high-throughput data sets can also been performed. For example, Gene Ontology (GO) analysis is commonly used to identify pathways and processes from a list of genes provided, for example from results obtained using gene expression microarray (136) or generated from a target prediction tools in the case of miRNA (137).

LIMITATIONS IN THE USE OF MIRNAS AS CIRCULATING BIOMARKERS

The use of miRNA signature as a novel diagnostic/prognostic tool is still in the descriptive phase. Numerous data have been collected so far, in various disease states; however their translation in clinical applicability requires much larger studies and universally implemented guidelines.

First of all, the lack of methodological details in published papers makes it difficult to directly compare the results, and lead to inconsistent or even contradictory results.

Standard protocols must be achieved for the different steps of miRNA analysis such as sample processing, RNA extraction and expression measurement/assessment methods as well as differences in specimen type, for example FFPE *vs.* fresh frozen samples, must be considered.

In addition, the research of miRNA profile in biofluids is particularly challenging as miRNAs, circulate either associated with proteins, lipoproteins or EVs, and this might require specific precautions during the extraction or analysis processes. Moreover, it is good practice to check the presence of small clots and hemolysis in plasma/serum which may contribute to the variability in miRNA expression.

Furthermore, we are not aware if miRNA expression varies at specific conditions such as: fasting or circadian rhythm, thus, standardization and annotation of these protocol details is necessary in order to minimize variability of unknown factors.

Data normalization, identification of well-characterized endogenous miRs specific to biofluid and pathology of interest, as well as characterization of baseline levels for miRs described as potential biomarkers are other crucial points in obtaining accurate results.

Certainly, a common information infrastructure for data exchange, analysis and protocols used would facilitate research in the miRNA biomarker discovery.

POINT-OF-CARE DIAGNOSTIC TOOLS TO DETECT CIRCULATING MICRORNAS AS BIOMARKERS OF DISEASE

Besides the challenge of biomarker discovery, there lies the challenge of rapidly detecting them with clinically relevant sensitivity and specificity using a low-cost and easy point-of-care injury test.

In the case of traumatic brain injury, a PoC technology would have several applications. This is particularly true for mTBI which represents a serious problem in military, and contact sports that has led to reduction in the sport participation in younger age groups.

The development of a pitch-side or "pre-hospital," portable TBI diagnostic devices, would implement the current guidelines in the management of mTBI (17, 138) in different ways:

1) In the initial pre-hospital assessment to determine whether patients should be transferred to a Major Trauma Centre or a local Trauma Unit.
2) In the Emergency Department (ED), to determine the need for a CT brain scan.
3) Pitch-side, to assist decision making as to removal from play and assessment of the need to take the player to the ED.
4) In sports clinics, to diagnose a concussive event and guide return to play.

5) In combat theaters, to determine the need to dispatch a rescue team.

So far, proteins were widely explored as biomarkers and immunoassays are extensively used as method of detection, although not often very sensitive and prone to false positives (139).

The PCR amplification method has played an important role in diagnostics over the last years because of its ability to detect few molecules (140) and the fact that microRNAs are particularly stable in biofluids, positions them as a new valid potential biomarkers to explore. MicroRNAs are also particularly suitable for these clinical applications as they are molecular switch regulators and for this reason their early expression anticipates the molecular mechanisms trigged by TBI.

Several companies are now working on point-of-care device that can measure microRNAs in the field. This is quite challenging, although not insurmountable, as microRNAs are present in femtomolar and picomolar concentrations and need to be extracted from the biofluid first.

Micorfluidics is another challenging problem. Transporting the methodology in a portable device, reducing the volume to few microliters over a few millimeters and mixing the rinsing solutions and all reagents are main issues.

Detection is another important point to discuss; various strategies were developed to improve the detection of miRNA (141).

Nanoparticles(NPs)-based biosensors, for example, are widely studied. The use of this biosensor has the potential to miniaturize the equipment and reduce the cost. In particular, carbon and metal-based NPs, such as gold nanoparticles (AuNPs) are excellent miRNA carriers that can be used to accelerate the signal transduction enhancing a rapid analysis and lowering the detection limit. Recently, a dual-function gold nanoparticle biolaben was used to detect miR-21 in serum (142).

Magnetic nanoparticles are also very popular. Wanunu et al. (143) developed a protocol using probe:miRNA duplex binded to p19-functionalized magnetic beads, which are first eluted and electronically detected using a nanopore (143).

Optical detection in combination with NP probes was also explored in the development of a novel highly specific and reproducible platform, the Scanometric MicroRNA (Scano-miR), to detect low concentrations of miRNAs (144).

Surface plasmon resonance (SPR) biosensors, is another example of label-free optical biosensing technologies. This method is based on optical measurement of refractive index changes given by the binding of analyte molecules present in samples to specific receptors immobilized on the SPR sensor. This method showed to be able to detect miRNA in <30 min at concertation down to 2 pM (145).

Finally, enzyme catalytic amplification-based electrochemical assay are also developed for this purpose (146).

However, hard work is still required to develop a reliable portable PoC device.

CONCLUSIONS AND PERSPECTIVES

miRNA profiling and detection provide valuable information on their essential roles in normal cellular function and disease, projecting their use in the clinical practice for the diagnosis and prognosis of several pathologies. With this review, our aim was to provide insights into the miRNA expression in TBI, the main commonly used detection methods to discover new biomarkers and the state-of-the art of the PoC development.

Despite their limited use as routine biomarkers, several companies already offer miRNA-based diagnostic assays.

In addition, there are new emerging classes of non-coding RNA such as piwi-interacting RNAs, and long non-coding RNA (lncRNA) that have important role in cellular physiology.

In the future, profiling methods that have the potential to detect all the RNA classes are likely to improve the understanding of the whole transcriptome and provide new valid information for the diagnosis, prognosis and therapy of several pathologies, including TBI.

AUTHOR CONTRIBUTIONS

VD drafting the article and final approval of the version to be published. KY drafting the article. US drafting the article. CD critical revision of the article. AB critical revision of the article.

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Does Severity of Alzheimer's Disease Contribute to Its Responsiveness to Modifying Gut Microbiota? A Double Blind Clinical Trial

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Alzheimer's disease (AD) is associated with cognitive dysfunction. Evidence indicates that gut microbiota is altered in the AD and, hence, modifying the gut flora may affect the disease. In the previous clinical research we evaluated the effect of a probiotic combination on the cognitive abilities of AD patients. Since, in addition to pathological disorders, the AD is associated with changes in oxidant/antioxidant and inflammatory/anti-inflammatory biomarkers, the present work was designed to evaluate responsiveness of the inflammatory and oxidative biomarkers to the probiotic treatment. The control (CON) and probiotic (PRO) AD patients were treated for 12 weeks by the placebo and probiotic supplementation, respectively. The patients were cognitively assessed by Test Your Memory (TYM = 50 scores). Also serum concentrations of nitric oxide (NO), glutathione (GSH), total antioxidant capacity (TAC), malondialdehyde (MDA), 8-hydroxy-2′ -deoxyguanosine (8-OHdG) and cytokines (TNF-a, IL-6, and IL-10) were measured. The cognitive test and the serum biomarkers were assessed pre- and post-treatment. According to TYM test 83.5% of the patients showed severe AD. The CON (12.86% ± 8.33) and PRO (−9.35% ± 16.83) groups not differently scored the cognitive test. Not pronounced change percent was found in the serum level of TNF-α (1.67% ± 1.33 vs. −0.15% ± 0.27), IL-6 (0.35% ± 0.17 vs. 2.18% ± 0.15), IL-10 (0.05% ± 0.10 vs. −0.70% ± 0.73), TAC (0.07% ± 0.07 and −0.06% ± 0.03), GSH (0.08% ± 0.05 and 0.04% ± 0.03) NO (0.11% ± 0.06 and 0.05% ± 0.09), MDA (−0.11% ± 0.03 and −0.17% ± 0.03), 8-OHdG (43.25% ± 3.01 and 42.70% ± 3.27) in the CON and PRO groups, respectively. We concluded that the cognitive and biochemical indications in the patients with severe AD are insensitive to the probiotic supplementation. Therefore, in addition to formulation and dosage of probiotic bacteria, severity of disease and time of administration deeply affects results of treatment.

Keywords: probiotics, Alzheimer's disease, cognition, inflammation, oxidative stress, microbiota

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease accounting for 60–80% of all dementia (1). It is characterized by progressive declines in cognitive and functional abilities, neuropsychiatric symptoms, caregiver burden and premature death (2). Particularly, it is accompanied with early impairment of episodic memory (3, 4) followed by progressive deficits in short term memory leading to a final procedural memory deficit (5). AD is etiologically resulted of the extracellular accumulation of amyloid-β plaques, formation of neurofibrillary tangles, neuroinflammation, neuronal injury, and synapse loss (6). In recent studies, neuroinflammation and oxidative stress are particularly considered for their important role in the pathogenesis of AD (7, 8). Especially, growing evidences indicate that both oxidative stress and inflammation are involved in the mechanisms associated with Aβ-induced neurotoxicity (9).

Cholinesterase inhibitors such as donepezil, rivastigmin, and galantamin are approved for treatment of mild to moderate degree of AD. Also the partial NMDA receptor antagonist memantin is prescribed in the moderate to severe of AD (10). However, in spite of fanciful advances in therapeutic aims, AD still lack effective medications. Therefore, novel and effective treatments research have focused on other resources of treatment (11). The microbiota, the ecological community of commensal, symbiotic, and pathogenic microorganisms literally sharing our body space, includes more than 10 times the number of host cells to human cells (12). Emerging studies indicate that the microbiota may contribute to the regulation of multiple neurochemical and neurometabolic pathways through a complex series of highly interactive and symbiotic host-microbiome signaling systems (13–15). This interconnection of the gastrointestinal tract and the central nervous system (CNS) is known as microbiota-gut-brain axis (16) that regulates brain function and behavior. Emerging data demonstrates that certain pathologies, related to an altered microbiome, are linked to mood, stress, behavior, and cognition (17). Some complications such as cognitive disorders, oxidative stress, neuroinflammation, which are also observable in AD, are identified to be influenced by the gut flora as well as probiotics. Probiotics are defined as the living microorganisms with the beneficial effects for humans and animals when administered in a sufficient number. The most commonly used probiotics are the strains of lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium*. Preclinical research shows that probiotics may improve cognitive performances in the animal models with impaired cognition (18). It is reported that probiotics inhibit oxidative stress via reducing inflammation and increasing antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (19).

Test Your Memory (TYM), introduced by Brown et al. (20), is a brief test designed to detect AD. It includes a series of 10 cognitive tasks consists of orientation, ability to copy a sentence, semantic knowledge, calculation, verbal fluency, similarities, naming, visuospatial abilities, ability to do the test and recall of a copied sentence giving a possible total of 50 scores. Importantly, the developers of the test believe that education and social class have only mild effects on the TYM score (20).

In our previous work we reported for the first time the effect of a probiotic supplementation on the cognitive as well as metabolic status of the patients with AD (21). In the present work using a different formulation of probiotic combination we applied the TYM test as a cognitive tool to assess the cognitive status of the people suffering from AD treated by a mixture of probiotic bacteria. Further, because of deep changes in balance of the oxidant/antioxidant and inflammatory/anti-inflammatory biomarkers in the AD, in the present work we also considered how the serum concentration of the inflammatory and oxidative biomarkers responds to the oral bacteriotherapy.

MATERIALS AND METHODS

Participants

This clinical study was performed as a randomized, double-blind, and placebo-controlled clinical trial. Participants included in this study were people with AD (65–90 years old) residing at Emam Ali (Tehran, Iran), Golabchi (Kashan, Iran), Miad (Kashan, Ravand, Iran), and Barekat (Aran and Bidgol, Iran) Welfare Organizations between June 2017 and August 2017. AD patients were diagnosed based on the NINDS-ADRDA criteria (22) and revised criteria from the National Institute on Aging-Alzheimer's Association (23). For further proof the AD patients were compared with the cognitively intact people based on the TYM cognitive test. Accordingly, the participants gaining TYM scores in level of AD (<45 out of 50 scores) were entered the study (see section "Results" for details). Patients with metabolic disorders, chronic infections and/or other clinically relevant disorders were excluded from the study. Standard formula for clinical trials was used to calculate sample size for the study. Based on a previous study (24), considering type one error (α) of 0.05 and type two error (β) of 0.20 (power = 80%) we used 1.3 as SD and 1.1 as the difference in mean (d) of TYM as key variable. Accordingly, we needed 25 persons in each group. Assuming 5 dropouts in each group, the final sample size was determined to be 30 persons per group. **Figure 1** explains flow of subject selection assigned as CON and PRO groups enrolled in the study.

Ethical Approves

This clinical trial was done in accordance with the Helsinki Declaration of 1975. Also, performing the study was approved by the ethics committee of Kashan University of Medical Sciences (KUMS) and registered in the Iranian Website for Registration of Clinical Trials IRCT (IRCT number: 2017061534549N1). Written Informed consent was received from all patients.

Study Design

At the onset of the study, all subjects were matched for disease severity based on gender, BMI, and age. Participants were then randomly divided into two groups: the control (CON) group ($n = 23$ including 13 females and 10 males) receiving placebo capsules containing 500 mg maltodextrine and, the probiotic (PRO) group ($n = 25$: 18 females and 7 males) receiving capsules containing a mixture of probiotic bacteria (see below for details). The treatment was lasted for 12 weeks. Participants were requested to have no change in their regular physical activity and consume no nutritional supplements during the clinical trial.

Intervention

It would be more appropriate to use strains of probiotics for human consumption derived from the human intestinal tract, well characterized, able to outlive the rigors of the digestive tract and possibly colonize, biologically active against the target as well as to be stable and amenable to commercial production and distribution (25). Very rare reports are evident about effectiveness of probiotic bacteria on cognitive phenomena (26–29). In our previous works we tested mixtures of probiotics containing both *Lactobacillus* and *Bifidobacterium* genera (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum,* and *Bifidobacterium bifidum*). In the present study we tested the effect of other multispecies probiotics containing different stains and species of the genera *Lactobacillus* and *Bifidobacterium*. We prepared two types of capsules each containing 3 bacteria (with a total dosage of 3×10^9 CFU) including either *Lactobacillus fermentum*, *Lactobacillus plantarum,* and *Bifidobacterium lactis* (provided by Zist Takhmir Company, Tehran, Iran) or *Lactobacillus acidophilus*, *Bifidobacterium bifidum*, and *Bifidobacterium longum* (provided by Milad Farmed Company, Tehran, Iran). The probiotic group received one of each capsule every other day.

Anthropometric Considerations

Weight and height of the participants were determined in an overnight fasting status using a standard scale (Seca, Hamburg, Germany) before and 12 weeks after the treatment. BMI was calculated as: weight (Kg)/height (m^2).

Outcome Evaluation

The TYM test was used to evaluate the level of cognition in the patients and the TYM results were considered as the primary outcomes. However, the two questions in the semantic knowledge section of the TYM were substituted by questions familiar to Iranian people. In the first question the subjects were asked to name the Iranian president rather than the UK prime minister. Also the second question of semantic knowledge "In what year did the 1st World War start?" uncommon knowledge in Iran, was changed to "In what year did the war between Iran and Iraq?" As a secondary outcome the findings of the oxidative stress and inflammatory biomarkers were assessed.

Assessment of Biochemical Parameters

Twelve-hour fasting blood samples were collected by venipuncture before and after the intervention. The blood samples were taken according to a standard protocol and centrifuged (Hettich D-78532, Tuttlingen, Germany). Then, the samples were stored at −80°C until analysis. Serum total antioxidant capacity (TAC) was quantified using the method of ferric reducing antioxidant power method developed by Benzie and Strain (30). The method of Beutler et al. was used for measuring total glutathione (GSH) (31). Plasma concentration of malondialdehyde (MDA) was measured by the thiobarbituric acid reactive substance method (32).

The serum high sensitivity concentrations of IL-6, IL-10, and TNF-α were measured using commercial ELISA kit (Diaclone, French). Serum high sensitivity 8-hydroxy-2′ -deoxyguanosine (8-OHdG) concentration was also quantified by use of commercial ELISA kit (Zelbio, Germany). Plasma nitric oxide (NO) was quantified by the Griess method (33).

Statistical Methods

Kolmogrov-Smirnov test was applied to the data to determine normal distribution of the variables. Possible differences in anthropometric measures were detected using unpaired student *t*-test. The effect of probiotic supplementation on the TYM cognitive test, oxidative stress biomarkers, and inflammatory factors, determined by one-way analysis of variance (ANOVA) followed by Tukey's post test. The change before and after the intervention was calculated as: % (post-treatment value–pre-treatment value)/ pre-treatment value. The differences between the healthy and AD participants, between the participants with moderate and severe AD, and between the changes in the CON and PRO groups were determined by unpaired student *t*-test. The data are reported as mean ± SEM. The differences were significant if P value was <0.05. All statistical analyses were performed using SPSS—version 18.

RESULTS

Anthropometric Characteristics of the Patients

The patients assigned to the CON and PRO groups weighed 80.57 ± 1.79 and 79.70 ± 1.72 kg, respectively at the onset of the study. BMI index of the subjects enrolled in the study showed no difference over 12 weeks. The change percent of BMI was 0.11 ± 0.29 and 0.74 ± 0.34 in the CON and PRO groups ($P > 0.05$), respectively (**Table 1**).

Confirmation of Alzheimer's Disease in the Patients Based on the TYM Cognitive Test

The TYM test was used to further confirm the healthy and Alzheimer's subjects. TYM is a cognitive tool designed for detection of AD that includes 10 cognitive tasks with a total of 50 scores. Therefore, the participants were firstly introduced to the TYM cognitive test. The subjects assigned as cognitively intact people gained an average of 44.55 ± 0.37 scores. This value is near to the criterion in the TYM test (34) to confirm lack of any dementia. On the other hand, the people assigned as AD patients committed an average of 14.51 ± 1.40 scores.

TABLE 1 | Anthropometric characteristics of the patients.

		CON group ($n = 23$)	PRO group ($n = 25$)	*P* value
Gender	Male	10	7	0.42
	Female	13	18	0.25
Age (year)		80.57 ± 1.79	79.70 ± 1.72	0.36
Weight (kg)	Before treatment	60.63 ± 1.26	60.12 ± 1.12	0.42
	After treatment	60.58 ± 2.36	60.32 ± 1.42	0.48
Height (cm)		156.43 ± 1.86	156.77 ± 1.23	0.38
BMI (kg/m^2)	Before treatment	24.44 ± 1.33	24.05 ± 1.07	0.47
	After treatment	24.56 ± 1.34	24.21 ± 1.06	0.45

CON, control; PRO, Probiotic.

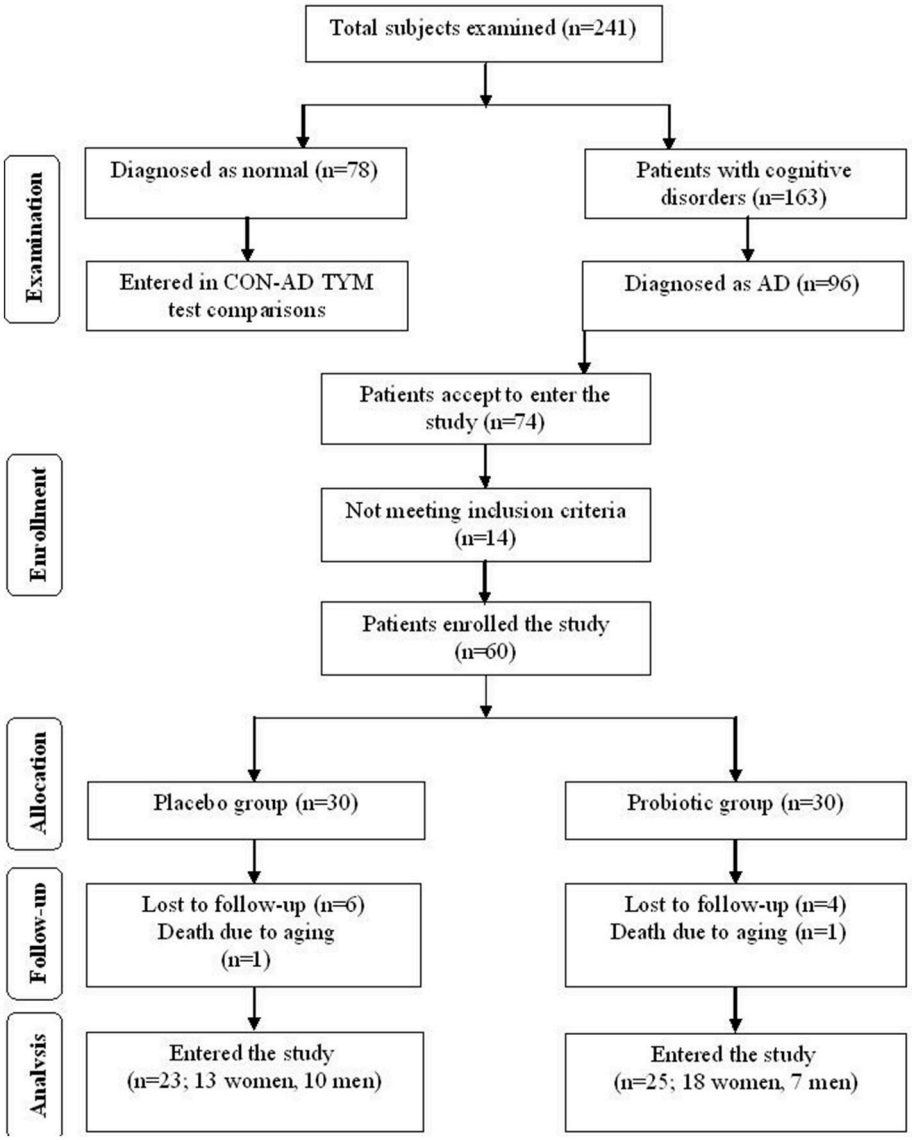

FIGURE 1 | Summary of patient flow.

Such a score in the TYM test fall in range of severe AD (scores <25). Unpaired student *t*-test indicated a considerable significant ($P < 0.0001$) between the cognitively intact people and those with AD (**Figure 2**). Then, the AD patients were divided in to two CON and PRO group treated by placebo (maltodextrine) and probiotic bacteria, respectively. Of them, a majority of 83.5% showed a severe AD and the rest (16.5%) had a moderate disease. The people with severe AD gained 10.63 ± 1.35 score and those with moderate AD gained 28.7 ± 4.47 score. The scores achieved by the moderate and severe AD patients entered the study are illustrated in the **Figure 3**. In the CON patients 17.86% were fallen in a range of moderate AD committing a mean score of 30 ± 4.62. The rest (82.14%) of the patients fell in a range of severe AD, gaining a score of 9.57 ± 1.37. In the PRO group, 16.13% of patients gained 27.14 ± 4.32 scores showing a moderate AD. The rest of patients ($n = 26$, 83.87%) gained 11.69 ± 1.33 scores confirming a severe disease. Consequently, the percentage of patients with mild and severe disease was almost the same in both CON and PRO groups.

The Effect of Probiotic Supplementation on TYM Test

The TYM cognitive test was taken from the CON and PRO group before and 12 weeks after the intervention. The pre- and post-treatment TYM scores in the PRO group were 14.64 ± 1.71 and 17.42 ± 2.42, respectively; the values in the CON patients were 14.35 ± 2.27 and 17.47 ± 2.89, respectively (**Table 2**). Analysis of variance indicated no difference between the two groups [$F_{(3, 114)} = 0.29$, $P < 0.82$]. The change percent between the scores achieved at the onset and offset of the trials was 12.86% ± 8.33 in the CON group and −9.35% ± 16.83

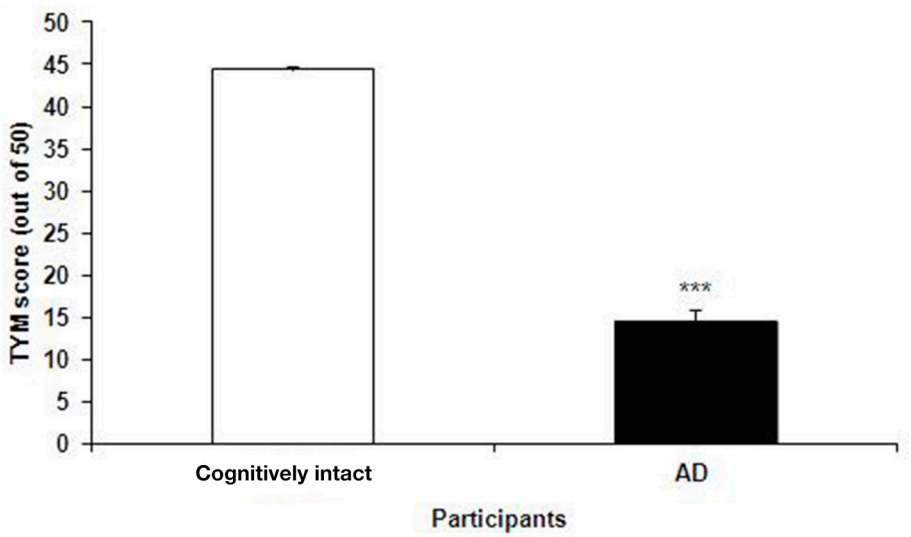

FIGURE 2 | The scores of the TYM test achieved by the cognitively intact people and those with Alzheimer's disease (AD). The scores gained by the cognitively intact and AD participants were 44.55 ± 0.37 against 14.51 ± 1.40, respectively. Unpaired student *t*-test indicated a significant difference between the two groups (***$P < 0.0001$).

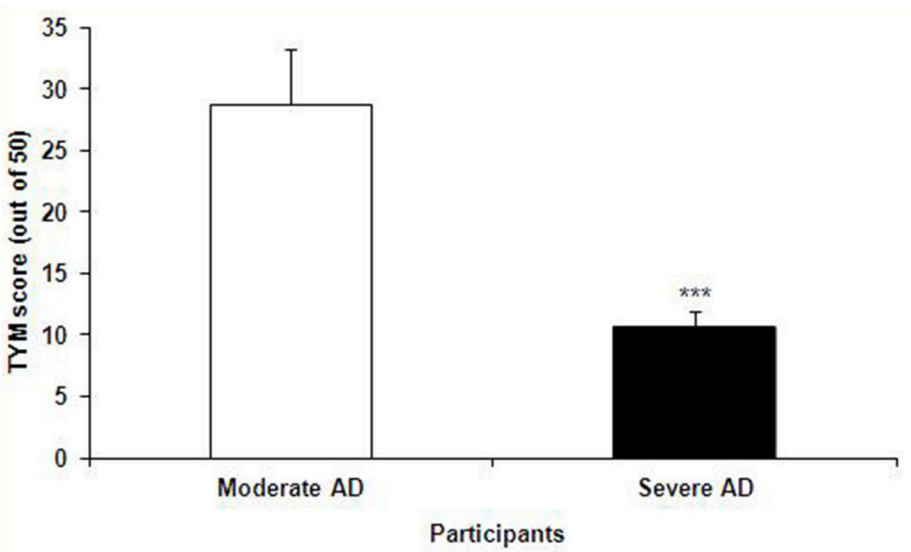

FIGURE 3 | The TYM scores achieved by the patients with moderate and severe Alzheimer's disease (AD); the former group gained 28.7 ± 4.47 score and the latter 10.63 ± 1.35 score. Unpaired student *t*-test showed a significant difference between the two AD groups (***$P <$ 0.0001).

in the PRO group, however, the unpaired student *t*-test indicates no statistical difference between the two groups (**Table 3**).

Inflammatory/Anti-Inflammatory Factors

Analysis of variance applied on the data taken from the CON and PRO groups showed that treatment of the AD patients with the probiotic supplementation not significantly affected TNF-α [$F_{(3, 63)} = 0.64$; $P > 0.05$], IL-6 [$F_{(3, 71)} = 0.27$; $P > 0.05$], and IL-10 [$F_{(3, 63)} = 0.64$; $P > 0.05$]. The change percent in the serum level of TNF-α, IL-6, and IL-10 in the CON group was 0.35% ± 0.17, −1.67% ± 1.33, and 0.05% ± 0.10, respectively. The values in the PRO group were −0.15% ± 0.27, 2.18% ± 0.15, and −0.70% ± 0.73, respectively. The pre- and post-treatment values are shown in the **Table 2** and the change percent are given in the **Table 3**.

Oxidants/Antioxidants Factors

A general statistical difference was observed concerning TAC between the two group [$F_{(3, 81)} = 4.42$, $P < 0.01$]. However,

TABLE 2 | Pre- and post-treatment cognitive scores and biochemical values in the CON and PRO groups.

	CON group		PRO group		Difference between the two groups
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment	*P* value
TYM (score out of 50)	14.35 ± 2.27	17.47 ± 2.89	14.64 ± 1.71	17.42 ± 2.42	0.82
TAC (μmol/L)	868.75 ± 42.45	885.72 ± 29.05	1029.97 ± 23.41	937.07 ± 16.06	<0.01
GSH (μmol/L)	640.78 ± 19.77	731.55 ± 37.33	727.82 ± 22.01	759.01 ± 28.29	>0.05
MDA (μmol/L)	3.07 ± 0.07	2.98 ± 0.07	3.20 ± 0.07	2.87 ± 0.06	<0.05
NO (μmol/L)	39.12 ± 1.81	47.75 ± 3.45	35.94 ± 2.43	38.06 ± 0.75	<0.01
TNF-α(pg/ml)	0.63 ± 0.03	2.13 ± 1.07	1.89 ± 0.72	1.56 ± 0.44	>0.05
IL-10(pg/ml)	0.59 ± 0.01	0.76 ± 0.17	0.73 ± 0.12	0.59 ± 0.01	>0.05
IL-6(pg/ml)	3.58 ± 1.04	3.37 ± 1.41	3.42 ± 0.61	4.51 ± 1.29	>0.05
8-OHdG(ng/ml)	43.72 ± 6.31	41.80 ± 6.19	43.25 ± 3.01	42.70 ± 3.27	>0.05

The values in the two groups are compared by one-way ANOVA followed by Tukey's post-test.
CON, control; PRO, Probiotic.

TABLE 3 | Comparison of the change percent of the biochemical factors between the control and probiotic groups.

	CON Group (Change%)	PRO group (Change%)	*P* value
TYM (score out of 50)	0.12 ± 0.08	−9.35 ± 16.83	>0.05
TAC (μmol/L)	0.07 ± 0.07	−0.06 ± 0.03	0.058
GSH (μmol/L)	0.08 ± 0.05	0.04 ± 0.03	0.26
MDA (μmol/L)	−0.11 ± 0.03	−0.17 ± 0.03	0.11
NO (μmol/L)	0.11 ± 0.06	0.05 ± 0.09	0.30
TNF-α (pg/ml)	0.35 ± 0.17	−0.15 ± 0.27	>0.05
IL-10 (pg/ml)	0.05 ± 0.10	−0.70 ± 0.73	>0.05
IL-6 (pg/ml)	−1.67 ± 1.33	2.18 ± 0.15	<0.001
8-OHdG (ng/ml)	−0.33 ± 0.37	−0.02 ± 0.12	0.21

The values between the two groups were compared by unpaired student t-test.
CON, control; PRO, Probiotic.

within group analysis indicated no considerable difference between the pre- and post-treatment values in the two groups. The change percent in the CON and PRO groups were 0.07% ± 0.07 and −0.06% ± 0.03, respectively.

We observed no variation in the serum level of GSH in the two testing groups [$F_{(3, 82)} = 4.25$, $P > 0.05$]. The change percent in the CON and PRO groups were 0.08% ± 0.05 and 0.04% ± 0.03, respectively.

Although ANOVA showed a significant difference in serum level of NO between the CON and PRO patients [$F_{(3, 81)} = 4.42$, $P < 0.01$, **Table 2**], however, the *post-hoc* test indicates that the change percent in the CON (0.11% ± 0.06) and PRO (0.05% ± 0.09) groups was ignorable (**Table 3**). Despite ANOVA confirms a general variation in the MDA concentration of serum in the CON and PRO groups [$F_{(3, 82)} = 3.50$, $P < 0.05$, **Table 2**] the post-test indicates a negligible change percent of MDA in the CON (−0.11% ± 0.03) and PRO (−0.17% ± 0.03) patents (**Table 3**). The pre- and post-treatment change in the serum level of the DNA degrading factor 8-OHdG showed not statistically to be considerable [$F_{(3, 71)} = 0.02$, $P > 0.05$]. The change percent values are −0.33 ± 0.37 and −0.02 ± 0.12, respectively. The **Table 2** explains analysis of pre- and post-treatment values in the CON and PRO AD patients based on ANOVA. The change percent in the CON and PRO groups analyzed based on unpaired student *t*-test are shown in **Table 3**.

DISCUSSION

The pathologic features of AD are multifaceted, including precipitation of extracellular amyloid-β plaques, formation of intracellular neurofibrillary tangles and damage to neuronal synapses (28). However, these aspects of AD are easily researchable in experimental studies on animal models. Therefore, in level of investigation, histological changes in the AD patients were not under focus of this study. Despite, some other indications which are also observable in AD patients are measurable in clinical researches. Hence, this study was devoted to evaluation of alterations in the oxidants/antioxidant and inflammatory/anti-inflammatory factors as well as cognitive status in the AD patients.

We asked if reinforcement of the gut microbiota by probiotic bacteria influences the cognitive as well as biochemical disorders in the AD patients. Using the TYM test, cognitive status of the AD patients was analyzed. As illustrated in the **Figure 2** the scores achieved by the AD patients are less than one third compared to the cognitively intact people, confirming a real dementia in the group assigned as AD. The AD patients were given a formula of probiotic supplementation consisting both Lactobacilli and Bifidobacteria. The cognitive TYM assessment indicated no difference between the CON and PRO groups. Our results showed that the intervention not pronouncedly influenced either inflammatory (TNF-α and IL-6) or anti-inflammatory (IL-10) factors. Also, our findings indicated that the oral bacteriotherapy had no considerable effect on the oxidants (MDA and 8-OHdG) or antioxidant (TAC, GSH) factors.

In healthy humans, there is a close mutualistic and symbiotic relationship between gut microbiota and the body. This normal state of the human intestinal microbiota is called eubiosis. Any distortion from eubiosis, linked with whether a decrease of intestinal biodiversity or increase of pathogenic bacteria, is called dysbiosis. The dysbiosis results in alteration of the immune system of the gut mucosa and the rise of inflammatory,

immune, metabolic or degenerative diseases (35). Emerging evidence indicates that change in gut microbiota composition may contribute in some neurological disorders. Therefore, intervenes like antibiotics, probiotics, pathogens, and nutrition are expected to affect the composition of gut microbiota and physiological function of gut and, thus, influence the host cognitive behavior and change the risk of AD (36). Particularly, nutritional interventions are now promising as strategies to treat or at least delay AD progression.

Very rare preclinical findings are evident on the effect of probiotic supplements on AD. Recently, Bonfili et al. reported that treating an animal model of AD (mice 3xTg-AD) with a probiotic formulation (SLAB51) positively interfere with inflammatory cytokines and concentration of some gut hormones, reduce amyloid-β plaques and improve cognitive function (26). In another study Nimgampalle and Kuna revealed that *Lactobacillus plantarum* MTCC1325 might have anti-Alzheimer properties against D-Galactose induced AD (27). Further support was provided by Peng et al. where they reported that the probiotic bacteria *Lactobacillus plantarum* NDC75017 improves the learning and memory ability in aging rats (37). Also Distrutti et al. demonstrated that a formulation of probiotic (VSL#3) positively affected inflammatory and neuronal processes and restored impaired synaptic plasticity (38). In a research on healthy humans Chung et al. reported that fermented milk containing *Lactobacillus helveticus* IDCC3801 improves cognitive function in healthy older adults (29).

We were the first to present a clinical work demonstrating a favorable effect of probiotic bacteria on the patients with AD. Three months treating the AD patients with a probiotic formulation containing four bacteria (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus fermentum,* and *Bifidobacterium bifidum*) slightly improved cognitive indices, increased some antioxidant factors and normalized some lipid profiles (21). However, compared to the previous work, more AD patients were fallen in severe stage in the present study (83.5 vs. 67%) and less in moderate stage (16.5 vs. 33%). In another clinical trial we also found positive effect of a probiotic supplementation on motor behavior, and plasma level and gene expression of some biochemical indices in the people with multiple sclerosis (39, 40).

Lack of inflammatory biomarkers measurement in the AD patients was considered as a limitation in our previous work. Hence, in the present study the inflammatory cytokines TNF-α, IL-6, and IL-10 were evaluated. Increased TNF-α is known as a key element in inflammatory cascade that in turn increases the amyloid-β and tau pathology in AD (41). Evidence indicates an enhanced expression of IL-6 in both the periphery (42) and the CNS (43) in AD patients. Concerning IL-10, both positive (44) and negative (45) effects of this cytokine on cognition of AD animal models are reported. However, the probiotic supplement used in this study had no effect on either inflammatory or anti-inflammatory parameters in the AD patients. Some other inflammatory biomarkers related to the cognitive disorders must also be considered. Two of them are S100A12 and neopterin, known as intestinal and systemic inflammatory biomarkers, respectively. In a recent work Leblhuber et al. found a close correlation between fecal S100A12 and serum neopterin in cognitive disorders like AD; signifying a role of gut inflammation as a possible pathogenic cofactor in cognitive deterioration and dementia (34).

A key mechanism associated with AD is oxidative stress (46) that might be modified through nutritional or antioxidant supplements (47). Our findings indicated no effect of the probiotic supplementation on either oxidant or antioxidant biomarkers. In a clinical trial Tramutola et al. reported that AD patients were irresponsible to the administered antioxidants (48). They concluded that clinical trials of drugs with only a single mechanism against AD at the dementia stage might be expected.

Findings of this clinical trial indicated that treatment of the AD patients with a formulation of the probiotic bacteria effectively influenced neither the cognitive function nor biochemical factors. In our previous work on the AD patients we found a favorable effect of a different probiotic supplementation on both the cognition and some of biochemical statuses. We think that the difference between results of the two studies could be attributed to the different probiotic formulation and severity of the disease. However, some other reasons might explain why application of supplements on aged patients such as people with AD no sufficiently affects the cognitive and biochemical factors. A reason could be the protocol including the duration of the supplement administration. In a recent clinical trial conducted on more than 7,000 men with AD Kryscio, et al found that supplements containing vitamin E, selenium, or vitamin E + selenium had no effect on dementia (49). Also, wrong timing of administration may be responsible for the failure of supplements to influence the age associated disorders where, in sever stage of AD, the loss of synapses and development of neurofibrillary tangles are irreversible pathological changes (50). Concomitantly, the use of probiotic at this stage may not be successful in reversing the disease process. Eventually, it is worthwhile to imply that inflammatory/oxidative stress pathways are not specific to AD and are sensitive pathways involved in many diseases. Thus, we have tested the effect of probiotic supplementation on cytokines and oxidative stress pathways that might be only a part of the pathophysiology of the AD.

Conclusively, inconsistent effects of supplements on neurological disorders in aged people must not be interpreted as paradoxical results. Other intervenes including age and severity of disease must be considered in future studies.

There were some limitations that influenced our study; importantly inclusion of people mostly in severe stage of AD, small number of subjects included in the study, the dosage and formulation of probiotic bacteria, and a sort supplement exposure time. Additionally, assessment of the impaired cognitive related inflammatory biomarkers, S100A12, and neopterin, are suggested in future investigations on AD patients.

AUTHOR CONTRIBUTIONS

MSa designed the project. AAg, AAl, MSo, and SE (general physician) performed the study. RD (neurologist) and MH (psychiatrist) visited the participants. GH analyzed the data. The

manuscript was written by MSa. Final edit was accomplished by MSa.

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CORRECTION
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Corrigendum: Does Severity of Alzheimer's Disease Contribute to Its Responsiveness to Modifying Gut Microbiota? A Double Blind Clinical Trial

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A Corrigendum on

Does Severity of Alzheimer's Disease Contribute to Its Responsiveness to Modifying Gut Microbiota? A Double Blind Clinical Trial
by Agahi, A., Hamidi, G. A., Daneshvar R, Hamdieh M, Soheili M, Alinaghipour A, et al. (2018). Front. Neurol. 9:662. doi: 10.3389/fneur.2018.00662

In the original article, there was an error. The term "normal people" was used instead of the term "cognitively intact people."

Corrections of this term have been made throughout the text in the **Materials and Methods, participants**; the **Results**, **Confirmation of Alzheimer's Disease in the Patients Based on the TYM Cognitive Test**; Figure 2 and in the **Discussion**, paragraph one.

Additionally, the determination of the sample size was incorrect. A correction has been made to the **Materials and Methods**, **Participants:**

"This clinical study was performed as a randomized, double-blind, and placebo-controlled clinical trial. Participants included in this study were people with AD (65–90 years old) residing at Emam Ali (Tehran, Iran), Golabchi (Kashan, Iran), Miad (Kashan, Ravand, Iran), and Barekat (Aran and Bidgol, Iran) Welfare Organizations between June 2017 and August 2017. AD patients were diagnosed based on the NINDS-ADRDA criteria (22) and revised criteria from the National Institute on Aging-Alzheimer's Association (23). For further proof the AD patients were compared with the cognitively intact people based on the TYM cognitive test. Accordingly, the participants gaining TYM scores in level of AD (<45 out of 50 scores) were entered the study (see section "Results" for details). Patients with metabolic disorders, chronic infections and/or other clinically relevant disorders were excluded from the study. Standard formula for clinical trials was used to calculate sample size for the study. Based on a previous study (24), considering type one error (α) of 0.05 and type two error (β) of 0.20 (power = 80%) we used 1.3 as SD and 1.1 as the difference in mean (d) of TYM as key variable. Accordingly, we needed 25 persons in each group. Assuming 5 dropouts in each group, the final sample size was determined to be 30 persons per group. Figure 1 explains flow of subject selection assigned as CON and PRO groups enrolled in the study."

Furthermore, there was an error in the use of the term "underpowered." A correction has been made to the **Discussion**, Paragraph 10:

"There were some limitations that influenced our study; importantly inclusion of people mostly in severe stage of AD, small number of subjects included in the study, the dosage and formulation of probiotic bacteria, and a sort supplement exposure time. Additionally, assessment of the impaired cognitive related inflammatory biomarkers, S100A12, and neopterin, are suggested in future investigations on AD patients."

Lastly, there was a missing translation of the TYM test. A correction has been made to the **Material and Methods**, **Outcome Evaluation**:

"The TYM test was used to evaluate the level of cognition in the patients and the TYM results were considered as the primary outcomes. However, the two questions in the semantic knowledge section of the TYM were substituted by questions familiar to Iranian people. In the first question the subjects were asked to name the Iranian president rather than the UK prime minister. Also the second question of semantic knowledge "In what year did the 1st World War start?" uncommon knowledge in Iran, was changed to "In what year did the war between Iran and Iraq?" As a secondary outcome the findings of the oxidative stress and inflammatory biomarkers were assessed."

The authors apologize for these errors and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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BRIEF RESEARCH REPORT
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24S-Hydroxycholesterol Correlates With Tau and Is Increased in Cerebrospinal Fluid in Parkinson's Disease and Corticobasal Syndrome

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24S-hydroxycholesterol (24OHC) and Tau are produced in neuronal cells and neurodegeneration leads to increased flux of both of them into cerebrospinal fluid (CSF). In the present study, CSF levels of 24OHC and 27S-hydroxycholesterol (27OHC) along with those of Tau, P-Thr^{181}-Tau and Aβ_{42} were measured in patients with early Parkinson's disease (PD), Corticobasal syndrome (CBS), Corticobasal Degeneration (CBD), and controls. Using mouse models with increased or no formation of Tau protein and increased production of 24OHC, we have also tested the hypothesis that there is a direct association between neuronal turnover of 24OHC and Tau. The levels of 24OHC are increased, at a group level, in patients with PD or CBS. We found significant correlations between levels of 24OHC and Tau or P-Thr^{181}-Tau in CSF from patients with PD, CBS or CBD. There were no similar correlations between 24OHC and Aβ_{42} in CSF from these patients. The neuronal levels of 24OHC were not altered in Tau knockout or Tau overexpressing mice. *Vice versa*, Tau species levels were not changed in Cyp46 overexpressing mice with increased neuronal levels of 24OHC. We conclude that the strongly correlative fluxes of 24OHC and Tau from neuronal cells to CSF are likely to be secondary to neurodegeneration and not due to direct interaction between the two factors. We suggest that this high correlation reflects a rapid neurodegeneration of specific neuronal subtypes with simultaneous release of 24OHC and Tau into the CSF.

Keywords: 24S-hydroxycholesterol, oxysterols, biomarkers, CSF, Parkinson's disease, corticobasal degeneration

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INTRODUCTION

In contrast to cholesterol itself its side-chain oxidized metabolites 24S-hydroxycholesterol (24OHC) and 27-hydroxycholesterol (27OHC) are able to pass the blood-brain barrier. 24OHC is exclusively formed in neurons and is continuously fluxed into the circulation (1). 27OHC is mainly formed in extracerebral tissues and organs, but there is a continuous flux of this oxysterol from the circulation into the brain (2). Neurodegeneration results in increased flux of 24OHC from neurons into CSF, possibly due to a direct release from the decomposing cells (1). Neurodegeneration also results in disruption of the blood-brain barrier and reduced capacity of the neuronal enzyme CYP7B1 to metabolize 27OHC resulting to increased 27OHC in CSF (2).

Evidence has accumulated that 24OHC in CSF can be used as a biomarker for neurodegeneration, particularly at early stages (3). Another commonly used biomarker for neurodegeneration is Tau. The role of this protein is to stabilize axonal microtubule by promoting tubulin assembly. Abnormal phosphorylation of neuronal Tau leads to destabilization and increased levels of Tau and phospho-Tau in CSF (4). In a previous study (5), we found a significant correlation between Tau or P-Thr^{181}-Tau and 24OHC in CSF from patients with Alzheimer's disease and mild cognitive impairment. This finding was confirmed in a later study by another group (6).

Here, we have compared CSF levels of 24OHC and 27OHC along with those of Tau, P-Thr^{181}-Tau and Aβ_{42} in patients with parkinsonism. Specifically, we have studied CSF samples from patients with Parkinson's disease (PD), which is a synucleinopathy, but associated with genetic polymorphisms regulation tau expression (7). We have also studied samples from patients with Corticobasal syndrome (CBS), a condition characterized by filamentous Tau inclusions in neurons and astrocytes (8, 9). Since CBS encompasses a diagnostically heterogeneous group of patients (8, 9), we also evaluated CSF from pathologically confirmed cases with Corticobasal Degeneration (CBD). Finally, we have tested the hypothesis that there is a direct association between neuronal turnover of 24OHC and Tau with use of mouse models with increased or no formation of Tau protein and increased production of 24OHC.

MATERIALS AND METHODS

Patients and CSF Sampling

This study involved CSF samples from patients from the Neurology clinic, Karolinska University Hospital and the Memory Clinic, University of California, San Francisco (UCSF) Memory and Aging Center. All the investigations of the patients and the analyses of their CSF were approved by the ethic committees of the respective institutions. Informed consent was obtained from the subjects.

In experiment 1, CSF from controls (i.e., subjects with tension headache or benign parastesia), patients with early PD [diagnostic criteria, see (10)] or CBS [diagnostic criteria see (8)] were analyzed. In experiment 2, CSF from patients with pathologically confirmed CBD from UCSF was studied. The CBD diagnosis was made according to a previously described procedure (9). Demographic information about the different grups are presented in **Table 1**.

The CSF samples were obtained by lumbar puncture and collected into polypropylene-tubes and subsequently centrifuged (1,300–1,800 × g, 4°C, 10 min). The supernatant was carefully pipetted off and dispensed in 500 µl aliquots before storage at −80°C. The time interval from collection to freezing was less than 60 min.

Animal Experiments

All the animal experiments were approved by the local Animal Experimentation Ethics Committee.

Mouse Models With Overexpressed or No Tau Levels

Twelve weeks old male wildtype, tau KO and hTau OE mice on a C57Bl6 background were used. Both mutant Tau lines are deficient of murine Tau, but hTau OE mice overexpress all six human Tau isoforms, leading to gradual Tau pathology and behavioral deficits (11). Mice were decapitated, cortical brain tissue samples dissected, immediately frozen at −80°C until analysis for oxysterol levels.

Mouse Model With Overexpressed CYP46 and High Levels of 24OHC in Circulation and Brain

Ten weeks old male mice with overexpression of CYP46 under the β-actin promotor on a C57Bl6 background were used (12). CYP46 is an enzyme synthetizing 24OHC and the CYP46 OE mice have a 2-fold increase of 24OHC in the brain and a 4–6-fold increase in serum (12). The mice have no obvious behavioral phenotype. Cortical brain tissue was obtained as above and analyzed for Tau species.

Analyses of Oxysterols

The analyses of 24OHC and 27OHC in CSF and mouse brain tissue were performed by isotope dilution mass spectrometry as described previously (2, 3, 13). In one CSF sample from a CBS patient could only 24OHC, and not 27OHC, be measured.

Analyses of Tau Species, Aβ_{42} and Neurofilament

Tau, P-Thr^{181} Tau (commonly referred to as phospho-tau) and Aβ_{42} analyzes in CSF samples from Karolinska were made with enzyme-linked immunosorbent assay (ELISA) kits from Innogenetics NV Ghent Belgium. CSF samples from USCF were analyzed with the INNO-BIA AlzBio3 (Innogenetics, Ghent, Belgium) platform to measure Tau, P-Thr^{181} Tau and Aβ_{42} and the Uman Diagnostics ELISA kit (Umea, Sweden) to measure neurofilament (NFL).

Levels of total Tau, 4R Tau and P-Ser^{202} Tau in cortical brain tissue from wildtype and CYP46 OE mice were determined by Western blotting and chemiluminiscence as previously described (14). The primary antisera were kind gifts from Drs Peter Davies and Rohan da Silva.

Statistics

Data are presented as mean ± S.D. Data was tested for normality using the Kolmogorov–Smirnov test. When two groups were compared, unpaired Student's *t*-test was used. When more than two groups were compared, statistical analyses were made with one-way ANOVA followed by Dunnet's test or Kruskal Wallis test followed by Dunn's test. Correlation analyses were made with Pearson's test followed by *t*-tests. All statistical analyses were made with GraphPAD Prism (GraphPAD Prism 5.0).

TABLE 1 | Demographics of examined controls (Ctrl) and patients with Parkinson's Disease (PD), Corticobasal syndrome (CBS), and Corticobasal degeneration (CBD).

	Age (years)	Gender (M:F)	Disease duration (years)	Levodopa equivalent dose (LED)
Ctrl ($n = 19$)	58.2 ± 9.3 (45–82)	14:5	n/a	n/a
PD ($n = 30$)	63.9 ± 10.8 (38–89)	21:9	0	0
CBS ($n = 11$)	68.9 ± 5.4 (60–81)	7:4	2.5 ± 1.7 (0–6)	144 ± 240 (0–764)
CBD ($n = 8$)	65.8 ± 3.1 (62–70)	6:2	n/k	0

Data are presented as the mean ± standard deviation and (range). n/a stands for not applicable and n/k for not known.

RESULTS

Levels of 24OHC and 27OHC in Patients With PD or CBS

Patients with PD or CBS had significantly [$F_{(2,61)}$ 10.6; $p < 0.001$] higher levels of 24OHC in CSF than those of the control subjects ($p < 0.05$ and $p < 0.001$, respectively) (**Figure 1A**). The data for 27OHC in CSF was not normally distributed, but showed significant (Kruskal Wallis value 10.5) difference. Pairwise comparisons showed that the levels were significantly higher in the patients with CBS compared to controls ($p < 0.01$) (**Supplementary Figure 1A**).

Correlations Between Levels of 24OHC or 27OHC and Tau, P-Thr^{181} Tau and Aβ42 in CSF From Patients With PD or CBS

Significant correlation was observed between levels of 24OHC and Tau ($r = 0.6$, $p < 0.001$) in CSF from patients with PD (**Figure 1B**). A similar correlation was observed between 24OHC and P-Thr^{181} Tau ($r = 0.62$, $p < 0.001$) (**Supplementary Figure 2A**), but not between 24OHC and Aβ42 ($r = 0.21$, $p = 0.27$) (**Supplementary Figure 2C**). There was a lower, but significant ($r = 0.38$, $p = 0.04$ vs. $r = 0.39$, $p = 0.03$), correlation between 27OHC and Tau and P-Thr^{181} Tau in CSF from PD patients (**Supplementary Figures 1B**, **2B**). There was no significant ($r = 0.27$, $p = 0.14$) correlation between 27OHC and Aβ42 in these patients (**Supplementary Figure 2D**).

There was a very high correlation between 24OHC and Tau ($r = 0.98$, $p < 0.0001$) as well as P-Thr^{181} Tau ($r = 0.89$, $p < 0.001$) in patients with CBS (**Figure 1C**, **Supplementary Figure 3A**). There was no significant ($r = -0.16$, $p = 0.64$) correlation between 24OHC and Aβ42 (**Supplementary Figure 3C**). There were no significant correlations between 27OHC and Tau ($r = 0.32$, $p = 0.37$), P-Thr^{181} Tau ($r = -0.37$, $p = 0.3$) or Aβ42 ($r = 0.46$, $p = 0.18$) in the CBS patients (**Supplementary Figures 1C**, **3B,D**).

Since CBS encompasses a diagnostically heterogeneous group of patients (8, 9), we also evaluated CSF from pathologically confirmed cases with CBD. In accordance with obtained data from CBS patients, CBD patients showed a significant correlation between 24OHC and Tau ($r = 0.84$, $p = 0.008$) (**Figure 2A**) and a strong trend with P-Thr^{181} Tau ($r = 0.69$, $p = 0.054$) (**Figure 2B**). There was no significant ($r = 0.62$, $p = 0.10$) correlation between 24OHC and Aβ42 (**Figure 2C**). Measures of NFL was also available from these CBD patients, but they did not show ($r = -0.35$, $p = 0.44$) a significant correlation to 24OHC (**Figure 2D**). There were no significant correlations between 27OHC and Tau ($r = 0.29$, $p = 0.48$), P-Thr^{181} Tau ($r = -0.14$, $p = 0.75$), Aβ$_{42}$ ($r = 0.17$, $p = 0.69$) or NFL ($r = -0.24$, $p = 0.61$) (**Supplementary Figures 4A–D**).

There was no correlation between 24OHC and Tau ($r = 0.29$, $p = 0.38$), P-Thr^{181} Tau ($r = 0.05$, $p = 0.89$) or Aβ42 ($r = 0.06$, $p = 0.85$) in control subjects. There was no significant correlation between 24OHC levels and age in controls ($r = 0.18$, $p = 0.45$), PD ($r = 0.01$, $p = 0.95$) or CBD ($r = 0.09$, $p = 0.83$) subjects, whereas there was a positive correlation in CBS patients ($r = 0.81$, $p = 0.003$).

Mouse Experiments Designed to Test the Hypothesis That There Is a Direct Interaction Between 24OHC and Tau Turnover in the Brain

Based on the strong correlation between 24OHC levels and Tau in CSF, we used mutant mouse models to examine whether there could be a direct relation in their neuronal production. The hypothesis that increased neuronal production of 24OHC affects levels of Tau protein was tested in mice overexpressing CYP46. It is known that these mice have a two-fold increased levels of 24OHC in the brain (10). However, there were no significant differences between wildtype and CYP46OE mice in their levels of total Tau, P-Ser^{202} Tau (CP-13) or 4R-Tau in cortical brain tissue (**Supplementary Figure 5**). *Vice versa*, the hypothesis that primary changes in the levels of Tau are able to affect levels of 24OHC in the brain was tested with use of mice with no (Tau KO mice) or increased (Tau OE) levels of Tau. As shown in **Supplementary Figure 6A**, the levels of 24OHC were not significantly [$F_{(2,15)}$ 0.07] different between these groups. There was neither any significant (Kruskal Wallis value 3.8) alterations in the levels of 27OHC in the genetically modified Tau mice (**Supplementary Figure 6B**).

DISCUSSION

There is a continuous production of 24OHC in neuronal cells and a flux of this oxysterol from the brain into the circulation (1). A neurodegeneration will reduce this production resulting in slightly reduced levels of 24OHC in the circulation (3). The changes are however small and are difficult to use diagnostically. In contrast a neurodegeneration results in increased levels of 24OHC in CSF most probably due to a release from dying neuronal cells. This increase is sufficiently high to be used diagnostically (15). Assuming that a considerable part of the 24OHC and Tau in CSF is released from dying neuronal cells, a correlation between these two parameters can be expected

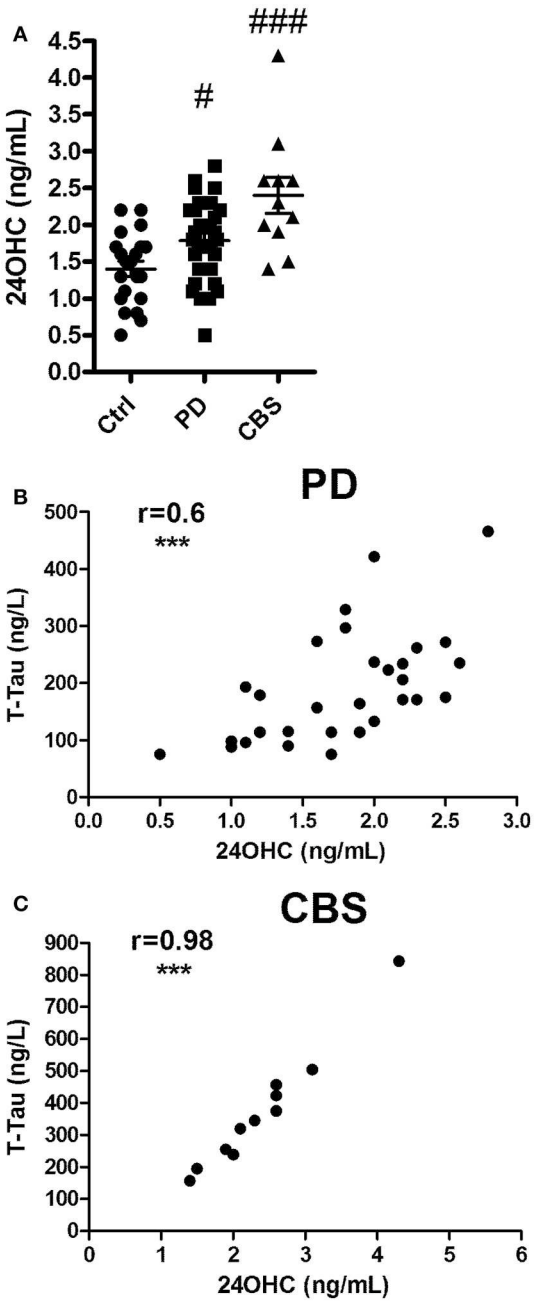

FIGURE 1 | (A) Levels of 24OHC in CSF from controls or patients with Parkinson's disease (PD) or Corticobasal syndrome (CBS). **(B,C)** Correlations between CSF levels of 24OHC and total Tau in PD **(B)** and CBS **(C)** patients. In **(A)**, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. control. In **B,C**, *r* values indicate Pearson correlations and $^{***}p < 0.001$ significance.

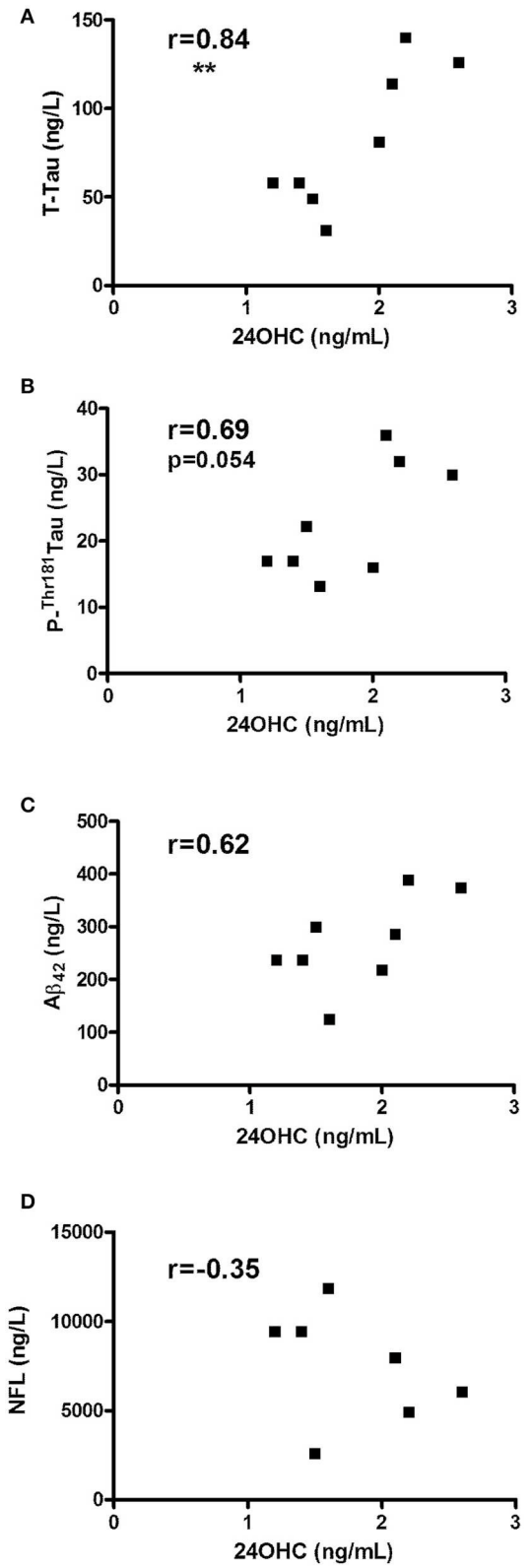

FIGURE 2 | Correlations between CSF levels of 24OHC and total Tau **(A)**, P-Thr^{181}-Tau **(B)**, Aβ_{42} **(C)** or NFL **(D)** in patients with corticobasal degeneration (CBD). *r* values indicate Pearson correlation and $^{**}p < 0.01$ significance.

in neurodegenerative disorders. Evidently, there was a strong correlation between 24OHC and Tau in CSF from PD patients. The CSF was collected at an early stage of PD and the patients had no medication against PD. There was also a significant but weak correlation between Tau and 27OHC in the PD patients. The flux of 27OHC is likely to be dependent upon the rate of

metabolism of this oxysterol by the enzyme CYP7B1 (2). The latter enzyme is mainly present in neuronal cells and a reduction of the number of these cells can be expected to increase the level of 27OHC into CSF. It should be pointed out that in contrast to 27OHC, 24OHC is not a substrate for CYP7B1. In view of this a considerably lower correlation between 27OHC and Tau can be expected. It should also be pointed out that due to the extensive metabolism, the levels of 27OHC in the brain are much lower than the corresponding levels of 24OHC (16).

The strongest finding here is the high correlation between 24OHC and Tau or P-Thr^{181}-Tau in CSF from patients with CBS and CBD. CBD is caused by accumulation predominantly of 4R Tau. The underlying CBD pathology gives rise to a variety of clinical presentations that encompass CBS, characterized by levodopa resistant asymmetric dystonia or rigidity, bradykinesia and myoclonus along with cortical symptoms such as apraxias, speech difficulties and alien limb phenomenona. However, CBD also includes a syndrome clinically similar to progressive supranuclear palsy (PSPS-CBD), a frontotemporal behavioral variant (FTD-CBD) and a variant with progressive non-fluent aphasia (PNFA-CBD). Due to this heterogeneity in presentation, a clinical diagnosis of CBD is often difficult and many patients will receive an *ante mortem* diagnosis that is altered upon *post mortem* examination (8, 9). In our first experiment we examined CSF from living patients with a typical CBS presentation, but could later verify the strong correlation between 24OHC and Tau in another cohort of pathologically confirmed CBD patients. Another difference between these cohorts were that the patients in the CBD cohort were not on any medication against parkinsonism which could potentially influence oxysterol levels.

The very high correlation between 24OHC and Tau led us to test the hypothesis that there is a direct interaction between the neuronal production of the two factors. Indeed, based on experiments with mouse models the possibility has been discussed that there may be a causal link between CYP46A1 protein content and memory impairment that result from Tau pathology (17).

However, experiments with a mouse model with high levels of 24OHC and mouse models with increased or no levels of Tau did not give support for this hypothesis. Thus, 24OHC is not likely to be a driving force for increased production of Tau and, *vice versa*, Tau is not likely to directly determine the production of 24OHC. It is noteworthy that young mice were used in our studies to avoid indirect influence of aging, but it may also turn out that reciprocal changes in 24OHC and Tau are only evident in older mice. Nonetheless, it seems likely that the correlation between the two factors in the patients is secondary to neurodegeneration, in PD as well as CBD patients. In theory, a high correlation between 24OHC and Tau can be expected if there is a very rapid decomposition of individual neuronal cells with a simultaneous release of both 24OHC and Tau. Accordingly, a higher correlation was seen in CBS/CBD than in PD patients, likely to reflect the more aggressive neurodegeneration in CBD/CBS.

In conclusion, CSF levels of 24OHC are elevated, at group level, in PD, CBS, and CBD, and show a very strong correlation to Tau. Future studies will evaluate whether 24OHC and Tau interact synergistically in pathophysiological events underlying PD or CBD. It will also be interesting to study whether there is a correlation between 24OHC and α-synuclein and to measure 24OHC in the same individual at different disease stages in longitudinal cohort studies.

ETHICS STATEMENT

This study was approved by the Research Ethics Committee of the Karolinska University Hospital and University of California, San Francisco. All subjects gave a written informed consent in accordance with the Declaration of Helsinki before the measurements.

AUTHOR CONTRIBUTIONS

IB: study planning, sample measurements, and manuscript writing. KP: sample measurements, data analysis, and manuscript writing. AB: patient and sample recruitment and manuscript writing. PS: study planning, data analysis, and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00756/full#supplementary-material

Supplementary Figure 1 | (A) Levels of 27OHC in CSF from controls or patients with Parkinson's disease (PD) or Corticobasal syndrome (CBS). **(B,C)** Correlations between CSF levels of 27OHC and total Tau in PD **(B)** and CBS **(C)** patients. In A, $^{\#}p < 0.05$ vs. control. In **B,C**, *r* values indicate Pearson correlations and $^{*}p < 0.05$ significance.

Supplementary Figure 2 | Correlations between CSF levels of 24OHC **(A,C)** or 27OHC **(B,D)** and P-Thr^{181}-Tau **(A,B)** or Aβ$_{42}$ **(C,D)** in patients with PD. *r* values indicate Pearson correlation and $^{*}p < 0.05$, $^{***}p < 0.001$ significance.

Supplementary Figure 3 | Correlations between CSF levels of 24OHC **(A,C)** or 27OHC **(B,D)** and P-Thr^{181}-Tau **(A,B)** or Aβ$_{42}$ **(C,D)** in patients with CBS. *r* values indicate Pearson correlation and $^{***}p < 0.001$ significance.

Supplementary Figure 4 | Correlations between CSF levels of 27OHC and total Tau **(A)**, P-Thr^{181}-Tau **(B)**, Aβ$_{42}$ **(C)** or NFL **(D)** in patients with CBD. *r* values indicate Pearson correlations.

Supplementary Figure 5 | Relative levels of total Tau, P-Ser^{202}-Tau ("CP13") and 4-repeat Tau in cortical brain tissue of wildtype (WT) mice and mice with an overexpression of CYP46 and increased levels of 24OHC.

Supplementary Figure 6 | Levels of 24OHC **(A)** and 27OHC **(B)** in cortical brain tissue of wildtype (WT) mice or with increased (Tau OE) or no (Tau KO) levels of Tau.

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REVIEW
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HMGB1: A Common Biomarker and Potential Target for TBI, Neuroinflammation, Epilepsy, and Cognitive Dysfunction

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High mobility group box protein 1 (HMGB1) is a ubiquitous nuclear protein released by glia and neurons upon inflammasome activation and activates receptor for advanced glycation end products (RAGE) and toll-like receptor (TLR) 4 on the target cells. HMGB1/TLR4 axis is a key initiator of neuroinflammation. In recent days, more attention has been paid to HMGB1 due to its contribution in traumatic brain injury (TBI), neuroinflammatory conditions, epileptogenesis, and cognitive impairments and has emerged as a novel target for those conditions. Nevertheless, HMGB1 has not been portrayed as a common prognostic biomarker for these HMGB1 mediated pathologies. The current review discusses the contribution of HMGB1/TLR4/RAGE signaling in several brain injury, neuroinflammation mediated disorders, epileptogenesis and cognitive dysfunctions and in the light of available evidence, argued the possibilities of HMGB1 as a common viable biomarker of the above mentioned neurological dysfunctions. Furthermore, the review also addresses the result of preclinical studies focused on HMGB1 targeted therapy by the HMGB1 antagonist in several ranges of HMGB1 mediated conditions and noted an encouraging result. These findings suggest HMGB1 as a potential candidate to be a common biomarker of TBI, neuroinflammation, epileptogenesis, and cognitive dysfunctions which can be used for early prediction and progression of those neurological diseases. Future study should explore toward the translational implication of HMGB1 which can open the windows of opportunities for the development of innovative therapeutics that could prevent several associated HMGB1 mediated pathologies discussed herein.

Keywords: HMGB1, RAGE, TLR4, TBI, epilepsy, neuroinflammation, cognitive dysfunction

HIGHLIGHTS

- The nuclear protein HMGB1 is a mediator for neurological conditions such as TBI, neuroinflammation, epilepsy and cognitive dysfunction.
- HMGB1 could be a common functional biomarker of TBI, neuroinflammation, epileptogenesis and cognitive dysfunction.
- Inhibiting the HMGB1/RAGE/TLR4 signaling axis could be a novel therapeutic strategy against several HMGB1 mediated conditions like TBI, neuroinflammation, epilepsy and cognitive dysfunction.

INTRODUCTION

Epilepsy is a serious neurological condition characterized by spontaneous and recurrent seizures (Liu et al., 2018) affecting people of all ages. Current anti-epileptic drugs (AEDs) only provides symptomatic relief rather than interfering with the disease mechanism, as well as one third of the patients are resistant to AEDs (Ravizza et al., 2017; Walker et al., 2017). Moreover, epilepsy imposes a burden by impacting several aspects of patients and family life. In addition, the burden is intensified due to the ranges of associated comorbidities such as cognitive dysfunctions, anxiety and depression. Hence, the development of novel biomarker which can predict and assess the disease condition as well as patient's outcome of the therapy against epilepsy is an unmet clinical need. As well as there is a pressing need of exploring new therapy against epilepsy which not only retard the seizure precipitation but also minimizes the associated comorbidities. In this regard, HMGB1 has emerged as a novel frontier and mounting number of preclinical studies targeting HMGB1 have been successful in diverse ranges of neurological conditions provoked by inflammatory responses (Wang et al., 2017; Zhao et al., 2017; Andersson et al., 2018).

High mobility group box 1 proteins are a family of DAMPs (Lotze and Tracey, 2005), which are highly conserved non-histone nuclear proteins and contributes to the architecture of chromatin DNA (Baxevanis and Landsman, 1995). HMGB1 acts as an inflammatory cytokine in response to epileptogenic insults (Kaneko et al., 2017). HMGB1 acts as a pathogenic inflammatory response to mediate ranges of conditions such as epilepsy (Maroso et al., 2010), septic shock (Wang et al., 1999), ischemia (Kim et al., 2006; Wang et al., 2015), TBI (Okuma et al., 2012), PD (Sasaki et al., 2016), AD (Fujita et al., 2016), and MS (Andersson et al., 2008). Structural evaluation of HMGB1 suggests that it exhibits two domains for DNA-binding, known as box A and box B, as well as C-terminal acidic tail comprised of repeating glutamic and aspartic acid residues (Venereau et al., 2016; Aucott et al., 2018b). DAMPs can influence synaptic function in the brain regions such as the hippocampus, which is involved in hyperexcitability and cognitive decline in epilepsy (Ravizza et al., 2017). It has been reported that immediately after neuronal injury, there is a passive release of significant amounts of HMGB1 from the nucleus into the extracellular space (Scaffidi et al., 2002).

High mobility group box 1 has several extracellular receptors such as RAGE, TLR9, TLR4, TLR2, integrin, a-synuclein filaments, proteoglycans, T-cell immunoglobulin and mucin domain (TIM-3), triggering receptor expressed on myeloid cells-1 (TREM1), cluster of differentiation 24 (CD24), C-X-C CXCR4, *N*-methyl-D-aspartate receptor (NMDAR) (Kang et al., 2014). Among these receptors, RAGE and TLR4 are the only receptors that are extensively studied and reported without doubt (Andersson et al., 2018). HMGB1 initiates several cell responses including inflammation as well as mediate the activation of inflammatory process via binding with RAGE and TLR4 (Bianchi and Manfredi, 2007; Iori et al., 2013). During neuroinflammatory conditions, HMGB1 is actively released by neurons and glia cells upon inflammasome activation and in turn activates at least two PRRs, namely TLR4 and RAGE on target cells (Ravizza et al., 2017). Once released extracellularly, HMGB1 binds to the TLR4 and RAGE expressed by immune cells which leads to nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) mediated production of pro-inflammatory cytokines (Iori, 2017). Role of HMGB1 in the development and disease of central nervous system (CNS) has been well described (Fang et al., 2012) where contribution of HMGB1 to the neurogenesis in the early phase of brain development, in neurite extension as well as its dual role in neural development and neurodegeneration is well discussed. HMGB1 promoted neuronal differentiation of adult hippocampal neural progenitors via activation of RAGE/NF-κB suggesting the role of HMGB1 in maintaining and sustaining hippocampal neurogenesis (Meneghini et al., 2013).

A previous study reported HMGB1 translocation as the main culprit for TBI (Li Y. et al., 2017). Recently, HMGB1 has received greater attention for its role in epilepsy (Zhao et al., 2017). It has been hypothesized that HMGB1 might be involved in epileptogenesis, especially through BBB disruption and induction of inflammatory processes though the precise mechanism remains still elusive. Several studies were previously conducted to determine the involvement of HMGB1 in the pathogenesis of epilepsy (Fu et al., 2017). HMGB1 plays a pivotal role in cognitive decline where HMGB1 is supposed to caused disruption of the BBB leading to cognitive deficits in aged rats (He et al., 2012). Interestingly, accumulating evidence suggests that neuroinflammation is highly associated with epilepsy and cognitive dysfunction after TBI and HMGB1 exhibits a key role

Abbreviations: AD, Alzheimer's disease; APOE-ε4, Apo lipoprotein E-ε4; BBB, blood–brain barrier; BDNF, brain derived neurotropic factor; BLA, basolateral amygdala; COX-2, cyclooxygenase-2; CREB, CAMP response element binding; CXCR4, chemokine receptor type 4; DAMPs, damage-associated molecular patterns; DNA, deoxyribonucleic acid; EEG, electroencephalogram; GABA, gamma amino butyric acid; GEPRS, genetically epilepsy prone rats; GLUR2, glutamate receptor 2; GRIA2, glutamate receptor ionotropic AMPA 2; HMGB1, high mobility group box 1; IHC, immunohistochemistry; KA, kainic acid; KIR4.1, inwardly rectifying potassium channel 4.1; LTP, long-term potentiation; MiR-129-5p, micro RNA-129-5p; MS, multiple sclerosis; MWM, Morris water maze; NORT, novel object recognition test; PAMPs, pathogen-associated molecular patterns; PD, Parkinson's disease; POCD, post-operative cognitive dysfunction; PRNCs, primary rats neural cells; PRR, pattern recognition receptor; PTZ, pentylenetetrazol; RAGE, receptor for advanced glycation end Products; RAS, retrovirus-associated DNA sequences; ROS, reactive oxygen species; SE, status epilepticus; TBI, traumatic brain injury; TEM, transmission electron microscopy; TGF-β, transforming growth factor-β; TLE, temporal lobe epilepsy; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α.

as an initiator and amplifier of neuroinflammation as well as in neuronal excitation (Frank et al., 2015). Annegers and Coan (2000) suggested that there is a high risk of epilepsy after TBI and epilepsy is associated with neurological comorbidities such as cognitive dysfunction (Pascente et al., 2016; Ravizza et al., 2017).

Treatments based on HMGB1 antagonists via targeting extracellular HMGB1 have generated encouraging results in a wide number of experimental models though the clinical studies are yet to be reported. Anti-HMGB1 monoclonal antibodies (mAbs) have demonstrated beneficial effects on epilepsy and TBI. The therapeutic benefits of anti-HMGB1 mAb on epilepsy have been previously demonstrated in animal model of epilepsy (Fu et al., 2017). Potential neuroprotective effects of HMGB1 has been reported in few studies where anti-HMGB1 mAb prevented intracerebral hemorrhage (ICH)-induced brain injury (Wang et al., 2017).

Interestingly, HMGB1 has been associated with all of the neurological conditions previously outlined (**Figure 1**). Neuroinflammation is the mediator of damage in TBI, epileptogenesis and cognitive decline are the post-TBI events. This makes worth further exploring the HMGB1 and the rationale behind the current review is to explore the potential of HMGB1 as a common biomarker and potential target for several neurological phenotypes discussed in this review. In spite of its proven role in TBI, neuroinflammation, epilepsy and cognitive decline there has been little interest in exploring HMGB1 as a common target and biomarker for those conditions. The preclinical and clinical evidences discussed herein strengthens HMGB1 to stand as a promising candidate to be the common biomarker and treatment target for the neurological conditions where neuroinflammatory pathway plays a central role. Hence, this review summarizes recent advances and discuss these emerging findings to explore the potential of HMGB1 as a common biomarkers and treatment target which could pave the way in developing therapies with broad application modifying the disease progression.

NEUROINFLAMMATION AS A PRIME DRIVER OF TBI, EPILEPSY AND COGNITIVE DECLINE?

Neuroinflammation is the key component of neuropathology after TBI and contributes to the chronic neurodegeneration and neurological impairments associated with TBI (Kumar and Loane, 2012). This is supported by gene profiling studies which show that genes related to neuroinflammation are upregulated after brain injury (Kobori et al., 2002) and elevated levels of the inflammatory cytokines TNF-α, TGF-β, and IL-1β are expressed after TBI (Morganti-Kossmann et al., 2002; DeKosky et al.,

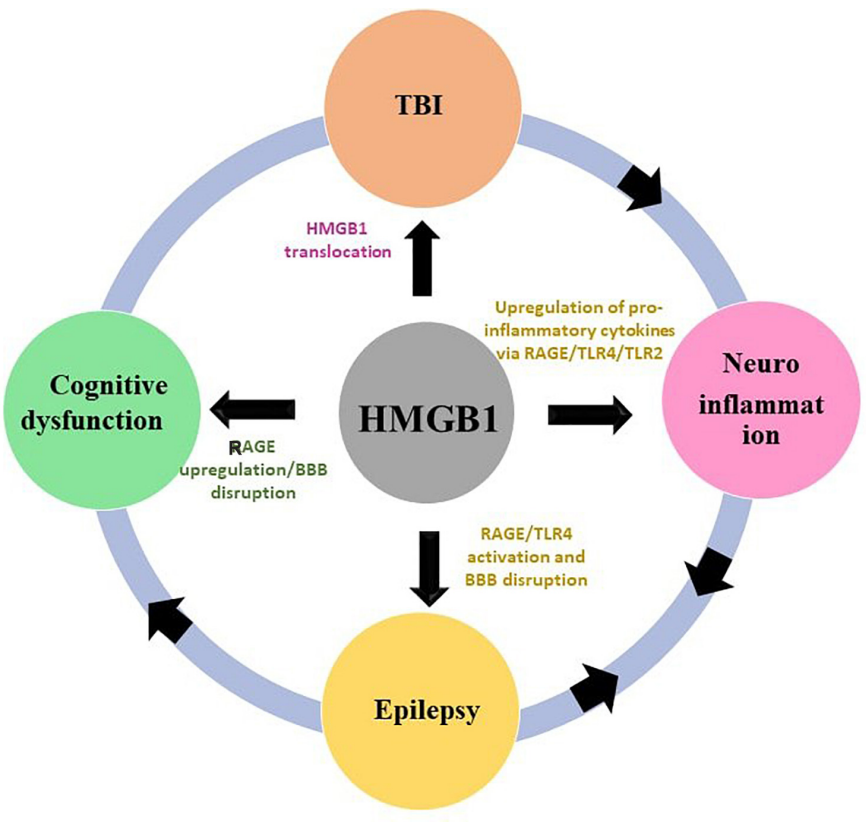

FIGURE 1 | Interlinkage of HMGB1 with TBI, neuroinflammation, epilepsy and cognitive dysfunction. The HMGB1 contributes to the pathogenesis of TBI, neuroinflammation, epilepsy, and cognitive dysfunction through a putative mechanism outlined in this figure.

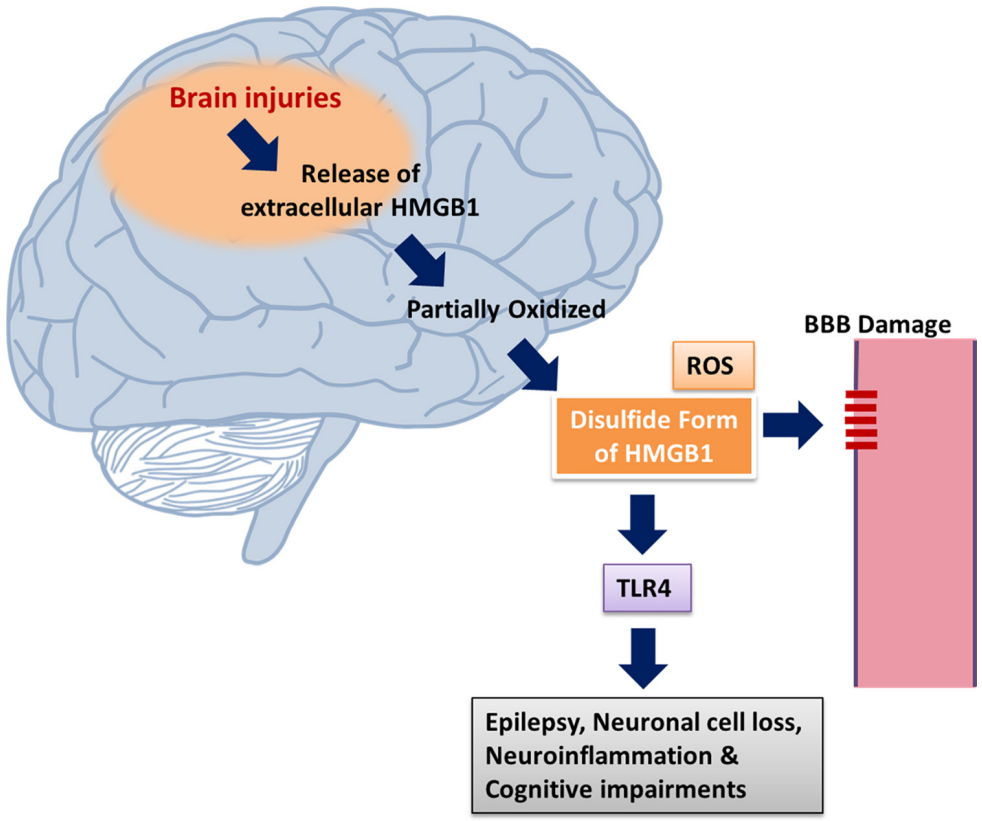

FIGURE 2 | Mechanism of HMGB1 release via brain injury. HMGB1 translocation results from brain injuries and epileptogenesis. Dying cells, neurons and glia are responsible for the release of extracellular HMGB1 which can be partially oxidized. Neuronal excitability is enhanced via the mediation of pro-inflammatory activities through the activation of TLR4 signaling by disulphide HMGB1. The stabilization of HMGB1 in its disulphide form is promoted by the generation of ROS. HMGB1 via activating receptors RAGE and TLR4 leads to neuronal cell loss, neuroinflammation, epilepsy, and cognitive dysfunction (Ravizza et al., 2017).

2013). Several experimental and clinical evidences support the role of inflammatory mediators as the origin of both seizures and epileptogenesis (Vezzani and Granata, 2005; Shimada et al., 2014). Brain inflammation contributes to the generation of individual seizures as well as cell death, which in turn contributes to the development of seizures via activation of inflammatory pathways (Vezzani et al., 2011). Moreover, there is evidence which highlights that inflammation can be a cause as well as consequences of epilepsy (Vezzani et al., 2011).

Traumatic brain injury incites a neuroinflammatory axis in the brain which perpetuates neurodegeneration and increases the chances of initiating epileptogenesis (Webster et al., 2017) However, the probability of developing epilepsy after TBI varies from 4.4% to 53% (Frey, 2003) and becomes more likely with a higher injury severity and a younger age at injury (Barlow et al., 2000). Neuroinflammation is a contributing factor to the pathophysiology of post-TBI epileptogenesis (Riazi et al., 2010). Neuroinflammation can alter the BBB permeability directly via cytokine aided activation of metalloproteinase or via disruption of tight junctions (Gloor et al., 2001; Utech et al., 2009), though the precise mechanism remains elusive. It is worthwhile to note that although neuroinflammation is typically provoked after a series of epileptogenic brain injuries, the proportion of patients developing the disease is small (Vezzani, 2015). The pivotal role of neuroinflammation in cognitive dysfunction has been reported but the underlying molecular mechanism are not yet known. However, there are insights regarding certain inflammatory pathways underlying hyperexcitability and excitotoxicity that can promote cognitive decline (Vezzani, 2015).

In recent days, research using experimental and clinical models has focused on the pathogenesis of how HMGB1 proteins contributes to TBI, neuroinflammation, epilepsy and cognitive decline. Such research has sought to pave the way to understand how these mechanisms can be interfered to develop therapies for the aforementioned neurological conditions. Moreover, accumulating evidence reported beneficial effects on evaluating anti-HMGB1 mAb and HMGB1 inhibitors against TBI, neuroinflammation, epilepsy, and cognitive decline. We will therefore discuss the outcomes of such experimental and clinical experiments in an individual pattern.

ROLE OF HMGB1 IN TBI

TBI is an insult to the brain through any external mechanical force (Webster et al., 2017), which makes TBI a devastating

and intractable cause of worldwide morbidity and mortality. Survivors live the rest of their lives with cognitive, motor, behavioral or speech and language disabilities (Richard et al., 2017). However, the pathophysiology of TBI is still elusive and a tremendous research must be made to explore the progression of neurodegeneration and the ensuing inflammatory processes (Parker et al., 2017). It is currently unavailable to attenuate the pathological process of TBI and improve neurological deficits (Jiang et al., 2018). TBI involves a primary insult known as structural damage due to any external mechanical force which is followed by a secondary injury including a multitude of neuroinflammatory phenomena such as excitotoxicity, oxidative stress and apoptosis (Webster et al., 2017). These processes begin within minute after TBI and can persist for months to years and is suspected to contribute to the expansion of tissue damage (Hinson et al., 2015).

During TBI, HMGB1 is released via the *N*-methyl D-aspartate receptor subtype 2B (NR2B)-mediated mechanism from necrotic neurons (Richard et al., 2017). HMGB1 mediates sterile inflammation and provokes macrophages and endothelial cells to release TNF-α, IL-1, and IL-6 by binding with RAGE and TLR4. This binding further activates the NF-κB pathway and facilitates the upregulation of HMGB1 and the expression of pro-inflammatory mediators (Gao et al., 2012). In addition, neuroinflammatory processes mainly mediated by activated microglia and astrocytes are crucial for the initiation and progression of TBI (Li D. et al., 2017). TBI induces a series of events including BBB breakdown, brain edema, upregulation of tight junction proteins (TJPs), expression of inflammation related molecules (Yang et al., 2018). TLR4 has been linked with TBI where TLR4 mediates glial phagocytic activity and inflammatory cytokines production (Jiang et al., 2018) and plays an important role in inflammatory response and brain injury (Fang et al., 2013). Once HMGB1 is released into the extracellular settings following TBI, it binds to transmembrane major mediators of the inflammatory response, TLR2, TLR4, and RAGE (Yang et al., 2005). Excessive inflammation resulting from activation of the HMGB1/TLR4 pathway in the brain has been implicated in TBI and ischemia-reperfusion injury (Yang et al., 2011). However, the mechanistic interlinkage between intracellular danger signaling, which involves the nuclear chromatin-binding factor, HMGB1 and inflammatory pathways after TBI is not yet fully understood (Parker et al., 2017). There is an increased understanding that TBI may induce activation of HMGB1/TLR4/RAGE/NF-κB signaling pathway and inflammatory cytokine expression, which would induce and/or aggravate the secondary brain injury where HMGB1 is supposed to implicate a critical role in promoting inflammation and aggravating damage after TBI (Xiangjin et al., 2014) (**Figure 2**). Several HMGB1 inhibitors have demonstrated protective effect against TBI via inhibiting HMGB1/TLR4/NF-κB pathway activation (Su et al., 2011), and by reducing HMGB1/RAGE interaction (Okuma et al., 2014).

Inhibition of HMGB1 expression and the TLR4/NF-κB pathways exhibits protective effects in animal model of TBI. HMGB1 inhibitors (glycyrrhizic acid) attenuated TBI by inhibiting the classically activated microglia/macrophages (M1) phenotype activation and promoting the alternatively activated microglia/macrophages (M2) phenotype activation of microglia/macrophages, via the inhibition of HMGB1 and suggest that targeting of HMGB1 to modulate the microglia/macrophage polarization might be a potential approach for TBI (Gao et al., 2018). Similar line of results has been reported where anti-HMGB1 mAb improved neurological deficits in ICH-induced brain injury. Anti-HMGB1 mAb inhibited the release of HMGB1 into the extracellular space in the peri-hematomal region, reduced serum HMGB1 levels and decreased brain edema by protecting BBB integrity, in association with decreased activated microglia and the expression of inflammation-related factors at 24 h after ICH (Wang et al., 2017). Neuroprotective effects of TLR4 knockdown has emerged as a promising approach for TBI. TLR4 knockdown ameliorated neuroinflammatory response and brain injury after TBI and suppressing autophagy induction and astrocyte activation is postulated the main mechanism behind the neuroprotective effects of TLR4 (Jiang et al., 2018). Data obtained from western blot analysis in an experimental study reported the release profile of HMGB1 and RAGE after TBI where HMGB1 was released as soon as 30 min after TBI and a decline in its expression was noted between 1 and 6 h after TBI. However, the expression level of RGAE was elevated at 6 h after TBI and reached its peak after 1 day (**Table 1**) (Gao et al., 2012). An immunostaining study reported that septic brain injury results in increased HMGB1 cytoplasmic translocation in neurons (**Table 1**) (Li Y. et al., 2017). A few studies have reported a noteworthy elevation of HMGB1, IL-1β, and TNF-α levels in serum as estimated by an enzyme linked immunosorbent assay (ELISA) kit in an experimentally induced TBI model in rabbits (Mohamed et al., 2017). However, ELISA analysis does not precisely differentiate between release pattern of HMGB1 either by necrosis, or from macrophages and monocytes, or a combination of both (Au et al., 2012).

High mobility group box 1 A-box fragment, an antagonist competing with HMGB1 for receptor binding, significantly ameliorated the BBB breakdown and brain edema induced by controlled cortical impact (CCI), and these effects were associated with the decrease in expressions of inflammation-related factors as well as improved neurological functions (Yang et al., 2018). Ethyl pyruvate (**Table 1**) (Su et al., 2011) and omega-3 polyunsaturated fatty acid supplementation (Chen et al., 2017b) has demonstrated its effectiveness against TBI via inhibition of the HMGB1/TLR4/NF-κB pathway. Evaluation of anti-HMGB1 mAb therapy against TBI in rats reported that anti-HMGB1 mAb remarkably inhibited fluid percussion-induced brain edema in rats, which was associated with an inhibition of HMGB1 translocation, protection of BBB architecture, downregulation of inflammatory molecule expression, and improvement in motor function (**Table 1**) (Okuma et al., 2012). Increased inhibition of the expression of HMGB1 signaling axis, with RAGE and TLR4, NF-κB DNA binding and downstream inflammatory cytokines were reported on glycyrrhizin treatment (Okuma et al., 2014).

The literature review ranging from human to animal studies suggests an important association between TBI and increased levels of HMGB1 in serum and cerebrospinal fluid (CSF) (Okuma et al., 2014). In addition, emerging data reported HMGB1

TABLE 1 | Summary of findings reporting HMGB1 in TBI.

S.N.	Intervention	Model	Mechanism	Observations	Reference
1.	HMGB1	Rat	• Inhibition of HMGB1 expression and TLR4/NF-κB pathway	• ↓ Reduced expression of HMGB1 and TLR4 • Improved motor function and lessened brain oedema	Su et al., 2011
2	Anti-HMGB1 mAb	Rat	• Protection against BBB disruption • Inhibition of the inflammatory responses	• Inhibition of translocation of HMGB1, protection of BBB permeability • Downregulation of inflammatory molecule expression • Improvement of motor function	Okuma et al., 2012
3	HMGB1	Rat	• Interference with HMGB1 and RAGE interaction • Inhibition of the expressions of TNF-a, IL-1β, and IL-6	• Inhibited the ↑ in BBB permeability and impairment in motor functions • Inhibition of translocation of HMGB1 in neurons at the site of injury	Okuma et al., 2014
4	Anti-HMGB1 mAb	Rat	• Protecting BBB integrity • ↓ Expression of inflammation-related factors	• ↓ Release of HMGB1 to the extracellular space in the peri-hematomal region • ↓ Serum HMGB1 levels and brain edema through maintaining BBB integrity	Wang et al., 2017
5	HMGB1	Rat	• Downregulation of sepsis-induced RAGE and NF-κBp65 expression	• HMGB1was ↑ in the cytoplasm via translocation • RAGE and NF-κβ p 65 were up regulated after brain injury • HMGB1 and its signaling transduction have a key role in the pathogenesis of septic brain injury	Li Y. et al., 2017
6	HMGB1	Human	• Targeting HMGB1/RAGE signaling	• HMGB1 disappeared or translocated from the nucleus to the cytoplasm at early stages after TBI • RAGE expression ↑ after TBI	Gao et al., 2012
7	HMGB1	Human	• Activation of microglial TLR4 and the subsequent expression of AQP4	• Peak CSF HMGB1 level in human TBI was within 0–72 h. • HMGB1 released from necrotic neurons through a NR2B-mediated mechanism	Laird et al., 2014

S.N., serial number; ↑, increased; ↓, decreased; HMGB1, high mobility group box 1; TRL4, Toll like receptor 4; RAGE, receptor for advanced glycation end products; NF-κB p 65, nuclear factor kappa-light-chain-enhancer of activated β cells p 65; IL-6, interleukin-6; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; BBB, blood–brain barrier; TBI, traumatic brain injury; CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; ICP, intra-cranial pressure; NR2B, N-methyl D-aspartate receptor subtype 2B; AQP4, astrocytic water channel aquaporin-4.

in the CSF of subarachnoid hemorrhage (SAH) (Nakahara et al., 2009) and in the serum of ICH (Zhou et al., 2010) highlighting as a potential biomarker of neurological outcome. Nevertheless, the clinical output of HMGB1 antagonist against several forms of brain injury is yet to be reported. Similar line of evidence were observed in which HMGB1 is associated with increased levels of intracranial pressure (ICP) in patients and promoted cerebral edema after TBI where the detrimental effects of HMGB1 are mediated through the microglial TLR4 activation and the expression of the astrocytic water channel aquaporin-4 (AQP4) (Richard et al., 2017). HMGB1 plasma levels were reported to increase within 30 min after severe trauma in humans and suggested a correlation between plasma levels of HMGB1 with early post-traumatic coagulopathy and severe systemic inflammatory response (Cohen et al., 2009). There was a 30-fold increment of plasma HMGB1 levels after trauma, as compared to normal controls during a 1-h period of injury which provides insights regarding the post-injury elevation levels of HMGB1 in human (Peltz et al., 2009) However, the study did not report any correlation between HMGB1 levels and the patients' outcome. Furthermore, a compelling relationship between plasma HMGB1 absorption and the severity of acute TBI was unraveled and correlation between Glasgow Coma Scale score and HMGB1 levels were reported, which can serve as prognostic information in patients with severe TBI (Wang et al., 2012). Higher CSF HMGB1s level are considered as an important

biomarker to predict outcome after pediatric TBI (Au et al., 2012).

The pathophysiology behind the complex inflammation cascades secondary to TBI is not yet fully understood. However, resulting injuries and outcomes after TBI have been studied in the past and have suggested HMGB1 to be a major player in disease progression as well as a potential therapeutic target to reduce the injuries and improve outcome following TBI. As well as the current biomarkers of TBI such as glial fibrillary acidic protein (GFAP) and S100B (Vos et al., 2010) are limited by low sensitivity, predictivity, and specificity (Metting et al., 2012). In this regard, due to its profound role in TBI pathology and inflammatory pathways post-TBI, HMGB1 appears to be a promising candidate which can be used as a prognostic marker of TBI. Moreover, downregulating HMGB1/RAGE/TLR4/NF-κB axis might be a novel strategy against TBI which could attenuate the neurological functions as well.

ROLE OF HMGB1 IN NEUROINFLAMMATION AND RELATED PATHOLOGIES

Neuroinflammation is considered as an innate immune responses in the CNS, which is triggered in response to several inflammatory signals such as pathogen infection, injury or trauma, which might ultimately result in neurotoxicity (Streit et al., 2004). Microglia are known as the predominant innate immune cell in the CNS and are thus considered a pivotal mediator of neuroinflammatory processes (Gehrmann et al., 1995). Microglia express TLRs, in particular TLR4 (Ransohoff and Perry, 2009), and are reported to mediate the pro-inflammatory effects of HMGB1 in peripheral innate immune cells (Yang et al., 2010). Disulphide form of HMGB1 (ds-HMGB1) potentiates the microglia pro-inflammatory response to an immune challenge suggesting that acute increases or exposure to ds-HMGB1, as may occur during acute stress or trauma, might induce a primed immune phenotype in the CNS, which may lead to an exacerbated neuroinflammatory response if exposure to a subsequent pro-inflammatory stimulus occurs (Frank et al., 2016). There is an increased understanding that HMGB1 mediates inflammatory and immune reactions in CNS and emerging evidence reveals that HMGB1 plays an essential role in neuroinflammation through receptors such as TLR, RAGE, and NMDAR (Wan et al., 2016). Moreover, HMGB1 induces RAGE and TLR4 mediated neuroinflammation and necrosis after injuries such as lesions in the spinal cord and brain (Fang et al., 2012). HMGB1 mediates inflammation by activating the innate immune receptors during sterile injury, in a similar manner to activation by PAMPs (Yang and Tracey, 2010). The BBB is a special microvessel structure in the CNS, consisting of microvascular endothelial cells sealed by tight junctions. Its permeability is closely related with degeneration, injury and inflammation of the CNS (Huang et al., 2011). Similar line of study shows that damaged BBB correlates directly with neuroinflammation involving microglial activation and reactive astrogliosis, which is associated with increased expression and/or release of HMGB1 (Festoff et al., 2016). In an experiment evaluating the contribution of extracellular, cerebral HMGB1 (in absence of other DAMPs) in its disulphide or fully redox form to neuroinflammation demonstrate that ds-HMGB1 and fully redox HMGB1 (fr-HMGB1) function as pro-inflammatory mediators in the CNS, promoting BBB disruption and cytokine production (Aucott et al., 2018a). Thus, anti-neuroinflammation and maintenance of BBB integrity may be potential targets for neuroprotection (Cheng et al., 2018). Increasing evidence suggests that selective targeting of CNS inflammation is a viable strategy for interfering disease onset or progression for a number of neurodegenerative disorders where neuroinflammation is the key player (Hong et al., 2016). Despite the deteriorating role of neuroinflammation in many neurological diseases, the number of existing anti-inflammatory drugs is quite limited because of insufficient efficacy or undesired side effect (Craft et al., 2005). HMGB1 has emerged has a novel frontiers due to its plausible role in neuroinflammation (Lee et al., 2014) as well as in inflammatory diseases (Harris et al., 2012) where the causal role for HMGB1 in a range of non-degenerative neuroinflammatory conditions has been well reported. Moreover, HMGB1 blocking therapies have proven to be highly beneficial, demonstrating remarkable neuroprotection in several neuroinflammation models (Kim et al., 2006).

Inflachromene (ICM), a microglial inhibitor possessing anti-inflammatory effects via binding with HMGB1 blocks the sequential processes of cytoplasmic localization and extracellular release of HMGBs by perturbing its post-translational modification as well as downregulates pro-inflammatory functions of HMGB and reduces neuronal damage *in vivo* demonstrating its potential against neuroinflammatory diseases (Lee et al., 2014). HMGB1 binds with lipopolysaccharides (LPS) and IL-1 to initiate and synergize TLR4-mediated pro-inflammatory response and immediately after pro-inflammatory stimulation by LPS, TNF-α, IL-1, IL-6, and IL-8, HMGB1 is released from activated monocytes and macrophages (Youn et al., 2008). The regulation of HMGB1 secretion is crucial for the regulation of HMGB1 mediated inflammation and is dependent on various processes such as phosphorylation by calcium-dependent protein kinase C (Oh et al., 2009). HMGB1 acts as a novel pro-inflammatory cytokine-like factor and regulates excitotoxicity-induced acute damage processes and delayed inflammatory mechanisms in the post-ischemic brain of Sprague Dawley (SD) rats (**Table 2**) (Kim et al., 2006). Elevation of HMGB1 in brain was measured in several non-degenerative neuroinflammatory condition such as ethanol exposure (Zou and Crews, 2014), and stress-induced neuroinflammatory priming (Weber et al., 2015). Neuroinflammation contributes to the progression of several neurodegenerative diseases including PD (Tansey and Goldberg, 2010) and AD (Heneka et al., 2015). Blocking the neuroinflammatory pathways in these neurodegenerative diseases will exerts neuroprotection against these diseases. Anti-HMGB1 mAb has inhibited the activation of microglia, prevents BBB breakdown, and inhibit the expression of inflammation cytokines such as IL-1β and IL-6 in an experimental model of PD demonstrating its neuroprotective effects possibly via suppressing neuroinflammation (Sasaki et al.,

TABLE 2 | Summary of findings reporting HMGB1 in neuroinflammation mediated conditions.

S.N.	Intervention	Model	Mechanism	Observation	Reference
1.	HMGB1	Rat	• Delayed inflammatory processes by extracellular HMGB1	• HMGB1 was released during the excitotoxicity-induced acute damaging process • Extracellular HMGB1 provokes inflammatory processes and acts like a novel pro-inflammatory cytokine-like factor	Kim et al., 2006
2	HMGB1	Mice	• Activation of NF-κB and NADPH oxidase by HMGB1 via binding with Mac1	• HMGB1-Mac1-NADPH oxidase signaling cascades connects chronic neuroinflammation and dopaminergic neurodegeneration	Gao et al., 2011
3	HMGB1	Rat	• HMGB1 acted as an early pro-inflammatory cytokine	• HMGB1 released into the cytoplasm soon after ICH • Mediate inflammation during the acute phase of ICH	Lei et al., 2013
4	HMGB1	Rat	• Inflammatory responses produced via HMGB1/TLR4/NF-κB signaling	• HMGB1 ↓ the release of IL-6 and TNF-α • HMGB1 inhibited activation of NF-κB in the developing brain	Tian et al., 2015
5	HMGB1	Rat	• Regulation of age-related priming of the neuroinflammatory responses by HMGB1	• HMGB1was ↑ in aged rodent brains and CSF • Blocking HMGB1 "desensitized" microglia in the aged brain and prevent pathological infection-elicited neuroinflammatory responses	Fonken et al., 2016

S.N., serial number; ↑, increased; ↓, decreased; HMGB1, high mobility group box 1; ICH, intracerebral hemorrhage; Mac1, macrophage antigen complex 1; CSF, cerebrospinal fluid; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TLR4, Toll like receptor 4; RAGE, receptor for advanced glycation end products; IL-6, interleukin-6; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen.

2016). Glycyrrhizin attenuated neuroinflammation, cognitive deficits, microglial activation related over-expression of pro-inflammatory cytokines in the hippocampus induced by LPS showcasing its therapeutic potential against neurodegenerative diseases like AD (Song et al., 2013).

Multiple sclerosis is an autoimmune-mediated chronic, inflammatory, demyelinating disease of CNS characterized by axonal damage (Compston and Coles, 2008). Experimental autoimmune encephalomyelitis (EAE) is the most reliable experimental model of MS (Miller et al., 2010). HMGB1 is receiving increasing attention in autoimmune disorders including MS. The very first study unraveling the role of HMGB1 in the pathophysiology of MS reported increased numbers of macrophages with cytoplasmic HMGB1 in active lesions (Andersson et al., 2008) and suggest HMGB1 as a novel biomarker of inflammatory demyelinating disease. Several researches has emerged on the base of earlier studies and suggest that the expression and release of HMGB1 are remarkably elevated in several stages of EAE where HMGB1 expression pattern is dynamically changed during the progression of EAE, as well as validated HMGB1 as a key mediator of EAE pathology (Sun et al., 2015). Targeting HMGB1 locally might exhibits therapeutic potential against EAE which can attenuate the disease severity and incidence as well as delayed disease onset time (Robinson et al., 2013). Neutralization of HMGB1 appears to be a novel strategy against MS as evidenced by an experimental study of MS, where anti-HMGB1 mAb ameliorated clinical severity, reduced CNS pathology, and blocked the production of pro-inflammatory cytokine (Uzawa et al., 2013).

Amyotrophic lateral sclerosis (ALS) is a non-demyelinating neurodegenerative disease characterized by increased neuronal loss and enhanced neuroinflammation, with extensive activation of glial cells and microglia stimulation releasing pro-inflammatory molecules, ROS, and nitric oxide (Ray et al., 2016). Neuroinflammation is postulated as a pathological hallmark of ALS (Lewis et al., 2012), and HMGB1 has been extensively studied in ALS due to its putative involvement in the pathology of ALS which is elusive yet. However, the elevated level of HMGB1 in the spinal cord of transgenic mice (SOD1G93A transgenic mice) were observed and reported that HMGB1 may have a role in the progressive inflammatory and neurodegenerative processes in response to the neurotoxic environment present in the spinal cord of SOD1G93A mice rather than to be involved as a primary event in the motor neuron death (Coco et al., 2007).

Withaferin A in an animal model of HMGB1-induced inflammatory responses suppressed the production of

IL-6, TNF-α and the activation NF-κB by HMGB1 (Lee et al., 2012). HMGB1 acts a pathogenic factor in many inflammatory conditions including experimental arthritis models (Schierbeck et al., 2011). HMGB1 has been observed to be a key mediator of intestinal inflammation in non-alcoholic fatty liver disease (NAFLD) via RAGE and redox signaling (Chandrashekaran et al., 2017). Another study reported that liver inflammation in diabetic mice was improved via regulation of the HMGB1/TLR4/NF-κB signaling pathway (Yin et al., 2018). Taken together, these results clearly highlight HMGB1 as a key mediator in several inflammatory diseases and suggest that HMGB1 exhibits therapeutic potential against these HMGB1 mediated inflammatory disease. Evaluating the ameliorative effects of glycyrrhizin on SAH in a rat model significantly improved neurological scores, reduced HMGB1-positive cells, downregulated mRNA and protein levels of HMGB1, inhibited BBB permeability, and attenuated neuronal cell death and apoptosis after SAH, suggesting it as a promising candidate for brain inflammation (Ieong et al., 2018).

The result obtained from human studies on MS patients corroborated with the experimental studies where HMGB1 and its receptors (RAGE, TLR2, and TLR4) were up-regulated in CSF of MS patients implicating that RAGE, TLR2, and TLR4 actively participate in an inflammatory, innate immune response driving and shaping the ensuing adaptive immune response during MS (Andersson et al., 2008). In clinical studies in patients with ALS, activation of TLR/RAGE signaling pathways were observed as evidenced by the elevated expression of HMGB1 and its receptors in reactive glia in human ALS spinal cord. The activation of these pathways might contribute to the progression of inflammation, resulting in motor neuron injury (Casula et al., 2011). In addition, serum HMGB1 auto antibody (Ab) has been suggested as a biomarker for the diagnosis of ALS and can be used to monitor disease progression (Hwang et al., 2013). High level of HMGB1, IL-6, and IL-17A has been detected in CSF of patients with an anti-NMDA receptor (NMDAR) encephalitis (neuroinflammatory disorder) (Ai et al., 2018), reflecting the underlying neuroinflammatory processes but does not report any precise role of HMGB1 in disease pathology. Clinical study performed on patients with AD and mild cognitive impairment (MCI) observed enhanced BBB permeability by HMGB1 and suggest HMGB1 as a clinical biomarker as well as validates HMGB1 as a non-invasive biomarker of BBB dysfunction and neuroinflammation which can assess the progression of neurodegeneration in AD and MCI patients (Festoff et al., 2016).

On the ground of evidences highlighted above, HMGB1 has been implicated in ranges of neuroinflammatory diseases as well as inflammation mediated disorders and HMGB1 exhibits huge potential to be a reliable biomarker for neuroinflammation related pathologies. On the positive note, beneficial effects of targeting HMGB1 in brain inflammation related pathologies is well documented. Extensive exploration to innovate therapeutic strategy to attenuate uncontrolled neuroinflammation triggered by HMGB1 is a pressing need.

ROLE OF HMGB1 IN EPILEPSY

Epileptogenesis is described as complex structural changes in the brain that convert a normal brain into a brain debilitated by recurrent seizure activity (Sloviter and Bumanglag, 2013). Neurodegeneration (Pitkanen and Lukasiuk, 2009; Reddy, 2013), disruption of BBB (Bar-Klein et al., 2017), the amygdala (Aroniadou-Anderjaska et al., 2008), the glutamatergic system (Aroniadou-Anderjaska et al., 2008), oxidative stress (Ashrafi et al., 2007), and epigenetic modification of DNA (Hauser et al., 1993) are all involved in epileptogenesis and aggravates the process (**Figure 3**).

Due to the lack of disease modifying effect in mainstream AEDs, precise understanding of diseases pathology and developing novel therapeutic approach for epilepsy-related hyperexcitability is a current need. Searching for molecular mediators of epileptogenesis in animal models, much attention has been paid to the potential pathogenic role of HMGB1 in the generation and recurrence of seizures (Kaneko et al., 2017; Yang et al., 2017). In spite of tremendous advancement in research, the pathogenesis of epilepsy is still complex, however, brain inflammation is supposed to play the role (Riazi et al., 2010). There is mounting evidence which report that neuroinflammatory processes in the pathophysiology of seizures/epilepsy and HMGB1 were found to behave like an inflammatory cytokine in response to epileptogenic insults (Kaneko et al., 2017). Glial cells activation has been reported to serve an important role in the development of epilepsy and HMGB1 may mediate microglial activation via the TLR4/NF-κB signaling pathway during seizures (Shi et al., 2018). HMGB1 activates IL-1R/TLR signaling in neurons and has a key role in seizure generation and recurrence via rapid sarcoma family kinases catalyzed phosphorylation of NMDA-NR2B receptors (Vezzani et al., 2012). As well as, HMGB1 serves a key role in epileptogenesis via microglial activation, via TLR4-NF-κB signaling pathway activation (Shi et al., 2018). An array of investigation has reported the role of HMGB1 in seizure but the precise mechanism on how HMGB1 leads to seizure generation is not documented well. HMGB1 released from glia and neurons and its signaling with TLR4 are suggested in generating and perpetuating seizures, the suggestion was based on the anti-convulsant activity of TLR4 inhibitors and Box A, a competitor of endogenous HMGB1 but the study lacks detailed mechanism how HMGB1/TLR4 axis leads to seizure generation (Maroso et al., 2010). Although HMGB1 activates both TLR4 and RAGE, the role of RAGE in seizures is less prominent than that of TLR4 (Iori et al., 2013).

Mesial temporal lobe epilepsy (MTLE) is a most common refractory focal epilepsy syndromes (Palleria et al., 2015) and role of HMGB1 in the pathogenesis of MTLE remains unknown. Experimental MTLE study reported significant upregulation of HMGB1 and TLR4 gene expression in the hippocampi of a rat and correlated this overexpression of HMGB1 and TLR4 to the pathogenesis of MTLE in immature rats (Yang et al., 2017). In addition, the role of HMGB1 and its receptors (RAGE and TLR4), including the pro-inflammatory cytokine IL-1β, in generating and perpetuating seizures is well documented

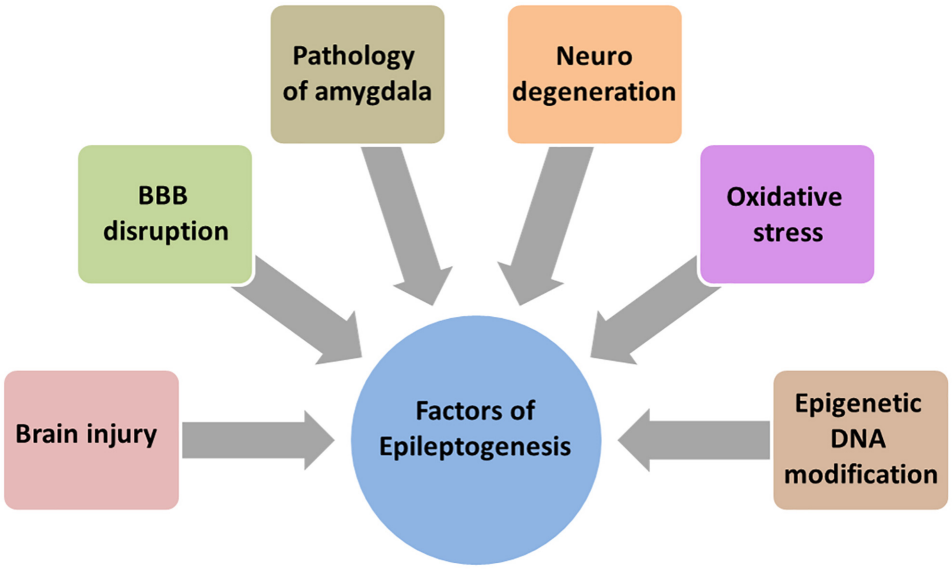

FIGURE 3 | Array of factors contributing to epileptogenesis. Much is unknown about the precise mechanism of epileptogenesis making it difficult to design and develop new therapies. But there is an increased understanding that brain injury, BBB disruption, neurodegeneration, oxidative stress, epigenetic DNA modification and pathology of amygdala contribute and aggravate the epileptogenesis.

(Zaben et al., 2017). Pharmacological and genetic studies on animal and clinical brain specimens showed that translocation and release of HMGB1 occurs in the pathological epileptogenic focus of different type of epilepsy (Maroso et al., 2010; Iori et al., 2013). It is worth noting that HMGB1/TLR4 axis not only reduced seizure frequency and duration, but also accelerated seizure onset, which usually occurs within minutes in kainite and bicuculline-induced seizure models implicating the important role of HMGB1 in the precipitation of the first seizure after a pro-convulsant administration (Maroso et al., 2010). Abnormal extracellular HMGB1 might contribute to the pathophysiology of epilepsy-related hyperexcitability as evidenced by study on PRNCs demonstrate a surge in extracellular HMGB1 approximated seizure initiation, establishing HMGB1 as a key pathophysiological contributor to the onset of epilepsy-related hyperexcitability (**Table 3**) (Kaneko et al., 2017).

TLR4 activation in neurons and astrocytes by HMGB1 proteins is a key mechanism of seizure generation and blocking TLR4 signaling using an antagonist could also reduce the severity of epilepsy (Iori et al., 2013). Investigation on post-surgery patients with intractable epilepsy revealed increased levels of HMGB1, TLR4, RAGE, NF-κB, p65 and inducible nitric oxide synthase (iNOS) in the brain of the epilepsy group as well as increased levels of IL-1, IL-6, TNF-α, TGF-β, and IL-10 in epilepsy patients (Shi et al., 2018).

Targeting HMGB1/TLR4/RAGE signaling for epilepsy has gained more attention in recent years. MicroRNA-129-5p inhibited the development of autoimmune encephalomyelitis (AE)-related epilepsy by HMGB1 expression and inhibiting the TLR4/NF-κB signaling pathway (Liu et al., 2017). HMGB1 and TLR4 antagonists slowed seizure precipitation, prevented acute and chronic seizure recurrence in C57BL/6 mice as well as reported increased expression of HMGB1 and TLR4 in human epileptogenic tissue, which is similar to a mouse model of chronic seizures and suggest a role for the HMGB1-TLR4 axis in human epilepsy (Maroso et al., 2010). Thus, HMGB1/RAGE/TLR4 signaling might contribute toward the generation and perpetuation of seizures (**Figure 4**) in humans and can be successfully targeted to attain anti-convulsant effects in epilepsies which are resistant to drugs. The expression level of HMGB1 was significantly elevated in the hippocampus and cortex after 24-h in a KA-induced model of SE, which suggests that the HMGB1 protein has a key role in epilepsy (Walker et al., 2014). HMGB1 enhances hypothermia induced seizures and contributes to the pathogenesis of febrile seizure. However, the precise mechanism of HMGB1 in febrile seizures remains unclear (Ito et al., 2017).

Limited data is available regarding evaluation of therapeutic benefits of HMGB1 inhibitors in animal models of epilepsy. However, glycyrrhizin demonstrates neuroprotection against lithium/pilocarpine-induced SE in rats, as well as ameliorates pilocarpine-induced oxidative injury and inflammatory responses via suppressing IL-1β and TNF-α, (González-Reyes et al., 2016) but did not demonstrate anti-epileptic activity. Dynamic changes in HMGB1 expression in the hippocampus of the mouse brain was reported after KA administration and glycyrrhizin exerts neuroprotective but not anti-epileptic effects via suppressing both acute and delayed HMGB1 inductions in the hippocampal cornu ammonis (CA)1 and CA3 region as well as its accumulation in serum (Luo et al., 2014).

Anti-HMGB1 mAb demonstrated an anti-seizure effect as evident by the lack of a disruption on the physical EEG

TABLE 3 | Summary of findings reporting HMGB1 in epilepsy.

S.N.	Intervention	Model	Mechanism	Observations	Reference
1	HMGB1	KA-induced seizure in mice	• Targeting HMGB1/TLR4 axis	• ↑ Frequency of seizure and total duration • Seizure can be ↓ by TLR4 and HMGB1 antagonists	Maroso et al., 2010
2	Anti-HMGB1 mAb	Acute seizure (MES and PTZ); Chronic seizure by KA in mice	• Inhibition of HMGB1 translocation	• ↓ Seizure threshold; ↓ time in tonic–clonic seizures and ↓ death • Delayed onset of generalized seizures; ↓ seizure stage; ↓ incidence of tonic seizures	Zhao et al., 2017
3	Molecular isoforms of HMGB1	Electrically induced Se in rats	• Activation of HMGB1/TLR4 axis	• ↑ level of HMGB1 and its acetylated and disulphide isoforms in blood	Walker et al., 2017
4	HMGB1	Pilocarpine-induced SE in rats	• Regulation of P-gp expression via RAGE/NF-κB inflammatory signaling pathways	• ↓ The expression levels of MDR1A/B mRNA and P-gp protein	Xie et al., 2017
5	HMGB1	KA-induced epilepsy in rats	• Modulation of glutamate metabolism	• ↑ Extracellular HMGB1 suggesting contribution of HMGB1 in epilepsy related hyperexcitability • Translocation of HMGB1from nucleus to cytosol after KA administration	Kaneko et al., 2017
6	HMGB1	Pilocarpine-induced epilepsy in rats	• Targeting HMGB1 via TLR4/NF-kB signaling pathway	• Inhibit the development of AE-related epilepsy • Suppression of HMGB1 expression • MiR-129-5p mediated TLR4/NF-kB signaling pathway ameliorated AE-related epilepsy	Liu et al., 2017
7	Anti-HMGB1 mAb	Pilocarpine-induced SE in mice	• Inhibition of HMGB1 release and inflammation	• Protection of BBB permeability; ↓ HMGB1 translocation • ↓ Latency and frequency of stage 5 seizures	Fu et al., 2017
8	Molecular isoforms of HMGB1	Human	• Evaluation of HMGB1 isoforms as mechanistic biomarkers of epileptogenesis in sera obtained from epileptic patients	• HMGB1 isoforms in the brain and blood were changed • Expression of disulphide HMGB1 in newly diagnosed epilepsy patients	Walker et al., 2017

S.N., serial number; ↑, increased; ↓, decreased; BBB, blood–brain barrier; Pgp, P-glycoprotein; MES, maximal electroshock seizures; KA, kainic acid; PTZ, pentylenetetrazol; HMGB1, high mobility group box 1; EEG, electroencephalogram; MDR1A/B, multidrug resistance protein 1A/B; RAGE, receptor for advanced glycation end products; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; miR-129-5p, micro RNA-129-5p; qRT-PCR, quantitative real-time polymerase chain reaction; AE, autoimmune encephalitis; mAb, monoclonal antibody; PRNCs, primary rats neural cells.

rhythm and basic physical functions as it prevents the translocation of HMGB1 from nuclei following a seizure. This anti-seizure effect was not observed in TLR4 knockout mice (**Table 3**) (Zhao et al., 2017). Moreover, anti-HMGB1 mAb also demonstrated a disease-modifying anti-epileptogenic effect on epileptogenesis after SE, as evidenced by a reduced seizure frequency and improved cognitive function (Zhao et al., 2017). The minimization of seizure frequency and duration can be achieved by inhibitors of HMGB1 which is supposed to act via targeting the HMGB1/RAGE/TLR4 axis and retard seizure precipitation via inhibition of HMGB1 translocation, protection of BBB integrity. In a similar line, anti-HMGB1 mAb exhibited inhibitory effects on the BBB leakage and pilocarpine-induced HMGB1 translocation. As well as prevented the BBB permeability and reduced HMGB1 translocation (**Table 3**) (Fu et al., 2017). Zhao et al. (2017) evaluated the anti-epileptic effect of anti-HMGB1 mAb on human brain slices from clinical drug-resistant epilepsy patients (**Table 3**)

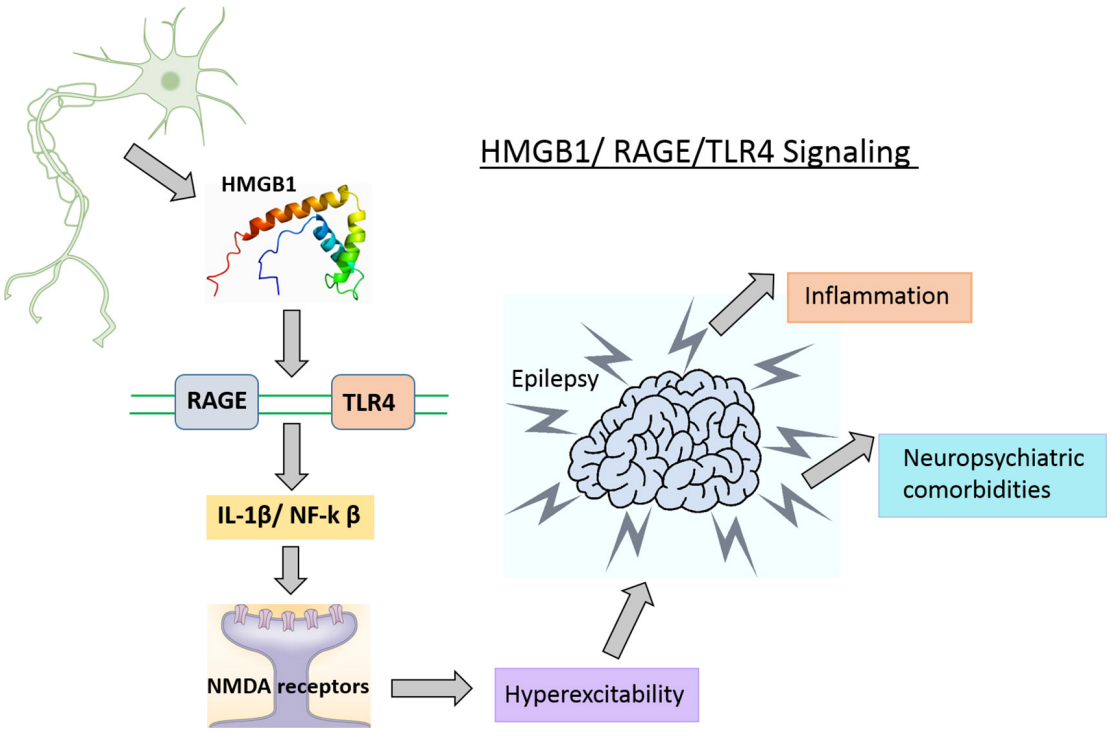

FIGURE 4 | HMGB1/TLR4/RAGE signaling in epilepsy. HMGB1 released from glia and neuronal cells in the CNS, activates its main receptor (TLR4 and RAGE) and contributes to epileptogenesis possibly via BBB disruption and activation of neuroinflammatory pathways.

where anti-HMGB1 mAb binds to HMGB1 and demonstrate long-lasting anti-epileptic properties, which is consistent with the previously estimated long half-time elimination in the brain (Zhang et al., 2011).

Extensive research highlights the putative role of HMGB1 in seizure generation, increased expression level of HMGB1 in epileptic brain (Chen et al., 2015) retardation of seizure precipitation by HMGB1 inhibitors (Zhao et al., 2017) implicating that HMGB1 is involved in all aspect from seizure generation to seizure retardation making HMGB1 as a strong candidate to be a reliable biomarkers for epileptogenesis. Moreover, the prevailing prognostic markers for seizure recurrence and seizure remission in patients diagnosed with epilepsy are solely based on supplementary factors including age, seizure type, EEG, and MRI, but are limited in their accuracy (Ravizza et al., 2017). Earlier study recommended HMGB1 isoforms as a mechanistic biomarkers for epileptogenesis where they investigate the value of blood HMGB1 in predicting epilepsy development as well as differentiating epileptogenic from non-epileptogenic rats after SE (Walker et al., 2017). HMGB1 as a biomarker of epileptogenesis will eventually provide a deeper insight on the normal biologic processes, pathogenic processes, or responses to an exposure or intervention, including therapeutic interventions with broad applications that are clinically able to arrest disease progression or to improve its clinical course. Moreover, precise understanding of mechanistic pathway on how HMGB1 induce seizure via inflammatory signaling will play a vital role in designing new therapies targeting inflammatory pathways to minimize seizures. However, the association between HMGB1 and seizure needs further exploration. Overall findings suggest that blocking the HMGB1/TLR4/RAGE regulatory axis may represent a novel method for treating epilepsy.

ROLE OF HMGB1 IN COGNITIVE DYSFUNCTION

Cognition refers to a collection of cognitive phenomenon such as learning and memory, attention, executive function, consciousness, and language (McAfoose and Baune, 2009). Cognitive dysfunction is among the most prevalent and debilitating features highly associated with epilepsy (de Krom, 2006), PD (Kalia, 2018), AD (Elgh et al., 2006). The precise mechanism of cognitive dysfunction is not well understood, though there is an increased understanding about chronic activation of cytokine-dependent inflammatory signaling contributing to neuronal dysfunctions manifesting as cognitive deficits (Cunningham and Sanderson, 2008). In addition, increased brain cytokine signaling impairs learning and memory (Dantzer et al., 2008). Moreover, neuroinflammation has been reported to cause memory impairments as evidenced by an experimental study where LPS administration cause memory impairment via inducing neuroinflammation (Lee et al., 2008). Several study has been reported where cognitive impairment has been ameliorated via alleviating neuroinflammation (Ganai and Husain, 2018).

The main focus of the topic is HMGB1, which is an initiator and amplifier of neuroinflammatory process. HMGB1 has been implicated in impairing memory via mediating RAGE and TLR4 (Mazarati et al., 2011) however, the exact mechanism of HMGB1 in cognitive decline is limited. HMGB1 exhibits pro-excitatory effects in the hippocampus by elevating the phosphorylation of NR2B-NMDA receptors (Maroso et al., 2010), and thus increasing the receptor calcium channel conductance (Viviani et al., 2003). NR2B-containing NMDA receptors prevent cell surface expression of the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Kim et al., 2005), which has a key role in both synaptic plasticity and memory (Sanderson et al., 2008), including the NORT (Schiapparelli et al., 2006). In the adult brain, NR2B decreases the span of retrovirus-associated DNA sequences (Ras)/extracellular signal-regulated kinases (ERK) activation pathway (Kim et al., 2005), which might also contribute to memory impairment (Weeber and Sweatt, 2002). Remarkably, the same mechanism that modulates the seizure-facilitating effect of HMGB1 (i.e., the activation of NR2B-containing NMDA receptor) might be simultaneously involved in facilitating learning deficits. Recombinant HMGB1 impaired memory encoding in wild type (WT), TLR4 knockout and RAGE knockout animals, but no effect was observed either on memory consolidation, nor retrieval. Moreover, memory deficits was not exhibited on TLR4 knockout nor RAGE knockout mice *per se*. Blockade of TLR4 in RAGE knockout mice using *Rhodobacter sphaeroides* LPS attenuated the memory function impaired by HMGB1(Mazarati et al., 2011). The upregulation of microglia and systemic HMGB1 levels were correlated with cognitive dysfunction (Terrando et al., 2010). IL-1 modulation has been implicated to ameliorate LPS-induced cognitive dysfunction, however, IL-1 blockade ameliorated cognitive decline by reducing microglia without affecting HMGB1 (Terrando et al., 2010).

Plausible detrimental effects of HMGB1 on memory may have broad clinical implications. In an experimental model of chronic cerebral hypoperfusion (CCH), HMGB1 neutralization attenuates hippocampal neuronal death and cognitive impairment where anti-HMGB1 neutralizing Ab exerts long-time positive effects on hippocampal CA1 neuronal survival and cognitive abilities in the chronic phase of CCH as well as preserves BBB integrity, and suppresses hippocampal glial activation, pro-inflammatory cytokine production (Hei et al., 2018). Anti-HMGB1 mAb has ameliorated the symptoms and phenotype of AD in an experimental model where mAb against HMGB1 completely rescued cognitive impairment in a mouse model via inhibiting neurite degeneration even in the presence of amyloid beta (Aβ) plaques. The recovery in the memory impairment was evidenced by Y-maze test (Fujita et al., 2016).

Post-operative cognitive dysfunction is probably the most frequent type of postoperative cognitive impairment and the pathophysiology of POCD remains incompletely understood (Grape et al., 2012). HMGB1 has been extensively studied against POCD. Possible role of neuroinflammation mediated by HMGB1, RAGE, and S100B (a class of DAMPs) was hypothesized in the pathophysiology of POCD, however, the relationship between HMGB1 or S100B or RAGE signaling and cognitive dysfunction was not completely confirmed (Li et al., 2013). HMGB1 and RAGE levels were remarkably upregulated after surgery and HMGB1 is supposed to cause cognitive decline via breaking BBB permeability, however, study did not conclude either BBB is disrupted after surgery and relationship between HMGB1 and cognitive decline cannot be ascertained as the study did not selectively block HMGB1 using mAb (He et al., 2012). Administration of endogenous HMGB1 proteins produced cognitive decline in mice and neutralized HMGB1 mAb ameliorated cognitive decline and inhibited the inflammatory response after tibial surgery, suggesting a initiating role for this mediator in POCD (**Table 4**) (Vacas et al., 2014). As well as clinical data obtained from patients undergoing gastrointestinal surgery showed that serum HMGB1 and IL-6 levels was elevated post-surgery, and the increased post-operative HMGB1 and IL-6 levels were associated with the cognitive decline the occurs 1-week post-surgery (**Table 4**) (Lin et al., 2014). Oral pretreatment of glycyrrhizin inhibited HMGB1 cytosolic expression, alleviates the surgery-Induced HMGB1 upregulation in the hippocampus of the mice and attenuated the severity of post-operative memory impairment, as evidenced by the shorter swimming latency and distance in MWM trials (Chen et al., 2017a). The therapeutic benefits of HMGB1 have been explored in sepsis survivors where HMGB1 mediates cognitive dysfunction in a murine model of severe sepsis survivors (Chavan et al., 2012). Administration of neutralizing anti-HMGB mAb to survivors, beginning 1 week after the onset of peritonitis, significantly ameliorate memory impairments and brain pathology.

Cognitive decline induced by epilepsy is supported by number of previous studies (Kundap et al., 2017). Earlier studies documented selective deficits in memory encoding in TLE (Schwarze et al., 2009). The plausible role of HMGB1 in epilepsy induced cognitive dysfunction has not yet been reported, though administration of anti-HMGB1 mAb in mice delayed epilepsy onset as well as ameliorated cognitive functions (Zhao et al., 2017). However, the precise role of HMGB1 protein in epielpsy induced cognitive dysfunction has not yet been reported. In order to obtain a precise understanding, it is necessary to perform a longitudinal studies to investigate the levels of HMGB1 in epileptic animal models and concurrently undertake behavioral studies to assess the cognitive function of the animals and evaluate the expression pattern of HMGB1 throughout the study.

There are very few clinical studies that have been conducted till date regarding the effectiveness of anti-HMGB1 mAb on ameliorating cognitive dysfunction in patients. Foo et al. (2017) earlier reported that an interaction between HMGB1 and APOE-ε4 is associated with cortical thinning in MCI. This interaction was observed by studying genomic DNA extracted from peripheral blood and the plasma HMGB1 was measured with an ELISA kit (**Table 4**) (Foo et al., 2017). In human brain slice studies obtained from surgical resection of clinical drug-resistant epilepsy patients, anti-HMGB1 mAb demonstrated an attenuation of cognitive function as well as a disease-modifying anti-epileptogenesis effect, which is indicated by reduction in seizure frequency (Zhao et al., 2017).

TABLE 4 | Summary of findings reporting HMGB1 in cognitive dysfunction.

S.N.	Intervention	Model	Mechanism	Observation	Reference
1	HMGB1	Mice	• Activation of inflammatory pathways by stimulating RAGE and TLR4	• ↑ Brain levels of HMGB1 induce cognitive abnormalities and are mediated by either TLR4 or RAGE.	Mazarati et al., 2011
2	HMGB1	Mice	• Activation and trafficking of circulating bone marrow-derived macrophages to the brain	• POCD can be prevented by minimizing the effects of HMGB1 • A neutralizing antibody to HMGB1 protein reduced memory dysfunction	Vacas et al., 2014
3	HMGB1	Mice	• Neuroinflammation mediated by HMGB1 and RAGE	• Expression of HMGB1, RAGE and NF-κB p6 ↑ after surgery. • HMGB1 and RAGE signaling modulate the hippocampal inflammatory response	Koeth et al., 2013
4	HMGB1	Rat	• HMGB1 and RAGE signaling	• HMGB1 and RAGE ↑ in the hippocampus of operated animals • HMGB1 interrupt and regulate the inflammatory response associated with the pathogenesis of POCD	He et al., 2012
5	HMGB1	Human	• Interaction between APOE-ε4 and HMGB1	• HMGB1 showed an association with ↓ cortical thickness • APOE-ε4 and HMGB1 are responsible for extensive cortical thinning in MCI.	Foo et al., 2017
6	HMGB1	Human	• POCD induced via inflammatory response	• HMGB1, IL-6 levels ↑ in patients after surgery • Elevated levels of HMGB1, IL-6 might be associated with cognitive dysfunctions after surgery	Lin et al., 2014

S.N., serial number; ↑, increased; ↓, decreased; HMGB1, high mobility group box 1; TRL4, toll like receptor 4; RAGE, receptor for advanced glycation end products; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; TEM, transmission electron microscopy; IHC, immunohistochemistry; MCI, mild cognitive impairments; APOE-ε4, Apo lipoprotein E ε4; IL-6, interleukin-6; POCD, post-operative cognitive dysfunctions.

HMGB1: TRANSLATIONAL IMPLICATION

Recent investigation shed more light on multiple roles of HMGB1 in a diverse range of pathologies such as brain injury, epilepsy, and neuroinflammation and cognitive decline. Treatments based on HMGB1 antagonists via targeting extracellular HMGB1 have generated encouraging results in a wide number of experimental models of aforementioned HMGB1 mediated pathologies, though the clinical studies are yet to be reported. However, complex biology of HMGB1 has not been fully understood yet and there is a notion that association between HMGB1 and brain injury, epilepsy, neuroinflammation mediated pathologies and cognitive decline requires deeper exploration, as the precise mechanism of on how HMGB1 mediates these neurological conditions are yet to be well documented. Despite of that, the identification of HMGB1 inhibitors results in significant experimental and clinical interest. Moreover, HMGB1 as a common biomarker of TBI, neuroinflammation, epileptogenesis and cognitive decline might be instrumental in assessing the disease progression, early prediction of disease as well as evaluating patient's response to therapy. Translational implication of HMGB1 will be a paradigm shift, which will not only overcome the limitation of currently available AEDs, improve the cognitive decline as well. Moreover, via inhibiting the neuroinflammatory pathways HMGB1 can ameliorate several brain injuries and neuroinflammation mediated pathologies. More precisely, inhibiting HMGB1/RAGE/TLR4 pathway represents a promising approach which can interfere with disease progression in epileptogenesis, neuroinflammatory disease, several forms of brain injury as well as memory impairment. The focus of the topic is TBI, neuroinflammation, epilepsy and cognitive decline, however, blocking HMGB1 might achieves significant neuroprotection in several forms of neurodegenerative disorders where neuroinflammation plays a crucial role. Future strategy should be focused on exploring several HMGB1 antagonist which can efficiently interact with the main HMGB1-receptor, RAGE, acting as competitive antagonists of HMGB1, such as recombinant box A (the truncated N-terminal domain of HMGB1) or S100P-derived RAGE peptide (Musumeci et al., 2014).

SUMMARY OF FINDINGS AND CONCLUSION

Neuroinflammation has been implicated in ranges of neurological disorders such as TBI, epilepsy and memory impairment. HMGB1 being the mediator of neuroinflammation has been reported to play crucial role in TBI, neuroinflammatory diseases and epileptogenesis via an unknown mechanism. As well as elevated level of HMGB1 in serum and CSF has been observed in these neuroinflammation mediated pathologies. These strengthens the rationale of our study in suggesting HMGB1 as a common biomarker in TBI, neuroinflammation, epilepsy and cognitive decline. Biomarker discovery together with investigations into novel therapeutic candidates would give a noteworthy headway in the treatment of TBI, epilepsy, memory impairment and neuroinflammation via acting on its mechanistic pathway rather than symptomatic control. In current review, an attempt was made to connect the dots between HMGB1 and its putative role in several forms of brain injury, neuroinflammation mediated conditions, epilepsy and cognitive decline using preclinical and clinical evidence.

Several important limitations regarding the topic should not be ruled out, such as feasibility and viability issues in making HMGB1 a common functional biomarker for neuroinflammation mediated pathologies discussed herein. Can inhibiting HMGB1/RAGE/TLR4 axis be a common target for these neurological conditions? Can therapeutic outcomes obtained from experimental evidence regarding the role of HMGB1 in all these neurological conditions be easily translated into clinical settings? These are the concerns that remains unsolved as more experimental data are yet to come.

Despite accumulating scientific evidence of HMGB1 in the neuroinflammation mediated conditions discussed in current review, no attempt has been made in portraying HMGB1 as common biomarker and target for these HMGB1 mediated neurological conditions. We suggest, HMGB1 proteins can be considered as a promising non-invasive, common biomarker of TBI, neuroinflammation, epilepsy and cognitive dysfunction as it meets many criteria to stand as a common biomarker. It is relatively stable in blood and can be rapidly and inexpensively measured in blood. Moreover, changes in the total HMGB1 levels in the brain during neurological conditions discussed herein, can be mirrored in the blood.

As a concluding remark, drawing evidence from earlier preclinical and clinical studies, the current review advances the concept of positioning HMGB1 as common functional biomarker that can significantly improve risk assessment, diagnosis and monitoring of the neurological diseases discussed in this review. As well as HMGB1 can emerge as a novel avenues against TBI, neuroinflammation, epilepsy and cognitive deficits which acts by blocking the neuroinflammatory pathway.

AUTHOR CONTRIBUTIONS

YP and MS carried out literature review, conceptualized, designed and drafted the manuscript. AC, YK, ÁA-S, KA, MA and IO provided critical revisions and contributed to the final manuscript. YK also designed the figures. All authors read and approved the final manuscript.

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Fourier-Transform Infrared Imaging Spectroscopy and Laser Ablation -ICPMS New Vistas for Biochemical Analyses of Ischemic Stroke in Rat Brain

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Objective: Stroke is the main cause of adult disability in the world, leaving more than half of the patients dependent on daily assistance. Understanding the post-stroke biochemical and molecular changes are critical for patient survival and stroke management. The aim of this work was to investigate the photo-thrombotic ischemic stroke in male rats with particular focus on biochemical and elemental changes in the primary stroke lesion in the somatosensory cortex and surrounding areas, including the corpus callosum.

Materials and Methods: FT-IR imaging spectroscopy and LA-ICPMS techniques examined stroke brain samples, which were compared with standard immunohistochemistry studies.

Results: The FTIR results revealed that in the lesioned gray matter the relative distribution of lipid, lipid acyl and protein contents decreased significantly. Also at this locus, there was a significant increase in aggregated protein as detected by high-levels $A\beta_{1-42}$. Areas close to the stroke focus experienced decrease in the lipid and lipid acyl contents associated with an increase in lipid ester, olefin, and methyl bio-contents with a novel finding of $A\beta_{1-42}$ in the PL-GM and L-WM. Elemental analyses realized major changes in the different brain structures that may underscore functionality.

Conclusion: In conclusion, FTIR bio-spectroscopy is a non-destructive, rapid, and a refined technique to characterize oxidative stress markers associated with lipid degradation and protein denaturation not characterized by routine approaches. This technique may expedite research into stroke and offer new approaches for neurodegenerative disorders. The results suggest that a good therapeutic strategy should include a mechanism that provides protective effect from brain swelling (edema) and neurotoxicity by scavenging the lipid peroxidation end products.

Keywords: photothrombotic, ischemic, brain, stroke model, FTIR imaging spectroscopy, LA-ICPMS, lipid peroxidation, neurodegeneration

INTRODUCTION

Stroke is the leading cause of adult disability in the world, leaving more than half of those affected dependent on daily assistance. Patients are often hospitalized and, or subjected to intensive rehabilitation programs for long periods, with their quality of life severely affected, socially and economically. Hence, a more thorough understanding of the molecular and cellular changes are required for the design of better therapies to treat this debilitating illness (Ramasubbu et al., 1998; Sarti et al., 2000; Snögren and Sunnerhagen, 2009; Lekander et al., 2017).

Ischemic stroke is caused by a reduction in blood circulation in the brain vasculature, resulting in decreased supply of oxygen and nutrients followed by an ischemic cascade, and if not reversed results in neurodegeneration and ultimately cell death (Deb et al., 2010; Xing et al., 2012; Hu et al., 2017). Acute ischemic stroke causes unregulated cation influx, mainly Na^+, which initiates the cytotoxic edema (Liang et al., 2007). Also, accumulation of Ca^{2+} inside forebrain neurons initiates glutamate mediated excitotoxicity (Lai et al., 2014; Prentice et al., 2015). This enhanced intra-cellular calcium-levels activates numerous pathways including proteases which can degrade proteins and membrane lipids and promote the generation of free radicals and reactive oxygen species (ROS) which can lead to neuronal damage, particularly of the cell membrane (Lee et al., 2000; Bretón and Rodríguez, 2012). Furthermore, mitochondria dysfunction can also result in apoptosis (Broughton et al., 2009; Xing et al., 2012). Stroke insult, also weakens and disturbs the blood–brain barrier (BBB) which can result in the development of vasogenic edema (Dostovic et al., 2016). Vasogenic edema causes disturbances in brain function, mass-effect (displacing surrounding brain tissues) with distortion, tissue shift and increased intracranial pressure and damage (Simard et al., 2007; Michinaga and Koyama, 2015).

Tissue in adjacent regions also undergo morphological, cellular and sub-cellular changes (Dirnagl et al., 1999; Lo et al., 2003; Shichita et al., 2014; Hu et al., 2017), such as gliosis (Garcia, 1984; Li et al., 2005; Huang et al., 2014), lipid peroxidation (Yamamoto et al., 1983; Yildirim et al., 2007; Zeiger et al., 2009) and protein malformation (Brouns et al., 2010; Zhao and Bateman, 2015). Perilesional changes are not necessarily detrimental, as neuronal plasticity and tissue reorganization in GM and WM may compensate and contribute to recovery post ischemic stroke (Carmichael, 2003; Cheatwood et al., 2008; Murphy and Corbett, 2009). Delay and ongoing tissue damage post ischemic stroke results in impeding brain tissue repair and recovery and contributes to brain dysfunctions. A model to study ischemic stroke is the photo-thrombotic model of focal ischemia, where photosensitive dye is excited by fluorescence causing a blood clot (Pevsner et al., 2001). Conventional techniques, such as magnetic resonance imaging (MRI) (Van Bruggen et al., 1992; Pevsner et al., 2001), auto-radiography (Dietrich et al., 1986), routine histology (Van Bruggen et al., 1992; Pevsner et al., 2001; Kuroiwa et al., 2009), and IHC (Van Bruggen et al., 1992) have been applied to characterize the biochemical and anatomical alterations after stroke in many studies (Dietrich et al., 1986; Pevsner et al., 2001; Kuroiwa et al., 2009). These techniques provide important data, but for example, MRI is not sufficient to study bio-chemical and bio-molecular changes at cellular and sub-cellular levels. Whereas, histology and IHC give high spatial resolution but again, only limited to certain biochemical markers as well as disrupting the tissue morphology.

Spectroscopic techniques such as FT-IR imaging spectroscopy and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) are potentially attractive bio-imaging platforms that can provide molecular (Kazarian and Chan, 2006; Petibois and Desbat, 2010) and elemental images (Becker et al., 2014), respectively. Both techniques offer high resolution (in the range of 5–10 μm), yielding detailed information about bio-chemical and chemical features at the cellular and/or sub-cellular level (Lasch et al., 2002; Kumar et al., 2014; Harrison and Berry, 2017) related to pathology. In contrast to standard histological staining methods, FTIR imaging has the ability to detect, simultaneously, discrete changes in molecular structure and composition of tissues. Direct biochemical analyses of all macromolecular components within tissue samples can be obtained from a single data acquisition, without the addition of chemical stains or reagents and without disrupting the tissue morphology (Petibois et al., 2009; Miller et al., 2013). Infrared (IR) spectra are dominated by macromolecular building blocks, such as proteins, lipids, cholesterols, phospholipids, carbohydrates, and nucleic acids (Carter et al., 2010; Srinivasan, 2010; Ami et al., 2013). The IR spectral absorptions provide readouts about these bio-molecules and produce a specific biochemical fingerprint of the sample (Ami et al., 2013; Baker et al., 2014; Kohler et al., 2015). FT-IR based techniques have been used to image molecular alterations associated with neuro-degeneration in animal models of Alzheimer's disease (AD) (Miller et al., 2006; Leskovjan et al., 2010; Liao et al., 2013; Benseny-Cases et al., 2014), Parkinson's disease (PD) (Szczerbowska-Boruchowska et al., 2007), amyotrophic lateral sclerosis (Kastyak et al., 2010), multiple sclerosis (Heraud et al., 2010), cerebral malaria (Hackett et al., 2015b), epilepsy (Turker, 2012; Turker et al., 2014) and hemorrhagic stroke (Ali et al., 2016; Caine et al., 2016; Balbekova et al., 2017). These studies have shown the potential of FTIR to assess molecular and neurochemical changes associated with brain pathology.

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been developed and established as a technique in the generation of quantitative images of metal distributions in thin brain tissue sections with applications in research related to neurological disorders. This technique detects trace elements that are essential for brain function, such as zinc (Zn), copper (Cu), and iron (Fe) (Becker et al., 2014). Simultaneous analysis of these trace elements in brain can yield important information on the processes of neuronal (dys) function for example after PD (Hare et al., 2009; Hare et al., 2010; Matusch et al., 2010, 2012), AD (Matusch and Becker, 2012), stroke (Becker et al., 2011) and

Abbreviations: CC, corpus callosum; CL-GM, contra-lesioned gray matter; CL-WM, contra-lesioned white matter; FTIR, Fourier transform Infrared; GM, gray matter; IHC, immunohistochemistry; LA-ICPMS, laser-ablation inductively-coupled-plasma mass-spectrometry; L-WM, lesioned white matter; PL-GM, perilesioned gray matter; PS-GM, primary stroke lesion gray matter; ROI, regions of interest; TC-GM, time control gray matter; WM, white matter.

brain tumor (Becker et al., 2011). LA-ICP-MS is suitable for the imaging of metals in thin cross sections of soft and hard biological tissues with very low concentrations such as sub μg/g level (Hare et al., 2009; Becker et al., 2011; Matusch and Becker, 2012; Becker et al., 2014). The advantages of LA-ICP-MS is that it is highly sensitivity, accurate, precise as well as being a unique technique to scan the elemental distribution in the whole brain section.

In the present study, we combined FTIR and LA-ICP-MS with routine histological/IHC to characterize the bio-chemical and elemental changes in PS-GM, PL-GM, L-WM, CL-WM, CL-GM, and CC following 1-week photothrombotic stroke in rat brain sections. Our goal was to use FTIR and LA-ICPMS imaging techniques with conventional histological studies to identify alterations of specific bio-molecules and elements associated with ischemic stroke. In addition, this study will advance stroke bio-diagnostics and treatment and provide links between tissue injury, plasticity and repair, as well as identifying novel therapeutic targets and interventions.

MATERIALS AND METHODS

Animal Model

All experimental protocols and animal handling procedures were in accordance with the National Institutes of Health (NIH) recommendations and with guidelines of the European Communities Council Directive. The experimental animals protocol was approved by the by the University of Utrecht Animal Research Ethics Board (DEC 2013.I.08.063), and adhered to the Netherlands Council on Animal Care guidelines for humane animal use (Ali et al., 2016; Balbekova et al., 2017). A total of 12 male (11 week-old, Sprague-Dawley rats; Charles River Laboratories International, Wilmington, MA, United States), from which six animals were used in the Photothrombotic stroke, which was induced in the right sensorimotor cortex (Ali et al., 2016) and remainder six rats served as age-matched controls. Data were analyzed by comparing the lesioned generated values with the unlesioned (contralateral) hemisphere.

Block and Tissue Preparation

One week after stroke induction, the animals were sacrificed with an overdose of isoflurane followed by transcardial perfusion. Brains were extracted, fixed in 4% paraformaldehyde (PFA) for 24 h and embedded in paraffin (Balbekova et al., 2017). Paraffin embedded tissue blocks were serially cut into 5 μm slices using a microtome (Leica RM 2155 semi-automated rotary microtome, Germany). Thin coronal sections of brain were taken from central location of the lesion (Bregma 1.20 mm). Serial and consecutive sections were obtained for FTIR measurements, LA-ICPMS, and hematoxylin and eosin (H&E) staining and IHC studies. The brain sections for FTIR imaging were mounted on a CaF_2 window and a transflective slide. The brain sections used for the histology and IHC were mounted on Flex IHC microscopic slides (Dako, Fisher Scientific, United States). Routine histology was performed on control healthy rats to confirm normal tissue morphology (Ali et al., 2016).

Immunohistochemistry

Slides of brain sections 5 μm sections were washed with phosphate buffered saline (PBS) and maintained in 0.1 M PBS with 0.2% Triton X-100, pH 7.4 (PBST) for 2 days at 4°C. For antigen retrieval, the slides were incubated in 10 mM sodium citrate containing 0.05% Tween 20, pH 6.0 for 1 h at room temperature. To block non-specific protein binding, sections were incubated in 6% bovine serum albumin (BSA) in PBST for 2 h at room temperature, then incubated for 3 days at 4°C with the primary antibodies. The primary antibodies (all Abcam, Ltd., Cambridge, United Kingdom, unless stated) were rabbit polyclonal anti-amyloid precursor protein (APP) (1:1000 dilution; ab2072), rabbit polyclonal anti-Tau (1:1000 dilution, ab47579), rabbit anti-GFAP (1:2000 dilution; ab7260), rabbit anti-MAP2 (1:2000 dilution; ab5622), or mouse anti-myelin basic protein (MBP) (1:2000 dilution; ab62631), or chicken anti-beta amyloid (1:2000 dilution; ab134022). The primary antibodies were diluted in 2% BSA in PBST. After the sections had been rinsed three times with PBST (10 min each), sections were incubated for 2 h at room temperature with Alexa Fluor 488-conjugated donkey anti-chicken IgG, Alexa Fluor 647-conjugated donkey anti-rabbit IgG, or Alexa Fluor 647-conjugated donkey anti-mouse IgG (1:500; Thermo Fisher Scientific, United States). Negative controls were subjected to the same protocol without primary antibodies. After three rinses with PBST, the mounting media containing DAPI (nuclear stain) were added to the slides and then overlayed with cover slips. The slides were kept away from light and examined using a fluorescence microscope.

Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy (FTIR) spectra were recorded using an Agilent FTIR Cary 620 micro-spectrometer in reflection mode within the range of 4000–700 cm^{-1} with 64 scans per spectrum, 4 cm^{-1} spectral resolution and spatial resolution 80 μm. Background single beam spectra were measured on a substrate without biological tissue by co-adding 64 scans. FTIR chemical images were recorded using a 64 × 64 Mercury Cadmium Telluride (MCT) Focal Plane Array (FPA) liquid nitrogen cooled detector in mosaic mode. FTIR imaging was performed with a 15× objective (numerical aperture = 0.62), yielding a pixel size of 5.5 μm × 5.5 μm. The average size of the brain is 1 cm × 1.2 cm. Three sections of the same anatomy per animal were examined. From each region of interest an area of 200 μm × 200 μm were analyzed. To ensure consistency in the data and negate technical errors, exemplar samples were mounted on CaF_2 slides and measured in transmission mode as well as in the *trans*-reflective mode using transflective slides. In both modes, we obtained very similar results. To circumvent the electric field standing wave effect (EFSW) reported for variations in section thickness, we repeated exemplar sections at 8 and 10 μm thickness, and here again, we obtained very similar spectral profiles as the measured samples. **Supplementary Table S1** (Stable 1) shows the FTIR assignments used in the medical and biomedical fields (Goormaghtigh, 2016).

Data Pre-processing and Analysis

All data processing and image generation were using either Resolution Pro. Software, (version 5.0), or Cytospec, (version 2.00.03) and Origin software (version 8). The representative FTIR spectrum acquired from brain WM region of a control rat brain in the spectral range of 4000–800 cm^{-1} is shown in **Supplementary Figure S1** and the detailed spectral band assignments are given in **Supplementary Table S1** in which different contribution to each band were presented (Kneipp et al., 2000; Cakmak et al., 2012). Baseline correction was performed on the full range of wavelengths for the spectra. FTIR images of the functional groups were calculated using the baseline areas under the FTIR absorption bands based on the assignments in (**Supplementary Table S1**) (Kneipp et al., 2000; Cakmak et al., 2012). In this study, the area under the curve from specific bands were calculated in order to measure different bio-chemical contents after photothrombotic ischemic stroke induction and values were normalized to the contralateral (unlesioned) hemisphere values. For example the total lipid was represented by (C–H stretching region) in the spectral range of 3000–2800 cm^{-1}; lipid acyl $\nu_s(CH_2)$ groups at 2860–2840 cm^{-1}; lipid ester ν(C = O) at 1755–1715 cm^{-1}; olefinic = CH band at 3027–3000 cm^{-1}; CH_3 asymmetric stretching $\nu_{as}(CH_3)$ (methyl concentration) at 2960–2950 cm^{-1}; amide I band (total protein) at 1700–1600 cm^{-1}; β-sheet aggregate at 1630 cm^{-1} and α-helix structures at 1655 cm^{-1} as detailed in **Table 1** and **Supplementary Table S1** (Kneipp et al., 2000; Cakmak et al., 2012).

The C-H spectral region is dominated by lipid contribution and a small contribution from proteins, carbohydrates and nucleic acids (Kneipp et al., 2000). Based on this understanding, the following CH_2 asymmetric, CH_3 asymmetric; olefinic = CH and lipid ester (C = O) lipid assignment were all good approximate values. Lipid molecular and structural alterations were investigated by evaluating the integrated area under specific lipid spectral bands as follows: CH_2 asymmetric (chain lipid length); CH_3 asymmetric (methyl concentration); olefinic = CH (unsaturated lipid) and lipid ester (C = O) (oxidative stress by-products) as shown in **Table 1**. Raw spectra were vector normalized and second derivative was computed by calculating forward difference twice and smoothed using spline fitting. The spline fitting used cubic spline interpolation of the second derivative to smoothen spectra. Resonance Mie scattering can affect the spectra from the border between a brain tissue and a substrate which is commonly existing in biological samples and spectra acquired from a substrate apart from brain tissue area were eliminated from analysis (Bassan et al., 2010). The contribution of different types of proteins was calculated by fitting a linear mixed model of Gaussian bands centered at the wavenumbers of the protein types. Relative distribution of β-sheet aggregate protein were determined at 1625 cm^{-1} from the second-derivative intensity spectrum (Pribic, 1994; Hackett et al., 2015b; Caine et al., 2016). The relative amount of the protein aggregation content was quantified from the curve fitting of original absorbance spectra (Hackett et al., 2015b). Curve fitting was performed with MATLAB (version 2014a, United States) over the spectral range 1700-1600 cm^{-1}.

TABLE 1 | The spectral regions used for analytical purposes.

Infrared band	Integrated spectral range (cm^{-1})
Lipid components	
CH_2 symmetric stretching	2852–2800
CH_2 asymmetric stretching	2915–2930
CH_3 asymmetric stretching	2950–2960
*C-H stretching	2994–2800
Olefin = CH	3000–3027
Carbonyl ester (C = O) stretching	1745–1731
Protein components	
Amide I	1700–1600
Amide II	1555–1535
β-sheet aggregate	1630
α-helix structures	1655

**Total lipid regions.*

Chemometric Data Pre-processing and Analysis

Chemometric analyses, incorporating principal component analysis (PCA) and hierarchical cluster analysis (HCA), were used to differentiate the spectral types with the FTIR spectra of the brain pre-processed prior to analysis using shape-preserving piece-wise cubic interpolation (Pribic, 1994). The infrared spectra were converted from Agilent system format to comma separated values (CSV) to create data sets that were suitable for PCA analysis using the "prcomp" function in the "stats" package in R (Becker et al., 1988; Sanchez, 2011). Three principal components (PCs) were used for analysis since they explained the highest percentage of variations. The 3D scores plot of the PCs that explain the majority of the variance in the dataset enabled spectra to be grouped according to the chemical information they contained (Pribic, 1994).

Hierarchical cluster analysis was applied using the A2R package in R to compare the three brain regions (PS-GM, PL-GM, and CL-GM) based on their PCA projections in the 3D score space (Becker et al., 1988; Pribic, 1994; Sanchez, 2011). HCA was used to group spectra that displayed the same degree of similarity by calculating the Euclidean distance between all the data sets using Ward's algorithm. The result was visualized in a dendrogram and the grouping of the three brain regions were presented as images consisting of colors clusters according to the heterogeneity scale (Becker et al., 1988; Sanchez, 2011).

Laser Ablation ICPMS

An ICPQMS (Agilent 7700) laser ablation system (CETAC Technologies, Omaha, NE, United States) with a Nd:YAG laser at wavelength 266 nm was used throughout this work. **Table 2** shows the laser ablation and ICP triggering operational conditions. The data were processed to generate elemental images using a macro, and image processing software 'ImageJ.' The scan speed of each line was 120 μms^{-1}, and the ICP-MS was

TABLE 2 | Parameters for measuring a brain samples by LA-ICPMS.

Parameters	Condition
Spot size	100 microns
Space between lines	4 micron
Laser energy	15%
Laser shot frequency	20 Hz
Shutter delay	10 s
Scan rate	160
Number of lines	120
Integration time	Varies from 0.02–0.06 s
Total integration time	0.614 s
Total acquisition time	67 s

configured in data-only mode to collect 185 readings per line scan at a rate of 2 s^{-1} (Hare et al., 2010; Matusch and Becker, 2012).

Statistical Analysis

A paired *t*-test was performed to assess significant differences between the molecular content of the PS-GM and PL-GM; PS-GM and CL-GM and PL-GM and the CL-GM tissue. For each group, a mean and standard deviation was generated for each animal. Then the individual animal values were averaged for each group. Mann–Whitney-*U* test was performed on all data to test for significant differences ($p < 0.05$) between control and treated groups. All *t*-tests were two tailed and the 95% confidence limit was used to test for significance.

RESULTS

Histological and Immunohistochemical Changes After Photo-Thrombotic Stroke

One of the major aims of this study was to correlate molecular alterations after ischemic photo-thrombotic stroke, by using FTIR spectroscopy and elemental distribution changes (LA-ICPMS bio-imaging) and complement these results with immunostaining analyses of specific biomarkers. Hematoxylin and eosin (H&E) staining of the brain sections from unlesioned rats (native time-matched controls) is presented in **Figure 1A**. The locus of the photo-thrombotic ischemic stroke was confirmed using H&E staining (**Figure 1B**). The image clearly depicts loss of brain structure and integrity at the stroke focus and results in an absence of H&E staining within the ischemic infarct. The rim of the stroke region is the characteristic pale expression of the H&E staining due to brain edema (see inset **Figure 1B**). The contralateral hemisphere of the affected brain shows H&E strong staining expression. The six ROI for this study were: (a) the PS-GM; (b) PL-GM; (c) L-WM; (d) CL-WM; (e) CL-GM; and (f) CC (**Figure 1C**).

To gain insights into the effects of stroke on nerve cells in the lesion as well as the healthy areas of the brain, IHC analyses using specific biomarkers was carried out. Immunohistochemical analysis of lesioned tissue sections (ipsilateral side) established neuronal degeneration in the infarct region with immunofluorescence analyses highlighting GFAP expression, the astrocyte marker, strongly localized within the PL-GM, indicating astrogliosis post stroke (**Figures 1D,E**). The infract region experienced neuronal loss, sections were tested for the principal neuronal marker proteins: MAP2 and Tau (**Figures 1F–I**). In both instances, we failed to detect these proteins within the infarct region- validating neuronal loss. Comparing the contralesion sections with the ischemic brain sections, we documented APP (**Figures 2A–D**) and aggregated $A\beta_{1-42}$ (**Figures 2C–H**) in the GM and the PL-GM. Mis-processed APP, ($A\beta_{1-42}$) its extracellular aggregation is one neuropathological marker used to classify Alzheimer's disease (AD) (Paul Murphy and LeVine, 2010; Esparza et al., 2016; Wildburger et al., 2017). Surprisingly in the L-WM there was an increase in $A\beta_{1-42}$ aggregates (**Figure 2G**). Examination of MBP showed loss in integrity and significant disorganization of the myelin sheaths in the WM, at the ipsilateral side, below the stroke point (**Figure 2G**). Interesting to note, that the contra-lesioned white matter (CL-WM, **Figure 2H**) shows uniform MBP staining throughout WM tracts as seen with the native control (**Figure 2E**).

Molecular and Elemental Changes Due to Stroke

FTIR Spectra and Images

Fourier transform Infrared bands assignments in this study were based on the specific spectral bands as defined in the Section "Materials and Methods" (**Supplementary Figure S1** and **Supplementary Table S1**). As an example, the FTIR spectrum for the WM we observed that spectral bands corresponding to lipid, protein, ester, nucleic acids, and carbohydrates dominate the spectra (**Supplementary Figure S1**). All samples were prepared, treated and processed in the same manner, it should be noted that lipid, protein and other biochemical component values from the lesioned (ipsilateral side) were normalized to the contra-lateral side. Again, the contralateral hemisphere bio-chemical components values were used as a base line values indicating the stroke induced changes to the cortical region and the surrounding WM.

In Hematoxylin and Eosin section captured by digital scanning microscope, a parallel-unstained image of a control brain section, captured by FTIR microscope bright field is depicted together with the corresponding chemical FTIR spectroscopic image (**Figures 3A–C**). Lipid and protein distribution, as found in native healthy rat brain sections,was represented in **Figures 3D,E**. In contrast to the even distribution of protein and lipid in the control, the lesioned brain lipid and protein show distinct distribution patterns (**Figures 3F,G**). For example, in the PS-GM both lipid and protein levels were greatly reduced and associated with increasing aggregated protein. The PL-GM and L-WM regions experienced decrease in the lipid and protein components associated with protein aggregation (**Figures 3F,G** and see also **Figure 3M**). **Figures 3H,I** depict the average FTIR spectra for different tissue regions and the analysis of these spectra were performed on $n = 6$ animals. In the stroke PS-GM region, the FTIR spectra revealed that the lipid, protein, lipid acyl bands $\nu_s(CH_2)$,

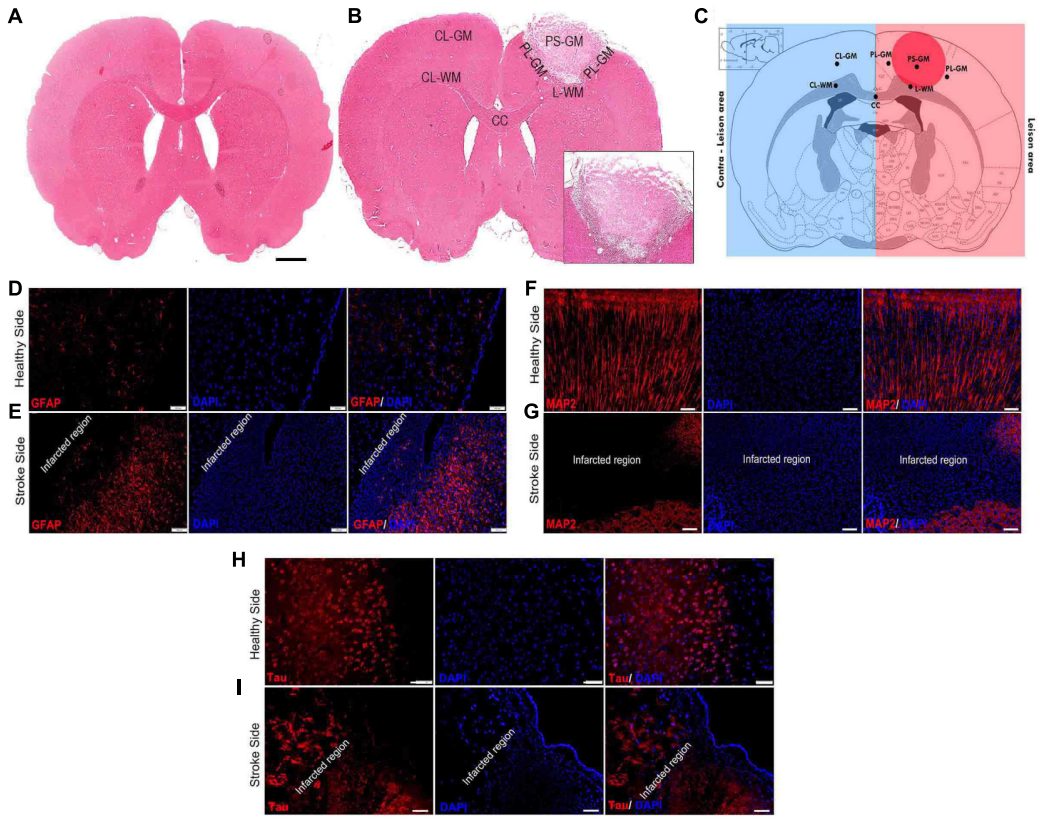

FIGURE 1 | Photothrombotic lesion in rat somatosensory cortex results in cell death and astrigliosis. **(A)** Hematoxylin and Eosin (H&E) staining brain section of native control brain (magnification 5×). **(B)** H&E stained image 1-week post-stroke brain (magnification 5×). **(C)** Brain section scheme for the six regions of interest (ROI): primary stroke lesion gray matter (PS-GM), perilesional gray matter (PL-GM), lesioned white matter (L-WM) and contra-lesioned white matter (CL-WM), contra-lesioned gray matter (CL-GM), and corpus callosum white matter (CC). **(D,E)** Healthy control and lesioned brain sections labeled with GFAP and DAPI: primary lesion gray matter (PS), perilesional gray matter (PL) indicated activated astrocytes (GFAP, red stain) around the ischemic region (peri-infarct region) and degenerated neurons with shrunken nuclei (DAPI, blue stain). Infiltration of the astrocytes around the primary lesion PS-GM region with GFAP (red). The DAPI labels (blue) degenerated cells. **(F,G)** Healthy control and affected stroke rat brains sections were labeled with MAP2 and DAPI. **(H,I)** Healthy control and affected stroke rat brains sections labeled with Tau and DAPI to assist in comparisons. Scale bar = 100 μm.

the lipid ester band ν(C = O) decrease compared with the PL-GM and CL-GM (**Figures 3H,I**). The stroke PS-GM FTIR spectrum had a significant reduction in the absorption band centered at 1227 cm^{-1} which arises from the P = O symmetric stretching vibrations of the phosphodiester bonds in DNA/RNA polysaccharide backbones (Carter et al., 2010; Srinivasan, 2010; Ami et al., 2013).

A more detailed representation of the average FTIR spectra of the PS-GM, CL-GM, CL-WM, and L-WM shown in **Figure 3H**. This highlights that lipid acyl bands were severely reduced in PS-GM and L-WM compared with CL-GM and CL-WM, respectively. The CH_3 (methyl concentration), olefinic = CH (unsaturated lipid) and the lipid ester (CO) (oxidative stress) were significantly increased while that of the CH_2 (chain lipid length) was decreased in the L-WM spectrum. The average FTIR spectra collected from the PS-GM, PL-GM, CL-GM and native control GM revealed marked reduction in lipid, lipid-acyl and protein in the ipsilateral compared with contralateral hemisphere; which were reduced when compared with the native control (**Figure 3I**).

Fourier transform Infrared analysis revealed that lipid content (n = 6 animals) was markedly reduced in the primary PS-GM (0.21 ± 0.010) when compared to the PL-GM (0.55 ± 0.028) and L-WM (0.89 ± 0.045) in the ipsilesional hemisphere. The amount of the lipid in PS-GM, PL-GM and L-WM were greatly reduced when compared with the contra-lesioned white matter (CL-WM) (1.1 ± 0.055) and contra-lesioned gray matter (CL-GM) (0.72 ± 0.036). These results were further compared with the healthy native control GM (0.79 ± 0.040) and WM (1.32 ± 0.066), where higher lipid content in comparison with the ipsilateral and contralateral hemisphere of the experimental animals (**Figures 3D,F** and see also **Figure 4L**). The results also revealed that the lipid acyl content (CH_2) concentration in the PS-GM (0.264 ± 0.019), PL-GM (0.522 ± 0.026), and L-WM (0.563 ± 0.028) decreased compared to the contralateral region (0.6012 ± 0.030) of the ischemic brain, and all values were less than the healthy native animal (0.649 ± 0.035) as presented in (**Figures 4C,D,M**).

In converse, the degree of unsaturated components olefinic = CH increased, in the PL-GM (0.0049 ± 0.0002) and L-WM (0.0062 ± 0.0003), compared to the contralateral

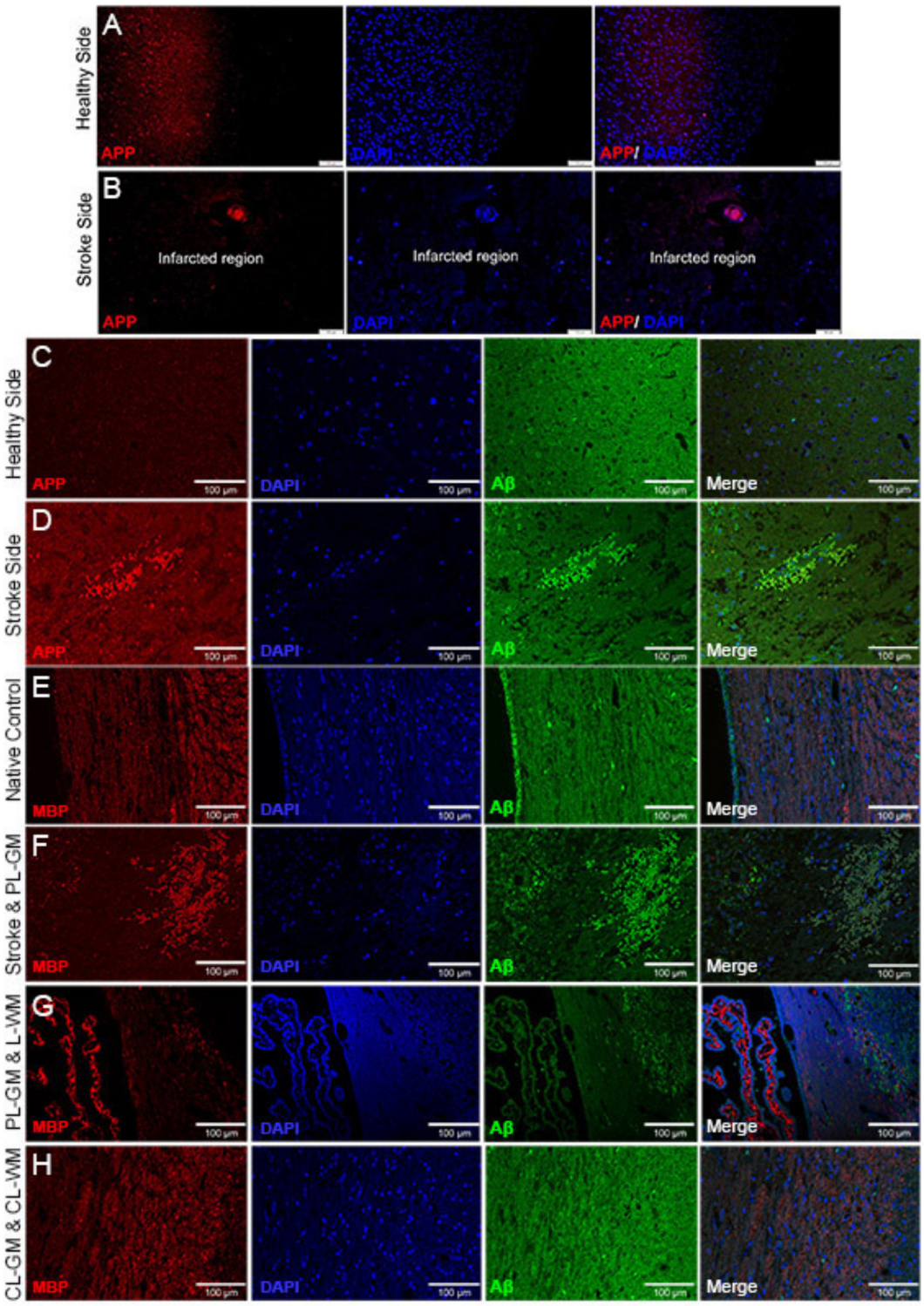

FIGURE 2 | Immunohistochemistry staining identifying altered APP and $A\beta_{1-42}$ in infarcted brain sections. **(A,B)** Contra-lesioned and lesioned hemispheres labeled with APP and DAPI: primary lesion gray matter (PS) shows amyloid precursor protein (APP) and contra-lesioned gray matter (CL-GM) with normal homogenous distribution of APP (red). **(C,D)** Contra-lesioned and affected stroke hemispheres were labeled with APP (red), DAPI (Blue) and $A\beta_{1-42}$ (green). **(E–H)** Native control and 1-week affected stroke rat brains sections labeled with myelin basic protein (MBP, red), DAPI (blue), and $A\beta_{1-42}$ (green). **(F,G)** Primary lesion gray matter (PS-GM) contains degenerated neurons (DAPI, blue) and disorganization of the myelin sheath (MBP, red) of the axonal neurons in the lesioned white matter (L-WM) scale bar = 100 μm for **(A–H)**.

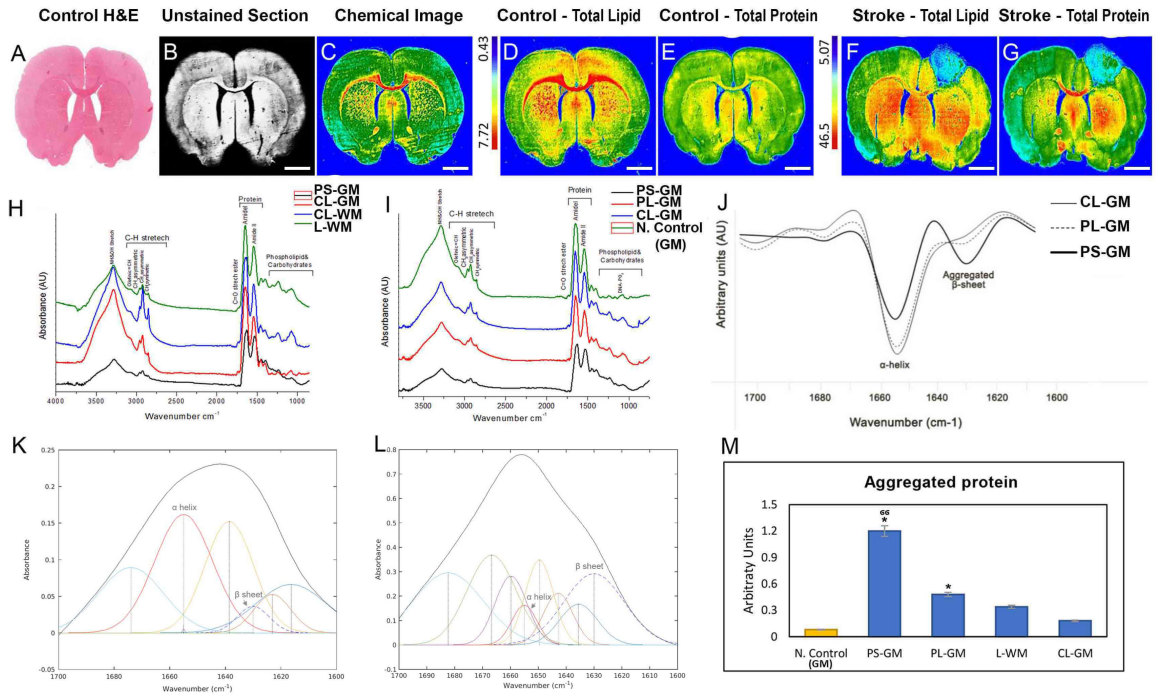

FIGURE 3 | Whole brain section-FTIR imaging of biochemical changes within contralateral and ipsilateral hemispheres following photothrombotic focal ischemic insult to the somatosensory cortex. **(A)** H&E stained brain section captured by Digital Scanning Microscope Bright field (20×). **(B)**. Representative unstained FTIR light microscopic image of the healthy control rat brain (10×). **(C)**. Representative FTIR chemical image of the unstained brain section showing main biochemical components: lipid, phospholipid, protein, carbohydrates and nucleic acids. **(D,E)** FTIR image that represent the total lipid and total protein distribution in the healthy control rat brain. **(F,G)** FTIR image that represent the total lipid and total protein distribution in the stroke affected rat brain. Scale bars = 100 μm. **(H)** Representative FTIR averaged spectra in the range of 4000–400 cm^{-1} acquired from the cortical region of the PS-GM, L-WM, CL-GM, and CL-WM. **(I)** Representative FTIR averaged spectra in the range of 4000–400 cm^{-1} acquired from the cortical region of the PS-GM, PL-GM, CL-GM and native control GM. **(J)** Representative average second-derivative spectra of the amide I band in the spectral range of 1700–1600 cm^{-1} acquired from the primary lesion gray matter (PS-GM), perilesional gray matter (PL-GM), and contra-lesioned gray matter (CL-GM) regions post ischemic. The spectra show α- helical secondary protein structure at 1655 cm^{-1} and β-sheets protein conformation at 1630 cm^{-1}. **(K,L)** Representative curve fitting of the amide I band in the spectral range of 1700-1600 cm^{-1} of the CL-GM and PS-GM, respectively, to quantify the aggregated protein relatively. **(M)** Histogram of the aggregated protein comparing different regions of interest: native control, PS-GM, PL-GM, L-WM with CL-GM. The FTIR images were colored-coded: red color corresponds to the highest content and blue color corresponds to the lowest content as shown on the color bars in the figures. Statistical significance was determined from six animals with a paired *t*-test and the 95% confidence interval. Bars represent mean ± SD. $^{*}p > 0.05$, $^{**}p > 0.01$ relative to CL-GM. $^{\sigma}p > 0.05$, $^{\sigma\sigma}p > 0.01$ relative to PL-GM.

hemisphere (0.0026 ± 0.0002) and the healthy native animal (0.0014 ± 0.0001) as shown in **Figures 4E,F,N**. These results also indicated that the CH_3 (methyl concentration) is severely reduced within the PS-GM (0.0048 ± 0.0002). On the other hand, the methyl concentration increased in the PL-GM (0.0494 ± 0.003) and L-WM (0.0627 ± 0.003) in the ipsilateral hemisphere in comparison to the contralateral hemisphere (0.0276 ± 0.0014) and the healthy native brain (0.0111 ± 0.0005) as represented in **Figures 4G,H,O**, respectively. Also documented was that the value of lipid ester (C = O) in the PS-GM region (0.11 ± 0.0055) was markedly reduced compared to PL-GM (0.64 ± 0.032) and L-WM (0.75 ± 0.0375) regions. Interestingly, lipid ester (C = O) amount in the contralateral hemisphere (0.28 ± 0.014) and native healthy brain (0.15 ± 0.0075) was lower than the PL-GM and L-WM as shown in **Figures 4I,J,P**.

The FTIR spectra revealed that total protein distribution in the cortex, especially in the PS-GM and PL-GM was greatly affected, due to stroke and, in the focal point of the stroke, there was protein aggregation. These analyses revealed that the protein level in the PS-GM (3.2 ± 0.16) was lower compared to PL-GM (12 ± 0.61). PS-GM and PL-GM experience severe reduction in protein in comparison to contralateral hemisphere (14.6 ± 0.73) and native healthy brain (17.8 ± 0.89) as presented in **Figures 3E,G** and see also **Figure 4K**. Moreover, the second derivative of the FTIR, range 1700–1600 cm^{-1}, specifically protein band at the spectral range of 1630–1625 cm^{-1}, indicates protein aggregation in the affected stroke region (**Figure 3J**), i.e., an increase in β-sheet and decrease in α-helix structures at 1660–1655 cm^{-1} as shown in the CL-GM and PS-GM spectra curve fitting (**Figures 3K,L**, respectively). The results indicate that the PS-GM (1.2 ± 0.06) region had increased aggregated protein content in comparison with the PL-GM (0.48 ± 0.024) and contralateral hemisphere (0.18 ± 0.009) and these two regions were having higher value than the native control (0.08 ± 0.004) (**Figure 3M**). (These findings correlate well with the histochemistry results see **Figure 2**). Here the results identified a decrease in the relative amount of lipid and protein bio-content in the PL-GM region of the ischemic brain

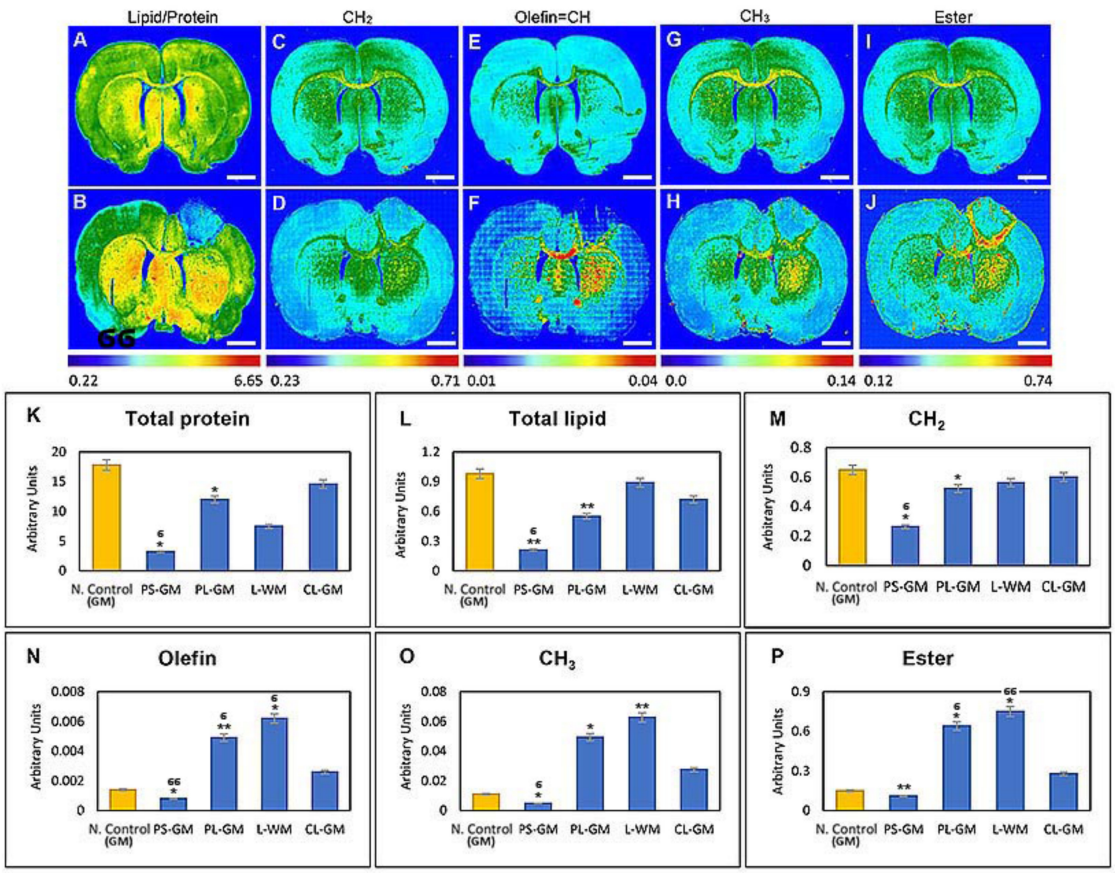

FIGURE 4 | Functional group (macromolecular/sub-cellular/biochemical) images obtained of the native control ischemic stroke brain sections. **(A–I)**. Representative FTIR images of lipid/protein, lipid acyl group (CH_2), olefin = CH, methyl (CH_3) and lipid ester (C = O) distribution in the native healthy control brain tissue sections, respectively. **(B–J)** The same function groups (above) obtained from the ischemic stroke brain sections. **(K–P)** Histogram of specific bio-molecules content such as total protein, total lipid, lipid acyl group (CH_2), olefin = CH, methyl (CH_3), and lipid ester (C = O), respectively. The regions of interest were native control rat brain GM and ischemic brain PS-GM, PL-GM, L-WM, and CL-GM. The FTIR images were color coded, red color corresponds to the highest content and blue color corresponds to the lowest content as shown on the color bars in the figures. Statistical significance was determined from six animals. Statistical significance was determined from six animals with a paired *t*-test and the 95% confidence interval. Bars represent mean ± SD. $^{*}p > 0.05$, $^{**}p > 0.01$ relative to CL-GM. $^{\sigma}p > 0.05$, $^{\sigma\sigma}p > 0.01$ relative to PL-GM.

in comparison to the native healthy control brain. Changes in the lipid/protein ratio (**Figures 4A,B**) suggest that these biochemical alterations associated with the stroke are due to triggering and initiating specific mechanisms of neurodegeneration.

The FTIR assays identified that the CC in the experimental animal (lesioned) brain, experienced reduction in lipid content (0.91 ± 0.0455) in comparison to the native healthy control animal (1.34 ± 0.067) (**Figures 3D,G**). The results also showed that that the lipid acyl (CH_2) amounts in the lesioned CC (0.582 ± 0.0291) decreased compared to the native animal (0.661 ± 0.033) (**Figures 4C,D**). There is also an increase in the unsaturated components olefinic = CH (0.0058 ± 0.0003) compared to the healthy animal (0.0040 ± 0.0002) (**Figures 4E,F**). These results also indicated that the CH_3 (methyl group) concentration in the lesioned CC (0.0574 ± 0.00278) increased compared to the native animal (0.0439 ± 0.0022) (**Figures 4G,H**). The lipid ester (C = O) concentration in the lesioned brain CC (0.64 ± 0.032) also experienced an increase in comparison to native animal (0.52 ± 0.026) ($p = 0.73$, $n = 6$) as shown in **Figures 4I,J**.

As a further analysis of the FTIR data, PCA was performed on the spectral second derivatives in the range of 4000–700 cm^{-1} (with the range of 2500–2000 cm^{-1} removed) for the native control and lesioned rat brain sections (**Figure 5A**). The differences were mainly significant in the region of 3994–2800 cm^{-1}, which correlates to the lipid bio-molecular changes in the primary lesion. Also, there were molecular changes in the spectral region of 1530–1680 cm^{-1} which is related to protein structure in the tissue section. The scores plot revealed that the spectral data collected from the rat cortices were clustered into two distinct groups that correlate with healthy (native) and stroke affected. The clear segregation between the two groups in the PCA plots revealed that the molecular makeup of the cortical tissue has changed due to ischemic stroke with the first 3PCs accounting for ∼98.5% of the total variation as shown in **Figure 5B**. The PCA analysis also resulted in clustering of the

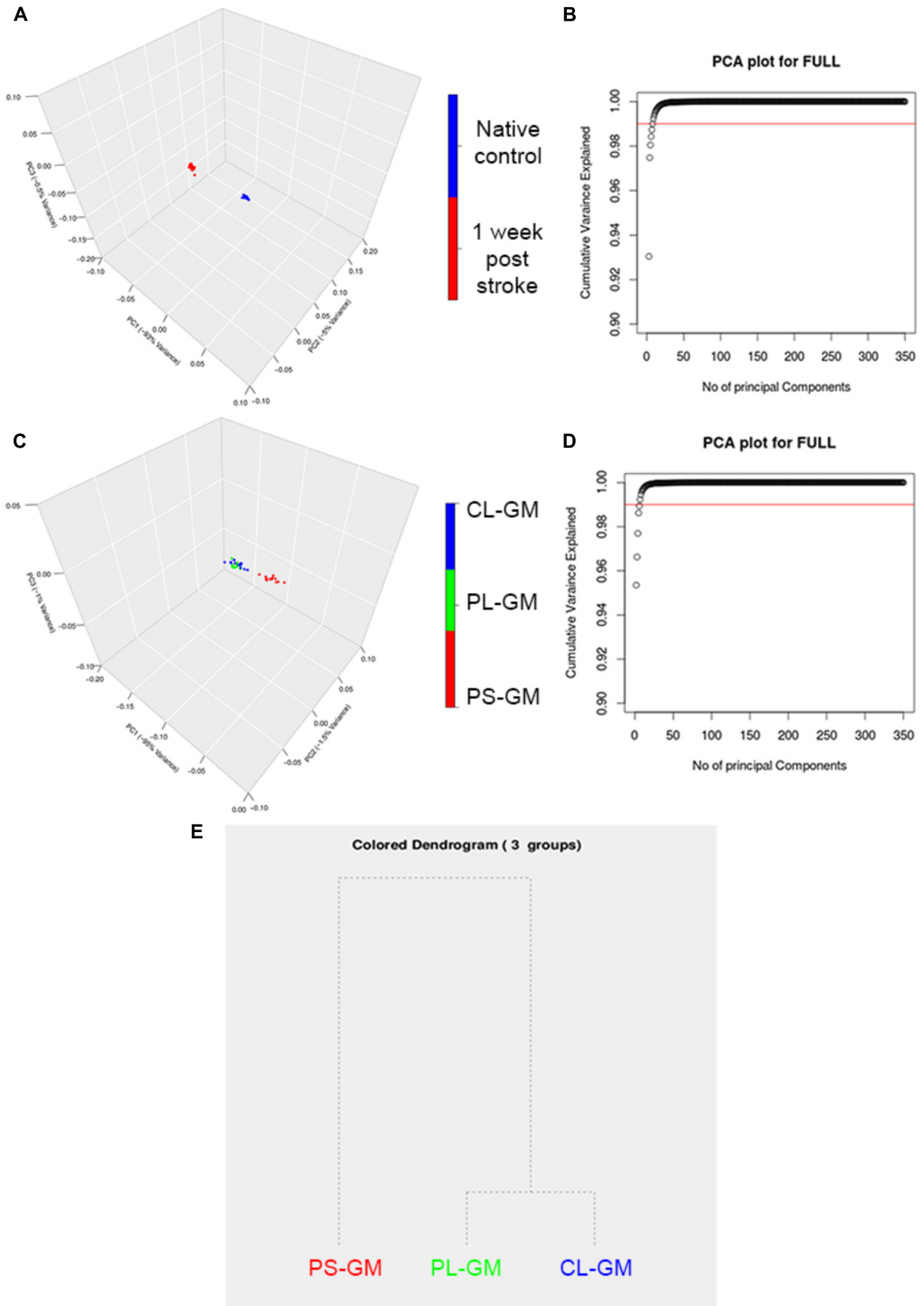

FIGURE 5 | Principle component analysis (PCA) analyses of averaged FTIR spectroscopic studies. **(A)** Representative 3D score plot for PCA analysis based on the average FTIR spectral data in the region of 4000–700 cm^{-1} (with the range of 2500–2000 cm^{-1} removed) collected from the time point of native healthy control and stroke affected rat brain. **(B)**. The score plot of the PCA shows that there are three main PCs that separate the time point native control healthy and the affected stroke rat brains and are accounted for PC1 (93%), PC2 (4%), and PC3 (1%). **(C)** Represents the PCs plot that shows that there are the three main PCs that separate PS-GM, PL-GM, and CL-GM spectra. **(D)** The score plot of the PCA and shows that there are three main PCs that separate the PS-GM, PL-GM, and CL-GM spectra are counted for PC1 (95%), PC2 (1.5%), and PC3 (1%). **(E)** Represents the hierarchical dendrogram that shows a clear separation PS-GM, PL-GM, and CL-GM regions in the ipsilateral side of 1-week post ischemic stroke. The hierarchical dendrogram shows that PS-GM is located in one group while the PL-GM and CL-GM regions are located in the second group.

PS-GM, PL-GM, and CL-GM (**Figure 5C**) from the scores plot of the three significant components: PC1 (95%), PC2 (1.5%), and PC3 (1%) as shown in **Figure 5D**. The first cluster contains the spectra from the PS-GM region, while the second group contains the spectra from PL-GM and CL-GM with this separation clearly shown in the hierarchical dendrogram (**Figure 5E**). The advantage of PCA is to allow the rat brain cortices to be clearly distinguished into healthy (control) and stroke region. Moreover, **Figure 5B** outlines an estimate that more than ∼98.5% of the variance in the spectra can be captured by the first three PCs. Furthermore, the PCA result makes it feasible to perform hierarchical clustering, thereby, combining PS-GM region with PL-GM and CL-GM regions using a dendrogram.

Elemental Analysis by Laser Ablation (LA-ICPMS)

Employing LA-ICP-MS can be used to assess the elemental distributions of C, P, S, Cu, Fe, Zn, and Ca in the rat brain section 1-week post-stroke (**Figures 6A–G**). Here element values from the ipsilateral cortex (lesioned hemisphere) were compared with those of the contralateral cortex. The LA-ICPMS results provide three main observations: (a) a reduction in all element concentrations (Ca, C, Fe, Cu, S, P, and Zn) inside the stroke focus point (PS-GM) region; (b) Ca, Cu, and Zn were mainly accumulated at the edge of the injured brain hemisphere; and (c) significant and high accumulation of Fe around the PS-GM and in the lesion rim, shown in detail in **Figures 6H–N**. Further, the results indicate that the distribution of the brain elements were greatly affected by the ischemic stroke. This abnormal distribution might also effect brain functions involved in muscle control, movement and memory (Prashanth et al., 2015; Chen et al., 2016).

DISCUSSION

In this study diverse techniques were applied to delineate biochemical and elemental alterations 1-week post-stroke in the PS-GM, PL-GM, L-WM, CL-WM, CL-GM, and CC. A combination of FTIR and LA-ICPMS with PCA analysis, yielded novel results: significant changes in lipid values; protein conformation and in elemental distribution in multiple regions, which may correlate with morphological changes. For example, FTIR studies identified significantly enhanced protein aggregation, which included $A\beta_{1-42}$ – one of the pathological markers of AD (Prashanth et al., 2015; Baldassarre et al., 2016; Chen et al., 2016). Moreover, we detected $A\beta_{1-42}$ in the ipsilateral lesioned WM – further proof that the site of stroke is more global than originally thought. It should be noted that we do not detect $A\beta_{1-42}$ aggregates in the contra-lateral WM indicative that the lesion induces APP/Aβ aggregation in the ipsilateral side. Furthermore, the breakdown of MBP and decrease in the myelin sheath thickness, post-ischemic stroke, which increases the vulnerability of exposed axons and leads to a decline in functional connectivity and behavioral deficits (Li et al., 2014; Baldassarre et al., 2016). Notably, other studies have reported that MBP dysfunction(s) were associated with physiological response to stress and emotional states, including anxiety and depression in adulthood, all possible indicators for commonalities in brain traumas (Drevets et al., 2008; Edgar and Sibille, 2012).

Ischemic stroke causes extensive brain damage through the generation of free radicals such as ROS O_2^- (Olmez and Ozyurt, 2012) which attack polyunsaturated fatty acid (PUFA) containing structures (Allen and Bayraktutan, 2009). FTIR imaging identified a change in the lipid/protein ratio in the PL-GM region an event triggered by specific processes that lead to bio-chemical changes. The histological data revealed that the insulted brain experienced edema. Previous studies reported that edema initiates oxidative stress and increased protein oxidation and protein mis-folding and cell degeneration (Hackett et al., 2015a). Edema and oxidative stress may be the driving factors in lipid fragmentation, protein aggregation (mis-folding) (Hackett et al., 2015a), neurodegeneration (Klein and Ackerman, 2003; Uttara et al., 2009) and cell death (Ryter et al., 2007). Current bio-diagnostic techniques, such as MRI and biochemical assays are not sensitive enough to detect oxidative stress. Whereas, FTIR imaging spectroscopy is, to our knowledge, the only technique sensitive enough to detect these molecular changes associated with oxidative stress (Kneipp et al., 2000; Wang et al., 2005; Ozek et al., 2010; Caine et al., 2016). Thus, in order to understand the lipid alteration associated with ischemic stroke, FTIR spectra of lipids were analyzed. In the brain sections, we found significant alteration in the lipid acyl (CH_2), lipid ester (C = O); olefinic = CH and methyl group (CH_3). When double bond sites of PUFA are attacked by free radicals this leads to degradation of lipids into smaller fragments (Allen and Bayraktutan, 2009; Olmez and Ozyurt, 2012; Caine et al., 2016). Lipid peroxidation is associated with increased methyl (CH_3) concentration and the formation of degradation products such as alkanes, carbonyl compounds, lipid aldehydes and alkyl radicals (Lamba et al., 1994; de Zwart et al., 1999; Manda et al., 2007; Yin et al., 2011).

Ischemic stroke caused reduction in the total lipid and lipid acyl contents at stroke focal PS-GM, PL-GM, and L-WM in the ipsilateral hemisphere. In PS-GM, total lipid decreased, as expected, due to tissue loss, evidenced also as protein loss. Hence, lipid acyl (CH_2), lipid ester (C = O), olefinic = CH, and methyl (CH_3) all declined significantly. However, in contrast to the PS-GM in the PL-GM, L-WM, and CC we observed a significant increase in lipid ester (C = O), olefinic = CH and methyl (CH_3). An increase in the olefinic = CH content at the ipsilateral hemisphere compared with contralateral and native controls suggests that stroke induced an increase in the unsaturated fatty acids. Although, lipid peroxidation should result in loss of olefinic = CH bonds, but interestingly in our study we observed an increase in the olefinic = CH bio-content, which can be due to accumulation of double bond end products through lipid peroxidation compensation mechanisms (Liu et al., 2002; Severcan et al., 2005). Our results are in good agreement with previous studies on the effect of the lipid peroxidation on diabetic rat liver microsomal membranes and diabetic patients' platelets (Liu et al., 2002; Severcan et al., 2005). These studies highlighted that lipid peroxidation induced an increase in the olefinic = CH content, which originated mainly from double

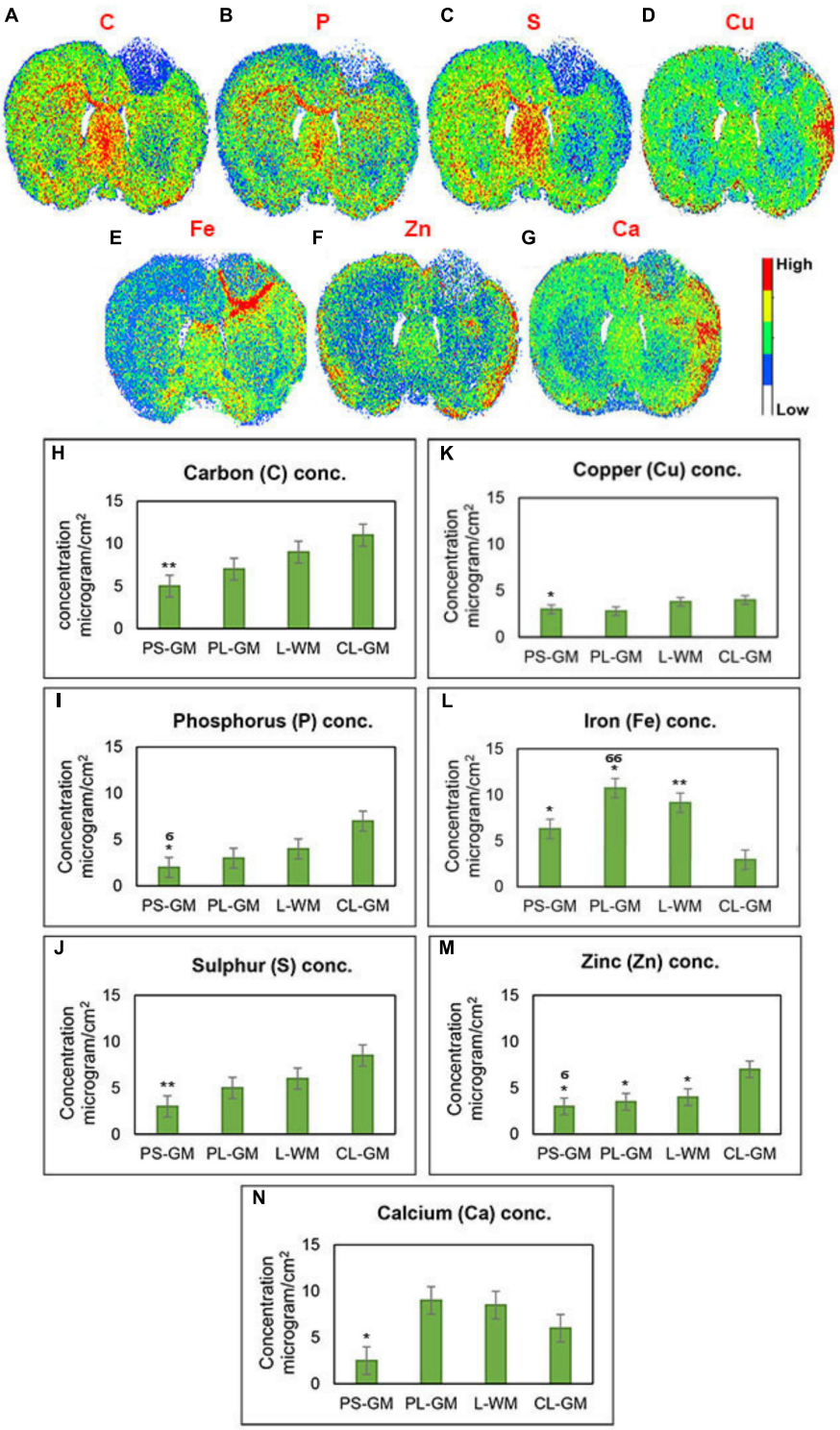

FIGURE 6 | Qualitative elemental maps for whole rat brain sections. **(A–G)** Represent images of the elemental distributions of C, P, S, Cu, Fe, Zn, and Ca, respectively, in the rat brain section 1-week post-ischemic stroke measured by LA-ICP-QMS. **(H–N)** Elemental concentration of C, P, S, Cu, Fe, Zn, and Ca, respectively, from four regions of interest such as PS-GM, PL-GM, L-GM, and CL-GM. These LA images were color-labeled according to the calculated concentration values, where red corresponds to the highest concentration and white corresponds to the lowest concentration as shown on the color bars in the figures. Statistical significance was determined from six animals with a paired *t*-test and the 95% confidence interval. Bars represent mean ± SD. $^{*}p > 0.05$, $^{**}p > 0.01$ relative to CL-GM. $^{\sigma}p > 0.05$, $^{\sigma\sigma}p > 0.01$ relative to PL-GM.

bond lipid peroxidation products (Kar et al., 2004; Gouras et al., 2010). Our results also show an increase in the lipid ester (C = O) post stroke. Thus, our results emphasize that ischemic stroke induced lipid peroxidation which results in marked decreases in the total lipid and lipid CH_2 contents associated with increases in the lipid ester (C = O), olefinic = CH and CH_3 bio-contents (Lamba et al., 1994; de Zwart et al., 1999; Manda et al., 2007; Cakmak et al., 2011). These bio-chemical alterations occurs at the PL-GM, L-WM and the CC but were prominent at L-WM and the CC due to higher lipid content.

From immunohistochemical studies we noted high GFAP expression, an indicator for astrogliosis, and from elemental data enhanced Fe levels-a possible marker for increased mitochondria; in all, a likelihood that glia are metabolizing lipids esters and olefins, including removing necrotic tissue. In the WM, below the site of the lesion, we again noted a decrease in total lipid content, which was associated with significant increase in lipid ester, olefin, and CH_3 indicative that possibly degradation of long chain fats was occurring, including cell membranes. Intriguingly at this locus, we detected only $A\beta_{1-42}$, which is thought to be formed due to exposure to reactive oxygen species.

Protein exposure to reactive oxygen results in protein cross-linking, aggregation, fragmentation, and denaturation, resulting in loss of function. The levels of the total protein and aggregated protein were calculated from the amide I band at 1700–1600 cm^{-1} and curve fitting at 1630 cm^{-1}, respectively. As expected from histological data the FTIR amide I data indicated a loss of protein from the lesioned GM (PS-GM) compared with the control samples. Interestingly, we observed that there is an increase in the band absorption of β-sheets at 1630 cm^{-1} and associated decrease of α- helical protein band absorption at 1655 cm^{-1} indicating protein aggregation. This protein conformational change was not detected on the contra-lateral side, which indicate that stroke induces protein alterations. The PS-GM and PL-GM regions experienced a decrease in total protein levels (amide values), with the most surprising and dramatic change in aggregated protein in these areas. Histochemical studies identified significant changes in APP in the infarcted area (closely associated with glial cells) and tests for $A\beta_{1-42}$- which has been previously reported to increase, not only in stroke, but also in other neurodegenerative disorders (Paul Murphy and LeVine, 2010; Esparza et al., 2016; Wildburger et al., 2017). It should be noted no Tau protein was detected in the lesioned area, thus it was inferred that the protein aggregation was exclusively APP derived and was $A\beta_{1-42}$. Moreover, it is tempting to speculate that $A\beta_{1-42}$ accumulation due to ischemic stroke might also trigger other neurological deficits and subsequent neurodegeneration (Kar et al., 2004; Gouras et al., 2010).

Elemental studies clearly defined significant loss of C, P, and S from the PS-GM, PL-GM, and the L-WM as these are the major components of nucleic acids, protein and lipids that make up cells. Sulfur concentration were greatly reduced in the ipsilateral hemisphere. Sulfur is a constituent of the anti-oxidant enzymes glutathione peroxidases (GP) and thioredoxin reductases (TR) and of the metabolic enzyme *S*-adenosylhomocysteine (S-ACH) (Zima et al., 1996; Birben et al., 2012; Li et al., 2013). In several neurodegenerative disorders loss of these enzymes has been postulated as a corollary of enhanced cell pathology (Zima et al., 1996; Birben et al., 2012; Li et al., 2013). Surprisingly, in the cortices we identified significantly high levels of Ca and Fe. From studies in the hippocampus, high Ca^{2+} levels were found to be excitotoxic (Dong et al., 2009) and in the brain Fe ions can increase the formation of reactive radicals and thus lipid peroxidation (Halliwell and Gutteridge, 1986). This change can also be attributed to an increase in to astroglial cells that metabolizing necrotic tissue. In our study, a small change in Cu values was found, but this could be linked to the fact that Cu and Zn are bound to synaptic termini and regulate exocytosis (Sadiq et al., 2012), thus the possibility is that release of these metals into the micro-environment may account for the increased neuronal excitability that has been reported by others after stroke (Li and Zhang, 2012). Our results indicate that ischemic stroke can induce significant changes in the stroke focus point, the GM, as well as in the adjacent WM of the affected hemisphere. Furthermore, the enriched lipid brain regions, especially brain WM are vulnerable to ischemic stroke and can be highly affected by the production of free radicals and bi-products of oxidation.

The chemometric analyses, PCA in the spectral range of 3500–900 cm^{-1} and the hierarchy dendrogram from HCA, delineated a clear separation between the control and the lesioned sections. From the PCA plot, it was clear that the primary stroke PS-GM clustered in one group while both PL-GM and CL-GM clustered together. This clear separation reveals that the stroke focus region PS-GM has significant molecular changes in its bio-chemical makeup. One of these major bio-molecular alterations was found by curve fitting of the second derivative of the protein bands in the PS-GM spectrum. It revealed an increase in the β-sheet content and an associated decrease in α-helical secondary structure- again the interpretation being that protein aggregation, a marker for neurodegeneration, occurred. The data also indicate that the effects of stroke are much more global on the ipsilateral side than originally thought. Thus, newer or more improved ischemic stroke management in the sub-acute phase should prioritize treating edema and the oxidative stress damage in order to have a successful outcome to maintain and improve the PL-GM and L-WM brain tissue.

Our findings suggest that edema and oxidative stress are the major factors that affect the integrity, structure and functionality of the brain as well as leading to bio-molecular and elemental alterations 1 week post-stroke. However, it has been found that 24 h post stroke, the molecular and elemental alterations are mainly due to tissue swelling following edema but oxidative-damage is not responsible for the these alterations especially at perilesional gray matter (PL-GM) region (Caine et al., 2016). Our results show that FTIR bio-spectroscopy is a non-destructive, rapid, and a refined technique to characterize oxidative stress bio-markers not obtained by routine approaches for lipid degradation. Also, we can detect secondary structure protein changes such as β-sheet formation. Normally, detection of these changes requires biochemical methods that include tissue homogenization and, or treatment for immunoblotting or IHC, destroying the spatial-temporal integrity of the brain. In summary, these results suggest that a good therapeutic strategy

should include a mechanism that provides protective effect from brain swelling (edema) and neurotoxicity by scavenging the lipid peroxidation end products. This strategy can include the protection of the blood–brain barrier (BBB) endothelial cells. Thus, we conclude that FTIR can complement and expedite research into stroke.

AUTHOR CONTRIBUTIONS

MA and FR performed all the FTIR and LA measurements in this study. MA, TA, KA-S, and EA were involved in data interpretation, drafting and editing the manuscript. AL was involved in elemental analysis studies. EA and NM were involved in IHC studies. RM and EU were involved in processing the experimental data and performing the multivariate and statistical analysis. All authors discussed the results and commented on the manuscript and in critical revision of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00647/full#supplementary-material

FIGURE S1 | The representative FTIR spectrum of white matter (WM) in a native control rat brain in the spectral range of 4000–700 cm^{-1} and mechanical properties of the native and lesioned brain sections. Representative FTIR spectra of WM in the native control rat brain from region 4000–700 cm^{-1}.

TABLE S1 | Fourier transform Infrared (FTIR) spectral bands and their corresponding bio-chemical assignment.

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Vacuolated PAS-Positive Lymphocytes on Blood Smear: An Easy Screening Tool and a Possible Biomarker for Monitoring Therapeutic Responses in Late Onset Pompe Disease (LOPD)

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Background: Primary aim was to investigate the diagnostic value of PAS-positive vacuolated lymphocytes on blood smear in Late Onset Pompe Disease (LOPD) patients and, secondly, to evaluate its potential utility in monitoring treatment effects.

Methods: We examined blood smear of 26 LOPD patients. We evaluated 10 treated and 16 untreated LOPD patients. Among the latter group, 7 patients later initiated ERT and were tested again 6 months after start. Blood smear was also sampled from 82 controls and 19 patients with other muscle glycogenoses (MGSDs). PAS staining was used to evaluate: (1) presence of lymphocytes with glycogen-filled vacuoles, (2) quantification of vacuolated lymphocytes.

Results: We found that PAS-positive lymphocytes were significantly higher in LOPD patients than in controls or other MGSDs ($p < 0.05$ and $p < 0.001$, respectively). ROC curve for discriminating between untreated LOPD patients and controls yielded an AUC of 1.00 (95%CI 1.00–1.00; $p < 0.0001$). PAS-positive lymphocyte cutoff level of >10 yielded sensitivity of 100% (95%CI 78–100%), specificity of 100% (95%CI 96–100%), and positive predictive value of 100%. Patients studied before and after ERT showed a dramatic decrease of PAS-positive vacuolated lymphocytes number ($p = 0.016$). In other MGSDs, PAS-positive lymphocytes were significantly lower that untreated LOPD patients but higher than controls.

Conclusions: Our data suggest that the Blood Smear Examination (BSE) for PAS-positive lymphocytes quantification could be used as a simple and sensitive test for a quick screening of suspected Pompe disease. The quantification of vacuolated lymphocytes appears to be also a valuable tool for monitoring the efficacy of treatment in LOPD patients.

Keywords: PAS-positive lymphocytes, blood smear, LOPD screening test, therapeutic monitoring, Pompe disease

INTRODUCTION

Pompe disease (glycogen storage disease type II, OMIM#232300) is a rare autosomal recessive lysosomal storage disorder caused by deficiency of acid alpha-glucosidase (GAA), a lysosomal enzyme that is responsible for the cleavage of the α-1,4- and α-1,6-glycosidic bonds of glycogen to glucose (1, 2).

GAA deficiency leads to the accumulation of glycogen in the lysosomes of several tissues, demonstrating a multisystemic disorder although cardiac and skeletal muscles involvement remains more prominent (3, 4).

Two different clinical forms are conventionally described: a severe infantile form (IOPD) characterized by muscular hypotonia, hypertrophic cardiomyopathy and respiratory failure, and a more heterogeneous late onset form (LOPD) with a predominant progressive proximal, axial and respiratory muscle weakness (5–7).

In LOPD, initial clinical manifestations as muscle weakness, exercise intolerance, myalgia, or even isolated hyperCKemia appear often unspecific and may mimic a large variety of other muscle disorders as limb-girdle muscular dystrophies (LGMD), congenital, metabolic or inflammatory myopathies (8–11).

[1]According to the recent European Pompe Consortium (EPOC) recommendations for a correct Pompe disease diagnosis, a rapid and appropriate Dried Blood Spot (DBS) test may detect reduced GAA activity (12–14). This method may allow a fast screening of LOPD high-risk populations, so providing an addressing role in the diagnostic algorithm (14–16). In case of positive result it is necessary to perform a second biochemical confirmatory test on a different tissue (leucocytes, fibroblasts, or skeletal muscle) and/or the molecular genetic analysis (12).

However, muscle biopsy remains an important tool in the evaluation of muscle disorders; in most of Pompe disease cases, the morphological study shows a pattern of vacuolar myopathy with glycogen storage but sometimes it can result unspecific (17).

Since 2006, Enzyme Replacement Therapy (ERT) with recombinant human α-glucosidase (rGAA) became available. Early initiation of ERT in symptomatic patients seems to be essential to limit the progressive muscle damage, emphasizing the need for an early diagnosis (12, 18–20).

Abnormal cytoplasmic vacuolation of lymphocytes, identifiable on blood smear examination (BSE), has been proposed as a possible screening tool in Pompe patients (21–23).

The aim of the present study was to primarily investigate the diagnostic value of BSE of vacuolated lymphocytes in a cohort of LOPD patients compared to sex- and age-matched healthy individuals and to other patients with different muscle glycogenoses (MGSDs). Further, we evaluated the possibility of using BSE of vacuolated lymphocytes as a biomarker for monitoring and assessing treatment effects in LOPD.

[1]Abbreviations: LOPD - Late onset Pompe disease; GSD glycogenoses - MGSD - muscle glycogenoses; LGMW - limb girdle muscle weakness; MGG - MayGrünwald/Giemsa; PAS - Periodic Acid-Schiff; GAA - acid alpha-glucosidase; DBS - dried bloodspot; BSE - Blood Smear Examination; GAA - acid alpha-glucosidase; IOPD – infantile onset Pompe disease; EPOC - European Pompe Consortium; ERT – Enzyme Replacement Therapy; ROC - Receiver operating characteristic; IQR - interquartile range; AUC - area under the curve.

METHODS

Study Population

The study was approved by the local ethics committee (University Policlinic of Messina). The research was conducted according to the revised Declaration of Helsinki (1998) and all participants provided written informed consent prior to participation in the study.

Between April 2015 and March 2017, we examined blood smears of 26 patients defined diagnosis of LOPD, followed at our Neuromuscular Unit. Subjects were 15 males and 11 females, aged from 3 to 78 years. In all patients, the diagnosis of Pompe disease was confirmed by GAA activity assay on skeletal muscle and genetic analysis as recently suggested by EPOC recommendations (12).

When the study began, a blood sample was obtained from 10 treated and 16 untreated LOPD patients. During the study course, 7 out of the 16 untreated patients initiated ERT and, to monitor the effects of therapy on lymphocytes vacuolations, patients were tested again 6 months after ERT start.

Control values were obtained from 82 age-matched healthy individuals —40 M and 42 F—aged from 12 to 90 years.

We also collected blood smears from 19 patients affected by other muscle glycogenoses: 9 with glycogenosis type V (GSD V), 2 with GSD VII, 5 with GSD III, 1 with GSD X, 1 with GSD XIII and another one with GSD 0.

Assessment of Vacuolated Peripheral Blood Lymphocytes

A blood sample was taken and two blood films were prepared for each subject. Using routine staining procedures for light microscopy, blood smears were stained by Periodic Acid-Schiff (PAS) stain to evaluate the lymphocytes with glycogen-filled vacuoles (23, 24). Laboratory staff performing sample analysis was blinded to clinical information.

The number of vacuolated PAS positive lymphocytes per 100 lymphocytes (percentage of PAS-positive lymphocytes) was counted.

Figure 1 shows PAS-stained blood smear of a healthy individual (A), of an untreated LOPD (B) and of the same LOPD patient after ERT (C).

Statistical Analyses

Data were assessed for equality of variance and distribution. Descriptive statistics with means and median, as appropriate, and proportions were used to describe continuous and categorical variables. The association between categorical variables and population group was evaluated using the chi-square test. Because of the skewed distribution, Mann–Whitney U test was used for 2 continuous group comparisons and or the Kruskal–Wallis test for 3 or more continuous group comparisons. To compare pre and post-ERT PAS-positive lymphocytes values, we used the Wilcoxon paired rank test. Receiver operating characteristic (ROC) curves were created to explore the ability of PAS-positive lymphocytes to distinguish between LOPD patients and controls. Estimates of the area under the curves were obtained (area under the curve = 0.5

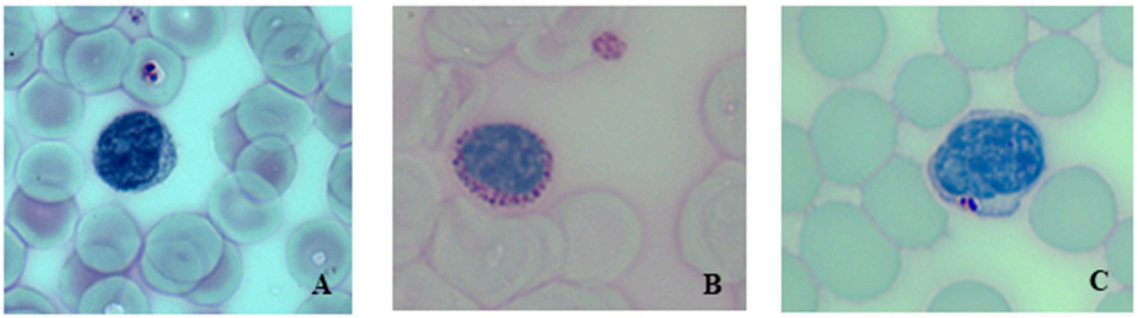

FIGURE 1 | Periodic acid-Schiff (PAS)-stained blood smear at 40 X magnification. **(A)** Healthy control, **(B)** Untreated LOPD patient showing a lymphocyte with a larger number of PAS-positive inclusions, **(C)** Same LOPD patient after 6 months of ERT.

indicates no discrimination and an area under the curve = 1.0 indicates a perfect diagnostic test). PAS-positive lymphocyte cut point was selected to maximize the sensitivity and specificity. Classification performance was assessed by sensitivity, specificity, and positive predictive values with 95% CIs. All statistical tests were two-sided and a p-value < 0.05 was considered statistically significant. Statistical analysis was carried out using SAS software package version 9.4 (SAS Institute Inc., Cary, NC) and R software (www.r-project.org; version 3.3.3).

RESULTS

We studied a total of 26 LOPD patients, 82 healthy individuals and 19 patients with other MGSD, enrolled from April 2015 until March 2017. The demographic and clinical characteristics of patients and controls are shown in **Table 1**.

In this LOPD cohort, 7 patients only showed presymptomatic hyperCKemia whereas 19 manifested with axial and limb girdle muscle weakness. Muscle biopsy, performed in 24 out of 26 LOPD patients, showed a variable amount of fibers with glycogen-filled vacuoles in 92% of patients.

The 19 patients with other MGSDs (10 M and 9 F) aged from 6 to 58 years. 9 GSD V and 2 GSD VII patients presented hyperCKemia, exercise intolerance and myoglobinuria without muscle weakness. GSD X and XIII patients complained of exercise intolerance, myalgia and contractures and rhabdomyolysis episodes. GSD III patients presented axial and limb-girdle muscle weakness. GSD 0 patient presented dysmorphic features with short stature, long face and low ears, exercise intolerance, and respiratory failure.

In all Pompe patients, we found, on a blood smear, a high percentage of vacuolated PAS-positive lymphocytes ranging from 10 to 57% in untreated and from 2 to 28% in treated patients that resulted significantly different than controls ($p < 0.01$ and $p < 0.001$, respectively) (**Table 2**).

GAA activity showed a strong negative correlation with PAS-positive lymphocytes in both treated and untreated LOPD patients ($r = -0.75$, $p = 0.01$, and $r = -0.60$, $p = 0.02$, respectively). On the other hand, PAS-positive lymphocytes weakly correlated with age in controls ($r = -0.27$, $p = 0.015$) and approached the significance in treated LOPD patients ($r = -0.58$, $p = 0.07$). Conversely, PAS-positive lymphocytes were not associated to clinical phenotype ($p = 0.53$) or any other correlation. No correlation with genotype was found.

TABLE 1 | Summary of demographic and clinical data of LOPD cases as well as healthy individuals included in this study.

	Healthy individuals ($n = 82$)	Patients with LOPD ($n = 26$)	p-Value[a]
Age, years, median (IQR) Range	53 (34–71) (12–90)	50 (37–55) (3–78)	0.09
Male, *n* (%)	40 (49%)	15 (57.69%)	0.63
Age at Onset years, median (IQR)	NA	32.5 (20–40)	
Age at Diagnosis, years, median (IQR)	NA	43 (34–51)	
CLINICAL PRESENTATION, *n* (%)			
– Isolated hyperCKemia	NA	7 (26.92%)	
– LGMW	NA	19 (73.08%)	
ERT, *n* (%)			
– Yes	NA	10 (38.46%)	
– No	NA	16 (61.54.08%)	

[a] *Mann–Whitney U test for continuous variables, cross-tabulations and 2-test for categorical variables.*
NA, not applicable.

PAS-positive lymphocytes in patients with MGSD, although they were significantly higher than in controls ($p < 0.05$), appeared to be statistically lower than untreated LOPD patients ($p < 0.01$), and tended to be lower compared to treated patients (**Figure 2**). Intriguingly, in GSD III cases, the presence of vacuolated lymphocytes was higher than in the other MGSD making the MGSD mean values in the lower level of the untreated LOPD patients.

ROC curve for discriminating between untreated LOPD patients and controls yielded an area under the curve (AUC) of 1.00 (95% CI 1.00–1.00; $p < 0.0001$) (**Figure 3**).

Classification performance at a PAS-positive lymphocyte cutoff level of >10 yielded a sensitivity of 100% (95% CI 78 to

TABLE 2 | Number of vacuolated-PAS positive lymphocytes in the study cohort of LOPD patients, in other MGSDs and in healthy individuals.

	Healthy individuals ($n = 82$)	Untreated LOPD patients ($n = 16$)	Treated LOPD patients ($n = 10$)	Other MGSDs ($n = 19$)	*P*-Value[a]
Number of vacuolated PAS positive lymphocytes (IQR)	1 (0–3)	37 (18–45)	9 (2–17)	3(1–9)	<0.0001
Range	(0–9)	(10–57)	(2–28)	(0–17)	

[a]*Kruskal-Wallis test for continuous variables, IQR, interquartile range.*

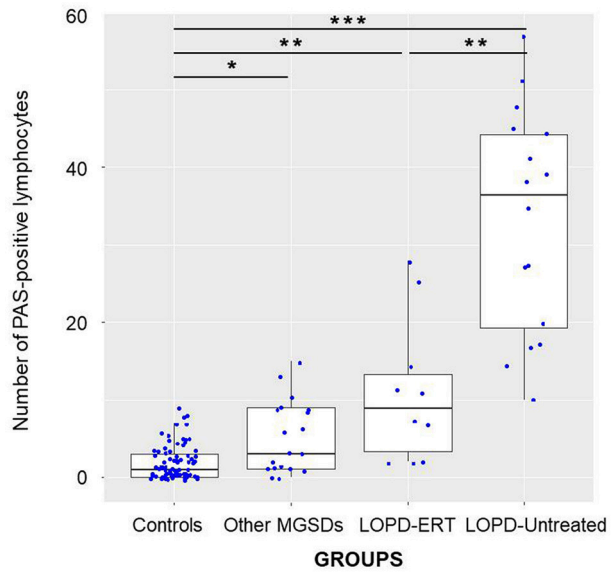

FIGURE 2 | A comparison of the number of vacuolated lymphocytes in patients with LOPD dichotomized into untreated and ERT-treated vs. controls and other MGSD. The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Significant differences are indicated with $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

100%), a specificity of 100% (95% CI 96 to 100%), and a positive predictive value of 100%.

We compared PAS- positive lymphocytes in blood samples, obtained from 7 patients before starting ERT and after 6 month of treatment. Patients showed a dramatic decrease in the number of PAS-positive lymphocytes after 6 months on ERT compared to baseline values ($P = 0.016$) (**Figure 4**).

DISCUSSION

Since the availability of ERT, as first line treatment for patients with Pompe disease, it became evident that an early diagnosis is crucial to achieve efficient therapeutic responses. The use of the DBS as a key screening method to identify patients with GAA deficiency has been proposed in several studies (14–16).

In Pompe disease, glycogen storage is present in different tissues including lymphocytes in the peripheral blood. It has been reported that on BSE, patients with Pompe disease may show vacuolated lymphocytes (21–23).

In 1977, von Bassewitz et al observed vacuoles in peripheral lymphocytes by light microscopy and, using electron microscopy, detected glycogen-filled lysosomes in 5 IOPD cases (21). In the following years, vacuolated lymphocytes have been described on blood smear also in other storage and metabolic disorders as Batten's disease (neuronal ceroid lipofuscinosis), Salla disease, β galactosidase deficiency, mucopolysaccharidoses, Niemann-Pick disease, fucosidosis, mannosidosis, and Wolman's disease. In 2005, a retrospective review of 2.550 blood films of patients with a clinical history suggestive of metabolic diseases, identified vacuolated lymphocytes in 156 cases, 23% were recognized as Pompe disease (15 IOPD and 8% LOPD) PAS staining was performed to better characterize glycogen storage in the lymphocytes vacuoles (22). In 2010, Hagemans et al collected peripheral blood films from patients with Pompe disease and controls showing that PAS-positive lymphocytes were more common in Pompe disease compared to controls and suggesting their possible role as diagnostic screening procedure (23). More recently Pascarella et al. suggested that quantification of PAS-positive lymphocytes in blood films is useful to identify autophagic vacuolar myopathies and should be routinely used for Pompe disease diagnosis (25).

In this study, we investigated the presence of glycogen-filled vacuoles in peripheral blood lymphocytes of LOPD patients to evaluate its use as screening test as well as surrogate biomarker to monitor therapeutic efficacy. Our data confirmed that PAS staining is a reliable marker of glycogen accumulation in LOPD patients lymphocytes. Comparing the number of PAS-positive lymphocytes of all 26 LOPD patients vs. controls or others MSGDs patients, we found that they were significantly higher in LOPD (**Figure 2**), proving that this method is quite specific to detect Pompe disease patients. A strong correlation was found between presence of vacuolated lymphocytes and GAA residual activity but not with other clinical parameters as age, disease duration or phenotype.

On the other hand, considering treated and untreated LOPD patients, we found that PAS-positive lymphocytes were significantly higher in untreated than in treated patients ($p < 0.01$). Diagnostic accuracy of the PAS-positive lymphocytes in blood was quite impressive showing an AUC of 100%. With a cutoff value of 10 as a percentage of PAS positive lymphocytes, a sensitivity of 100% and a specificity of 100% were reached (**Figure 3**). Thus, these results indicate that this test may play a role as valid and reliable indicator of LOPD.

It is worthwhile to outline that, in 7 LOPD patients, the percentage of PAS-positive lymphocytes, counted in blood smears before ERT and 6 months after, was significantly lower

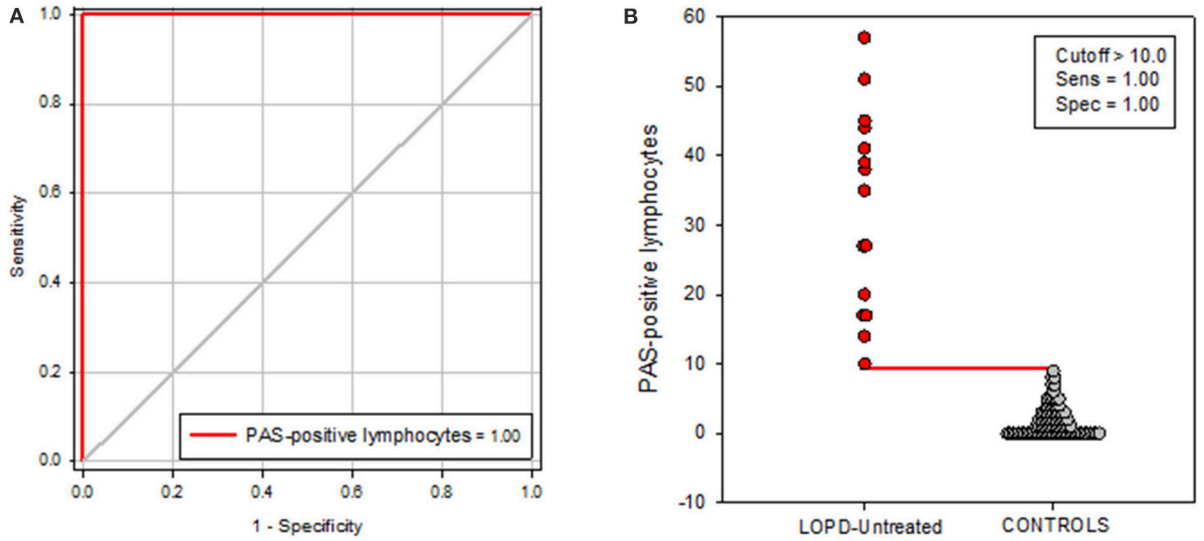

FIGURE 3 | (A) Receiver operating characteristic (ROC) curve for distinguishing untreated LOPD patients vs. controls. The area under the receiver operating characteristic curve (AUC) demonstrates that the levels of PAS-positive lymphocytes assessed in serum are able to discriminate between untreated LOPD patients and controls, with an AUC of 1.00 (95% CI 1.00 to 1.00). **(B)** Levels of PAS-positive lymphocytes in untreated LOPD patients and in controls. The red horizontal line represents the optimal cutoff value for distinguishing between untreated LOPD patients and controls.

after treatment (**Figure 4**). Although number of samples is quite limited, the latter finding could suggest that the percentage of the PAS-positive lymphocytes could be utilized as a surrogate biomarker to check therapeutic efficacy, even in future trials. Similarly in Fabry disease, another lysosomal disorder which share several commonalities with Pompe disease, GB3 and Lyso-GB3 in plasma and urine are considered as reliable biomarkers for staging the disease and monitor ERT response (26).

PAS-positive lymphocytes values of patients with other MGSDs were significantly reduced than in untreated LOPD patients. They also tended to be lower compared to treated LOPD patients (**Figure 2**), suggesting that BSE could be usefully applied as a screening tool in LOPD high-risk population, even in combination with DBS. Of course, it is worthwhile to outline that in the LOPD diagnostic algorithm, BSE as well as DBS results need to be confirmed by biochemical and genetic testing.

Being based on a simple histochemical test, BSE could be even easier to be applied in patients screening rather than DBS methods that require a fluorimetric or a tandem mass spectrometry, equipment that could be not universally available in the setting of diagnostic laboratories.

However, GSD III patients seem to have higher PAS-positive lymphocytes than other MGSDs; they appeared quite similar to the lowest Pompe disease untreated values in this cohort although GSD III clinical features are usually distinctive from Pompe disease (27). A possible explanation of a similar morphological appearance of vacuolated lymphocytes in GSD II and III, should take into account the fact that even debrancher enzyme is located in lymphocytes and its deficiency may lead to glycogen accumulation (28).

Our results have shown that residual GAA activity strongly correlated with PAS-positive lymphocytes in both treated and untreated LOPD patients. One limit of the study could be considered the relatively small sample size that precluded meaningful multivariate analyses.

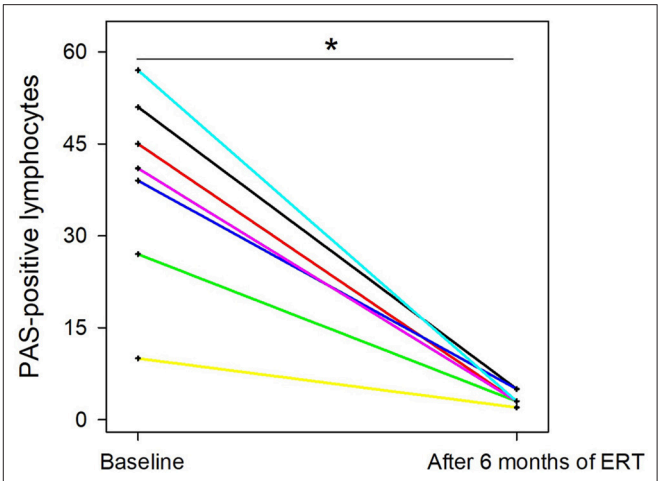

FIGURE 4 | Trends over time in the number of vacuolated lymphocytes *$P = 0.016$. Individual patient values are showed.

CONCLUSIONS

Our data suggest that quantification of PAS-positive lymphocytes in peripheral blood films could be used either as a simple screening method to support a diagnosis in patients with a suspected Pompe disease or also as surrogate biomarker for therapeutic management purposes.

COMPLIANCE WITH ETHICS GUIDELINES

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1978. Informed consent was obtained from all patients for being included in the study.

AUTHOR CONTRIBUTIONS

OM had full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of data analysis. DP, OM, AT, TM, CR, GV contributed to study design. DP, OM, TB, RO, AC, AM, AT contributed to data collection. DP, OM, AT drafted the manuscript. OM, TB, CR, TM provided clinical information. SM performed statistical analysis. All authors read and approved the final manuscript.

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Conflict of Interest Statement: In the last 3 years, AT has received from Sanofi Genzyme some reimbursement for talking in teaching courses and because he also is member of Global Pompe Registry committee. OM received reimbursement for participation in invited lectures by Sanofi Genzyme.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Longitudinal Associations Between Serum Cytokine Levels and Dementia

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Background: The purpose of this study was to investigate whether long-term inflammation is related to the incidence of dementia in a prospective observational study.

Methods: In total, 732 Korean community-dwelling elderly people >65 years were evaluated at baseline. Of the 625 without dementia, 518 (83%) were followed over a 2.4-years period, and the incidence of dementia was determined. Cytokine [interleukin (IL)-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α] levels were measured at baseline and follow-up. The individual and combined effects of cytokine levels on dementia were evaluated after adjusting for potential covariates (lifestyle factors, demographics, disability, cognitive function, and presence of the APOE e4 allele) and a Bonferroni correction.

Results: Incident dementia was associated with increased serum cytokine levels after 2 years; the association remained significant for TNF-α, IL1-α, and IL-1β concentrations even after applying a Bonferroni correction. The analysis of the combined effects of the five cytokines showed independent associations between increases in the summed number of higher cytokine levels, between baseline and follow-up. However, incident dementia was not expected based on higher baseline pro-inflammatory cytokine levels.

Conclusion: Our results suggest that dementia may precede changes in serum cytokine levels and inflammatory processes, rather than resulting from elevated pro-inflammatory cytokines.

Keywords: cytokines, dementia, geriatric psychiatry, inflammation, longitudinal studies

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INTRODUCTION

Dementia is a group of symptoms involving impairments in attention, memory, language, executive function, perception, and social cognition (1). As dementia has a high prevalence and a progressive, irreversible course in older adults (2, 3), it has become a global challenge for public health (4, 5). Understanding the etiology of dementia is essential for early diagnosis and treatment. Dementia has complex and heterogeneous etiologies, including amyloid plaques, tau-pathy, and cerebrovascular disease (6–8). Recently, the inflammatory process has received attention in the pathogenesis of dementia (9, 10).

Cytokines play a pivotal role in regulating the inflammatory response. The involvement of cytokines in dementia is supported by studies (11–13) showing that the levels of pro-inflammatory

cytokines [e.g., tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, and IL-8] are altered. A recent meta-analysis study reported significantly higher levels of the proinflammatory cytokines TNF-α, IL-6, IL-1β, IL-2, and IL-18 in peripheral blood samples of patients with Alzheimer's disease (AD) compared with a control group (14). However, causal relationships cannot be confirmed from case-control and cross-sectional investigations because increases in pro-inflammatory processes could be caused by disease pathophysiology (15, 16). Therefore, longitudinal studies are needed for clarification, although such studies have been scarce and have reported inconsistent findings. Some previous longitudinal studies have reported that the incidence of dementia is associated with TNF-α, IL-6, and C-reactive protein (CRP) concentrations (10, 17, 18), whereas other studies have found no relationship between baseline inflammation and future risk of dementia after adjusting for confounding factors (19, 20). These studies of inflammation-related dementia risk were conducted at a single time point, providing only the temporality of disease progression. Therefore, whether increased cytokine levels at a baseline evaluation can predict dementia risk remains unknown. To address these limitations, we analyzed data from a 2.4-years longitudinal cohort study to investigate prospectively the associations between five serum cytokine levels estimated at baseline and at follow-up with the incidence of dementia.

METHODS

Study Overview and Participants

This prospective cohort study was carried out from 2001 to 2003 in Kwangju, South Korea. Details of the study design have been described previously (21). Briefly, 732 community dwelling subjects aged >65 years from Kwangju, South Korea, were recruited from national resident registration lists. Of the 732 participants, 625 did not receive a dementia diagnosis at the baseline evaluation. Among these, 518 (83%) finished all assessments at follow-up. A secondary analysis was carried out with these 518 participants. The study was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained for the study, and this study was approved by the Chonnam National University Hospital Institutional Review Board.

Baseline Sampling and Assessments

Examinations included a fully structured diagnostic interview for AD and vascular dementia, peripheral blood sampling for five serum pro-inflammatory cytokines, and a formal evaluation of covariate factors.

Dementia Evaluation

Dementia assessments were performed at the baseline and follow-up. Examinations included the Mini-Mental State Examination (MMSE) (22), the Instrumental Activities of Daily Living Scale (23), the Clinical Dementia Rating scale (24), history, and a neurological examination. Clinical researchers assigned consensus diagnoses using standard criteria for dementia (1), AD (25), and vascular dementia (26). If AD and vascular dementia pathologies were mixed, they were diagnosed as either, using the criteria. In this study, we divided the participants into two different groups of incident dementia and no incident dementia.

Biochemical Analyses

Blood samples were collected from the participants in a fasting state and were taken in the morning when possible. The blood samples were collected in EDTA tubes, centrifuged, separated into plasma aliquots, and frozen at −70°C within 2 h of collection. Biochemical assays were conducted for five serum cytokines: IL-1α, IL-1β, IL-6, IL-8, and TNF-α. Serum cytokine levels were analyzed using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. To reduce assay variation, all specimens were analyzed on the same day, in duplicate, in random order, by a technician blinded to the participant's status. The inter-assay coefficients of variation were 4–8% for IL-1α, 5–9% for IL-1β, 6–9% for IL-6, 3–6% for IL-8, and 5–7% for TNF-α. The intra-assay coefficients of variation were 4–6% for IL-1α, 4–7% for IL-1β, 4–5% for IL-6, 2–4% for IL-8, and 3–6% for TNF-α.

Covariates

Several potential factors that may be associated with serum cytokine levels were investigated based on previous studies (6–8). Age, gender, and education were recorded. Depression was evaluated by the community version of the Geriatric Mental State schedule (GMS) (27). Functional disability was measured by the Korean version of the World Health Organization Disability Assessment Schedule II (WHODAS II) (28). Smoking status was investigated and participants were categorized into current smokers or not current (ex- and never) smokers. History of alcohol use was ascertained from the participants, and verified by family members. Low risk alcohol drinking was defined, based on consuming more than 14 drinks per week during the previous 3 months for men or more than 7 drinks per week for women, following the guidelines from the National Institute of Alcohol Abuse and Alcoholism (29). Physical activity was checked at baseline by asking about work and leisure activities on a 4-point scale (not at all active, not very active, fairly active, and very active) according to the standard protocol (30). A summary vascular risk score was calculated by summing self-reported disorders (stroke, heart disease, hypertension, and diabetes), hypercholesterolemia (fasting cholesterol >200 mg/dl), and obesity (body mass index >25 kg/m^2). The apolipoprotein E (APOE) genotype was reflected as a covariate in analyses of the relationship with AD.

Follow-Up Assessments

The follow-up was carried out in 2003 (31). Attempts were made to follow up 2 years after the baseline appointment (mean interval [standard deviation (SD)], 2.4 (0.3) years). Blood samples were collected to determine five serum pro-inflammatory cytokines, centrifuged within the hour, and stored at −70°C. The same biomedical assays were carried out using the ELISA method described above. Clinical researchers

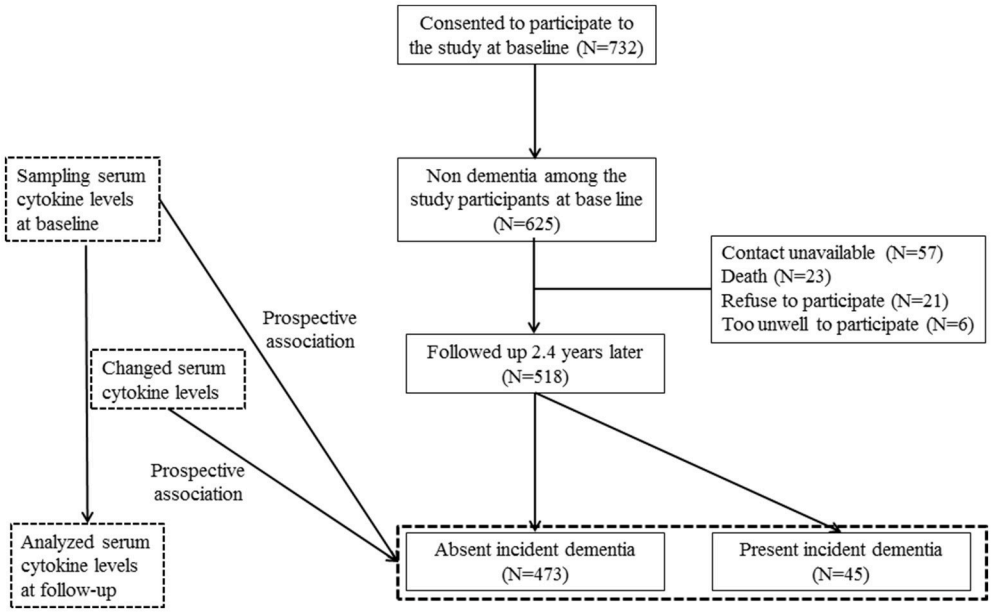

FIGURE 1 | Flow chart of the study.

assigned consensus diagnoses using standard criteria (1, 25, 26).

Statistical Analysis

The study design is outlined in **Figure 1**. Associations between baseline characteristics, demographics, assessment scales (MMSE, WHODAS II, and GMS depression) (27, 28, 32), smoking, alcohol, physical activity, and vascular risk score with incident dementia were analyzed using t- or χ^2-tests as appropriate. Results with significant associations (p-value < 0.05) were reflected in subsequent multivariate analyses. Unadjusted associations between the incidence of dementia and baseline serum cytokine concentrations and changes (from baseline to follow-up) were investigated using the Mann–Whitney U-test. The baseline levels and changes in levels were recorded as binary values of "lower" or "higher" (i.e., below or above the median value). Odds ratios and 95% confidence intervals were calculated for the associations between the baseline cytokine values and increases in cytokine levels during the follow-up in subjects with dementia. These associations were analyzed before and after adjusting for significant baseline covariates using logistic regression models. Because cytokines have demonstrated additive and synergistic effects on AD (21), the combined effects of the pro-inflammatory cytokines were calculated by summing the number of cytokines with higher levels at baseline and the number of cytokines with higher levels at the follow-up, and then assigning ordinal values (0, 1, 2, 3, 4, 5) to each. Associations between baseline values and the summed number of higher cytokine levels with the incidence of dementia were assessed initially by the χ^2-test for a linear trend (i.e., 1 df), and then with the same logistic regression models to adjust for covariates. The Bonferroni correction was applied to maintain an overall type I error rate of 0.05 against the multiple comparisons. All statistical analyses were performed with SPSS software (ver. 21.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Participant Characteristics

The recruitment procedure for the baseline and follow-up aspects of the study and distribution of dementia diagnoses are shown in **Figure 1**. Among the 625 subjects without dementia at the baseline evaluation, 518 (83%) completed all evaluations. At follow-up, incident dementia [mean interval (SD), 2.4 (0.3) years] was diagnosed in 45 participants (8.7%): 34 (6.6%) had AD, seven (1.4%) had vascular dementia, and four had "other" dementia (0.8%). Median [interquartile range (IQR)] IL-1α, IL-1β, IL-6, IL-8, and TNF-α levels were 40.3 (32.3–48.3), 12.0 (11.1–12.9), 9.5 (8.9–10.5), 20.1 (18.7–23.1), and 40.7 (37.5–43.9) pg/ml, respectively. Baseline characteristics are compared by the dementia diagnosis in **Supplementary Table 1**. Incident dementia was associated with older age, lower education, lower MMSE score, higher WHODAS II score, lower physical activity, and presence of the APOE e4 allele. The median (IQR) changes from baseline to follow-up for IL-1α, IL-1β, IL-6, IL-8, and TNF-α levels were +0.3 (0.3–1.9), +0.6 (0.4–0.8), +0.6 (0.6–4.0), +0.3 (0.1–1.8), and +1.2 (1.0–3.9), pg/ml, respectively. Baseline levels of and follow-up changes in serum cytokine concentrations according to the dementia diagnosis are shown in **Supplementary Table 2**.

Baseline and Follow-Up Concentrations of Serum Cytokines by Dementia Incidence

Unadjusted associations between serial serum cytokine levels and incident dementia are summarized in **Table 1**. Incident dementia was associated with increases in all five serum

TABLE 1 | Baseline levels of and follow-up changes in serum cytokine concentrations by incident dementia status.

	No incident dementia (*N* = 473)	Incident dementia (*N* = 45)	*P*-value*
BASELINE VALUES			
Tumor necrosis factor-α	40.3 (32.5–48.5)	40.7 (31.7–50.4)	0.921
Interleukin-1α	12.1 (11.1–12.9)	11.9 (11.2–14.1)	0.505
Interleukin-1β	9.5 (8.9–10.5)	9.3 (8.8–10.5)	0.981
Interleukin-6	20.0 (18.0–22.3)	21.0 (17.7–25.0)	0.146
Interleukin-8	40.4 (37.3–43.5)	43.0 (40.5–50.3)	**<0.001**
FOLLOW-UP VALUES			
Tumor necrosis factor-α	42.1 (36.5–47.7)	42.1 (37.1–47.1)	0.469
Interleukin-1α	13.2 (12.2–14.2)	13.9 (13.2–14.6)	**0.001**
Interleukin-1β	10.1 (9.5–11.1)	11.2 (10.0–12.6)	**<0.001**
Interleukin-6	22.1 (19.5–24.7)	24.6 (22.6–28.1)	**<0.001**
Interleukin-8	41.1 (38.4–44.6)	45.0 (38.5–53.5)	**0.002**
CHANGED VALUES			
Tumor necrosis factor-α	+1.2 (1.0–1.4)	+4.5 (−0.3–6.0)	**0.002**
Interleukin-1α	+0.3 (0.3–1.9)	+1.8 (1.1–2.0)	**0.001**
Interleukin-1β	+0.6 (0.6–0.7)	+2.2 (0.0–2.6)	**<0.001**
Interleukin-6	+0.6 (0.6–3.3)	+4.8 (0.8–6.0)	0.005
Interleukin-8	+0.3 (0.0–0.6)	+3.6 (−3.9–7.6)	0.100

**Mann Whitney test, Bold character denotes statistical significance after Bonferroni correction.*
Data are median (IQR).

cytokines during the follow-up, and the strength of the significance remained for TNF-α, IL1-α, and IL-1β after applying the Bonferroni correction. Incident dementia was associated with a higher IL-8 level at baseline, and the strength of the significance remained after applying the Bonferroni correction. The same analyses were repeated by dementia diagnosis and are presented in **Supplementary Table 2**. Incident AD was significantly associated with increases in IL1-α, IL-1β, and IL-6 levels but not with baseline cytokine levels after applying Bonferroni corrections. Incident vascular dementia was not associated any cytokine value.

Binary Higher Baseline Levels and Increasing Follow-up Level Categories of Serum Cytokines by Incident Dementia Status

The logistic regression results show a positive relationship between baseline and follow-up cytokine levels with the incidence of dementia (**Table 2**). Incident dementia was associated with IL-8 levels at baseline in unadjusted analyses. However, the association was not significant after adjusting for covariates. Incident dementia was significantly associated with higher levels of all five cytokines before and after adjustment, even after applying the Bonferroni correction. Incident dementia was not associated with higher pro-inflammatory cytokine level category at baseline before or after adjusting for covariates.

TABLE 2 | Associations of incident dementia with (binary) higher baseline cytokine levels and (binary) more pronounced increase in levels over follow-up.

	Unadjusted	Adjusted[a]
BASELINE HIGHER LEVELS		
Tumor necrosis factor-α	1.05 (0.57–1.93)	0.83 (0.43–1.60)
Interleukin-1α	0.97 (0.47–1.59)	1.29 (0.65–2.56)
Interleukin-1β	0.78 (0.42–1.45)	1.09 (0.56–2.14)
Interleukin-6	1.16 (0.63–2.14)	1.22 (0.63–2.36)
Interleukin-8	2.67 (1.37–5.21)†	2.56 (1.25–5.23)*
MORE PRONOUNCED INCREASE IN LEVELS		
Tumor necrosis factor-α	**7.83 (3.92–15.63)‡**	**8.44 (4.02–17.70)‡**
Interleukin-1α	**7.26 (3.31–15.95)‡**	**8.20 (3.58–18.82)‡**
Interleukin-1β	**7.16 (3.64–14.09)‡**	**7.19 (3.47–14.94)‡**
Interleukin-6	**5.23 (2.63–10.40)‡**	**5.01 (2.43–10.32)‡**
Interleukin-8	**3.80 (2.02–7.16)‡**	**3.73 (1.90–7.32)‡**

[a] *Adjusted for age, education, scores on Mini-Mental State Examination and World Health Organization Disability Assessment Scale II, APOE and physical activity.*
**p-value < 0.05; †p-value < 0.01; ‡p-value < 0.001.*
Bold character denotes statistical significance after Bonferroni correction.
Data are displayed as odds ratios (95% confidence intervals).

Sums of Higher Baseline Cytokine Levels and Increases in Cytokine Levels by Dementia Incidence

Figure 2 shows the combined effects of baseline and follow-up cytokine levels on dementia incidence. Incident dementia was associated with increases in the number of changes to higher cytokine levels during follow-up in unadjusted analyses ($\chi^2 = 50.91$; p-value < 0.001). However, no association was found between the incidence of dementia and increases in the summed number of higher cytokine levels at baseline ($\chi^2 = 0.604$; $p = 0.438$). After adjusting for older age, lower education, lower MMSE score, higher WHODAS II score, lower physical activity, and presence of the APOE e4 allele, the incidence of dementia increased significantly with increases in the summed number of changes to higher cytokine levels at follow-up. No association was found between incident dementia and increases in the summed number of higher cytokine levels at baseline.

DISCUSSION

The principal findings of this study were that the incidence of dementia was significantly associated with increases in all five serum cytokines, considered as both continuous and dichotomized variables, at follow-up, and the strength of these associations remained significant after applying the Bonferroni correction and relevant adjustments. The analysis of the combined effects of the five cytokines showed independent associations between increases in the summed number of higher cytokine levels between baseline and follow-up before and after adjustment. The incidence of dementia was only predicted by higher IL-8 levels at baseline, but this association was no longer significant in a multivariate analysis after applying the Bonferroni correction.

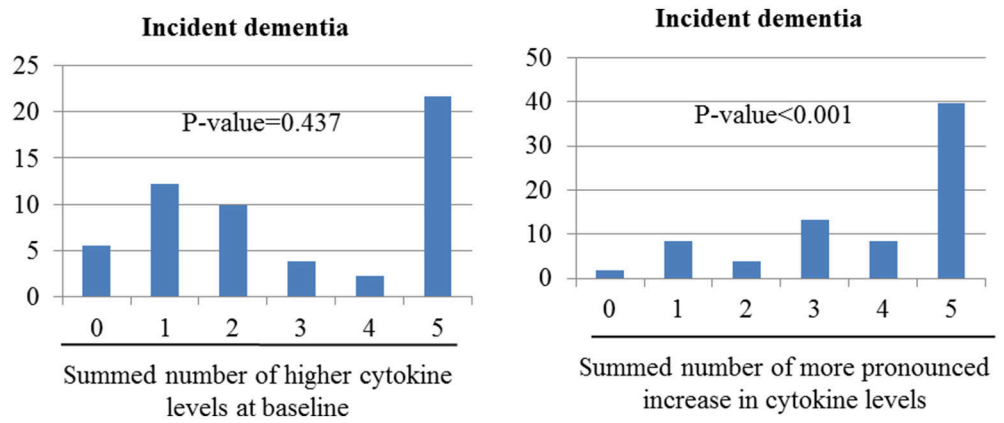

FIGURE 2 | Associations of incident dementia with number of cytokine levels categorize as high at baseline or as showing more pronounced increase over the follow-up period. Odds ratios (95% confidence intervals) for the association between incident depression and increased summed number of higher cytokine levels at baseline was 1.08 (0.88–1.33), *p*-value = 0.437; and between incident dementia and increased summed number of changed higher cytokine levels was 1.91 (1.55–2.34), *p*-value < 0.001 after adjustment for sex, cognitive function, disability, physical activity, and presence of the APOE e4 allele.

Inflammation may be an important mechanism underlying dementia (33, 34); however, it is unclear whether the inflammatory response is associated with the onset of dementia or is an outcome of dementia. To understand how inflammatory cytokines change, and possibly exert negative effects in dementia, a prospective cohort study with numerous inflammatory markers is needed. Very few studies have incorporated data from multiple time points in a clinical sample (35–37). Among them, only one study reported no association between AD and longitudinal inflammation (35), and two studies described an association between increases in inflammatory markers from baseline to follow-up and subsequent AD (36, 37). In this study, incident dementia was significantly associated with increases in cytokine levels during the study period. Several potential mechanisms might explain these findings. First, the level of inflammation may serve as a marker of active pathological processes. Hyperphosphorylation of the tau protein forming neurofibrillary tangles and amyloid beta (Aβ) accumulation in plaques are trademarks of AD progression (38). The abnormal Aβ clearance process results from the distortion of astrocytes or microglia to a pro-inflammatory state. This process is represented by elevated levels of pro-inflammatory cytokines and dysregulation of clear Aβ, and it leads to Aβ accumulation and worsened immune activation (39). In this study, 41 of 45 (91.1%) participants with dementia had AD and vascular dementia. Although the etiologies of AD and vascular dementia may be different, and thus lead to different disease outcomes, they may begin as a similar cascade of cytokine production in response to neuronal injury (8, 38, 39). Increased cytokine levels were significantly associated with incident AD but not with vascular dementia. However, due to the small sample size, there is a possibility of a type II error in the non-significant associations with vascular dementia. Second, it is also possible that cytokines increased due to causes other than dementia during the follow-up period. For example, normal aging can cause an increase in peripheral cytokines (33, 40). Our longitudinal observations support this hypothesis. Five cytokines increased during the follow-up period in both the dementia and control groups. The term sensitization is often used to describe induction of an immune response. Here, the incidence of dementia was associated with an increase in cytokine levels, but not with the absolute concentrations of baseline cytokine levels. Therefore, when conditions other than aging occur that can accelerate inflammation, such as amyloid plaques and neurofibrillary tangles (9), depression (9), genetic conditions (41), infections (42), trauma, and vascular events (8), immune sensitization can occur, which can accelerate cognitive impairment.

Previous studies of cross-sectional cytokine levels as predictors of the incidence of dementia have resulted in heterogeneous findings (35, 43–45). Those studies were limited by differences in study design, such as participant characteristics. In our study, baseline IL-8 level was associated with incident dementia in unadjusted analyses, but was not significant in the multivariate analysis after applying the Bonferroni correction. Furthermore, the impacts of cytokines have been revealed to be synergistic and, given the inconsistent outcome of a single cytokine level, the combined effect of multiple cytokines represents a more practical approach (21, 46). No associations were detected when high cytokine levels were summed. Together, these data do not support the hypothesis that cytokines result in dementia-related dysfunction but, instead, suggest that cytokine levels are elevated by dementia-related processes.

This study has several strengths. Cytokine levels were assessed serially, which could help to clarify causal relationships. In addition, the follow-up rate was reasonable and a number of potential covariates were considered in the analyses. This study also has several limitations. First, due to limited resources, we only measured five cytokines, and other important anti-inflammatory (e.g., IL-4 and IL-10) and pro-inflammatory (e.g., IL-13 and IL-18) cytokines were not evaluated (47). It is important to determine the exact function of various cytokines and disease progression. Overproduction of several

cytokines could result in damage to neurons, and these cytokines may play a role in the progression of dementia, but their protective effects have also been considered (47). Second, we collected blood samples from the periphery to measure inflammation in the central nervous system. Several studies have been conducted using imaging technology, including positron emission tomography, to measure neuroinflammation directly in subjects with dementia (48, 49). More studies are required to demonstrate the relationships between these cytokines and dementia. Third, due to protein degradation, it is recommended that blood samples be examined immediately. In this instance, due to technical difficulties, they were centrifuged at 3,000 rpm for 10 min, and sera were stored at −70°C until we finished specimen collection. However, most studies follow this procedure (20, 50). Fourth, the small set of covariate-adjusted analyses leaves the possibility of confounding bias. For example, we adjusted for age, education, MMSE, WHODAS, and physical activity but not for alcohol, smoking, or body mass index, which did affect the associations between cytokine levels and dementia, although these factors were not significantly associated with incident dementia at the follow-up (**Supplementary Table 1**). In particular, we could not provide an accurate diagnosis for smoking and alcohol use. Varying the cutoff points may lead to a different interpretation of the results of a trial. Thus, our adjustment might have been suboptimal. Fifth, conclusions regarding dementia that fully reflect the complex disease status are improbable, as stated above. In this study, due to a limitation in the study design, the probability that increases in cytokines may arise transiently, as in acute infectious disease, or continuously because of chronic inflammatory disease, may have influenced the heterogeneity, and both diseases may contribute to incident dementia. In addition, it was difficult to diagnose mixed dementia due to a lack of brain imaging data. However, this limitation is common in most epidemiological studies. Finally, because this study did not specify the timing of the dementia diagnosis, it was not possible to state clearly that dementia preceded inflammation. Furthermore, only 2 years of follow-up visits were carried out. Because dementia progresses over decades, having a longer follow-up is crucial to identifying the developmental trajectory of the disease. Additional follow-up studies will be needed to address these limitations.

Our findings suggest that incident dementia may lead changes in serum cytokine levels and inflammation, rather than resulting from elevated pro-inflammatory cytokines. It has been reported that patients with dementia, particularly those at more advanced stages, are at a higher risk for developing stroke and depression (51, 52), which could, theoretically, be mediated by inflammation. As mentioned above, our findings must be replicated in future studies with more cytokines. Furthermore, interactions between cytokine levels and genes need further assessment, as there is sufficient evidence that the transcriptional activity of specific polymorphisms influences cytokine production (53).

AUTHOR CONTRIBUTIONS

J-WK conducted the data analysis and drafted the article. RS and H-JK helped to analyze the data and to draft the article. K-YB, S-WK, I-SS, and J-SY helped to recruit the participant and perform dementia assessment and management. All authors approved the final version of manuscript to be published. J-MK had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2018.00606/full#supplementary-material

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Neurofilament Light Chain: Blood Biomarker of Neonatal Neuronal Injury

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Background: Neurofilament light chain (NfL) is a highly promising biomarker of neuroaxonal injury that has mainly been studied in adult neurodegenerative disease. Its involvement in neonatal disease remains largely unknown. Our aim was to establish NfL plasma concentrations in preterm and term infants in the first week of life.

Methods: Plasma NfL was measured by single molecule array immunoassay in two neonatal cohorts: cohort 1 contained 203 term and preterm infants, median gestational age (GA) 37.9 weeks (interquartile range [IQR] 31.9–39.4), in whom venous and arterial umbilical cord blood was sampled at birth and venous blood at day of life (DOL) 3; cohort 2 contained 98 preterm infants, median GA 29.3 weeks (IQR 26.9–30.6), in whom venous blood was sampled at DOL 7.

Results: Median NfL concentrations in venous blood increased significantly from birth (18.2 pg/mL [IQR 12.8–30.8, cohort 1]) to DOL 3 (50.9 pg/mL [41.3–100, cohort 1]) and DOL 7 (126 pg/mL [78.8–225, cohort 2]) ($p < 0.001$). In both cohorts NfL correlated inversely with birth weight (BW, Spearman's rho −0.403, $p < 0.001$, cohort 1; R −0.525, $p < 0.001$, cohort 2) and GA (R −0.271, $p < 0.001$, cohort 1; R −0.487, $p < 0.001$, cohort 2). Additional significant correlations were found for maternal age at delivery, preeclampsia, delivery mode, 5-min Apgar, duration of oxygen supplementation, sepsis, and brain damage (intraventricular hemorrhage or periventricular leukomalacia). Multivariable logistic regression analysis identified the independent predictors of NfL in cohort 1 as BW (beta = −0.297, $p = 0.003$), delivery mode (beta = 0.237, $p = 0.001$) and preeclampsia (beta = 0.183, $p = 0.022$) and in cohort 2 as BW (beta = −0.385, $p = 0.001$) and brain damage (beta = 0.222, $p = 0.015$).

Conclusion: Neonatal NfL levels correlate inversely with maturity and BW, increase during the first days of life, and relate to brain injury factors such as intraventricular hemorrhage and periventricular leukomalacia, and also to vaginal delivery.

Keywords: cerebral injury, neuropathology, biomarker, infant, parturition, prematurity

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INTRODUCTION

As direct access to the central nervous system (CNS) is almost impossible, neuronal biomarkers have been investigated for decades in order to improve early diagnostics, monitor disease progression and optimize care. Neurofilaments (Nf) are highly specific major neuronal scaffolding proteins comprising 4 subunits: the triplet of Nf light chain (NfL), Nf medium chain, and Nf heavy chain (NfH), and α-internexin in the CNS, or peripherin in the peripheral nervous system (1). Acute or chronic neuronal damage, including traumatic brain injury, stroke, dementia and multiple sclerosis, releases Nf fragments into the cerebrospinal fluid and eventually the blood compartment (2–6). Recent advances using highly sensitive single molecule array (Simoa) immunoassay have improved NfL detection, particularly in peripheral blood, making it a promising and readily accessible biomarker for neuroaxonal injury (7).

Whereas, circulating Nf has been extensively characterized in adults and older children with neurologic disease, data in infants and particularly newborns are sparse. One study reported raised serum NfH in children older than 6 months with febrile seizures lasting >30 min, suggesting that prolonged seizures cause some degree of neuronal damage (8). Plasma NfH in newborns with hypoxic-ischemic encephalopathy (HIE) was also higher than in healthy neonates (9, 10). Moreover, NfL levels in infants undergoing therapeutic hypothermia for HIE were significantly higher in those with unfavorable vs. favorable brain magnetic resonance imaging (MRI) outcome (11). As for mode of delivery, serum NfH levels at day of life (DOL) 2 in a small cohort of newborns did not differ between those born vaginally and those born by cesarean section (12).

Given the potential of Nf in adults with acute or chronic CNS damage and promising results in infants with HIE, we aimed to measure NfL levels by Simoa in two cohorts of preterm and term neonates in umbilical cord blood at birth and in venous blood a few days after birth.

MATERIALS AND METHODS

Study Participants

The study was based on data and blood samples prospectively collected from two neonatal cohorts. Cohort 1 comprised data and blood samples from 203 preterm and term neonates, median gestational age (GA) 37.9 weeks (interquartile range [IQR] 31.9–39.4), born and cared for at the University Hospitals of Zurich and Basel, Switzerland. More specifically, it comprised 89 preterm infants (GA < 37 weeks), including 52 with GA < 32 weeks, and 114 term infants (GA ≥ 37 weeks). The study was approved by the institutional review boards of both university hospitals (Ethikkommission beider Basel, EKBB07/09, Kantonale Ethikkommission Zurich, KEK08/09). Cohort 2 comprised data and blood samples from 98 very preterm neonates (GA < 32 weeks), median GA 29.3 weeks (IQR 26.9–30.6), born and cared for at the University Hospital of Basel, Switzerland. The study was approved by the institutional review board (Ethikkommission beider Basel, EK233/13) and was carried out in accordance with the declaration of Helsinki. Written informed consent was obtained from the parents prior to enrollment.

Abbreviations: Nf, Neurofilament; NfL, Neurofilament Light Chain; GA, Gestational Age; BW, Birth Weight; DOL, Day of Life; MPT, Moderate Preterm and Term.

Clinical Characteristics (Table 1)

Details of pregnancy (presence/absence of preeclampsia, amniotic infection, preterm labor, maternal age, premature rupture of membranes), delivery (umbilical artery pH, delivery modality), birth (GA, BW, sex, 5- and 10-min Apgar scores), and postnatal course to discharge home (presence/absence of sepsis and/or necrotizing enterocolitis, ultrasound brain damage with periventricular intraventricular hemorrhage [PIVH] or periventricular leukomalacia [PVL], duration of oxygen) were collected from the charts. Definitions of clinical characteristics, including preeclampsia, clinical chorioamnionitis, PIVH, and PVL, have been described previously (13), based on standardized definitions of the Swiss Neonatal Network.

Sample Preparation and Assessment of NfL

In cohort 1, venous blood (0.5 mL) was collected from the umbilical cord at birth ($n = 185$) and simultaneously with mandatory neonatal metabolic screening at DOL 3 ($n = 39$); 68 paired umbilical arterial samples were also collected at birth. In cohort 2, venous blood was collected with diagnostic blood samples at DOL 7 ($n = 98$). All samples were handled according to standard operating procedures for blood sampling in EDTA tubes, subsequent sample transfer to the central laboratory service, centrifugation, preparation of aliquots, and storage at −80°C until batch-wise analysis as described previously (14). Assay technicians were blinded to clinical information and pregnancy outcome.

NfL levels were measured by Simoa immunoassay using capture monoclonal antibody (mAB) 47:3 and biotinylated detector mAB 2:1 (UmanDiagnostics, Umea, Sweden), as previously described (15). Calibrators (neat) and serum samples (1:4 dilution) were measured in duplicate. Bovine lyophilized NfL was obtained from UmanDiagnostics. Calibrators ranged from 0 to 2,000 pg/mL. Batch-prepared calibrators were stored at −80°C. Intra- and interassay variabilities were < 10%; the few samples with intra-assay coefficients of variation >20% were remeasured.

Data Analysis

Statistical analyses were performed using SPSS for Windows version 24 (IBM) and included descriptive statistics, Spearman's rank-order correlation analyses and multiple linear regressions (MLR) using NfL as dependent variable. NfL variables were log10 transformed for the correlations and MLR. The independent variables included for MLR were based on significant correlations and significant non-parametric univariate analyses such as the Mann-Whitney U (2 levels) and Kruskal-Wallis tests (>2 levels). For cohort 1 these variables were: BW, 5-min Apgar, delivery mode (3 levels), preeclampsia, sepsis, and oxygen duration. For cohort 2 they were: BW, 5-min Apgar, sex, brain damage, sepsis,

amniotic infection, and oxygen duration. Due to collinearity between BW and GA, we used only BW in MLR, where it showed stronger correlation with NfL than GA.

RESULTS

Baseline NfL Levels

In cohort 1 overall median venous NfL concentrations were 18.2 pg/mL (IQR 12.8–30.8) at birth and 50.9 pg/mL (41.3–100.1) at DOL 3; in cohort 2 they were 128.5 pg/mL (78.8–224.8) at DOL 7.

We split cohort 1 into a very preterm group (GA < 32 weeks; $n = 52$) and a moderate preterm and term (MPT) group (GA ≥32 weeks; $n = 151$) with fewer prematurity complications ($n = 1$ in our sample). This also enabled us to compare the first group with cohort 2. NfL levels were significantly higher in very preterm infants than in the MPT group at birth (median 32.5 pg/mL, $n = 47$ vs. 15.3 pg/mL, $n = 138$; $p < 0.001$), but not at DOL 3 (median 48.5 pg/mL, $n = 16$ vs. 51.4 pg/mL, $n = 23$; $p = 0.668$). Moreover, levels increased significantly from birth to DOL 3 in both the very preterm and MPT groups (median 32.5 vs. 48.5 pg/mL, $p = 0.002$; and median 15.3 vs. 51.4 pg/mL, $p < 0.001$), and from DOL 3 to DOL 7 in the very preterm group (median 48.5 vs. 128.5 pg/mL, $p = 0.001$) (**Table 2**). This increase was confirmed in cohort 1 when comparing paired samples from same infants (MPT group $n = 16$, very preterm group $n = 11$) at birth and DOL 3 (median 18.2 pg/mL vs. 49.4 pg/mL). Out of these, only in 2 very preterm infants NfL levels remained unchanged, in all other infants they increased from birth until DOL 3. Paired umbilical cord arterial and venous plasma were closely related ($R = 0.875$, $p < 0.001$). Given this close correlation and the greater number of subjects ($n = 185$), we performed all further analyses using the venous blood samples collected at birth.

TABLE 1 | Descriptive statistics.

	Cohort 1 $n = 203$		Cohort 2 $n = 98$
	Moderate Preterm and Term (≥32 weeks GA) $n = 151$	Very preterm (< 32 weeks GA) $n = 52$	Very preterm (< 32 weeks GA) $n = 98$
NEONATAL CHARACTERISTICS			
GA (weeks)	38.3 (37.0–40.0)	30.1 (28.3–31.3)	29.3 (26.9–30.6)
BW (g)	3270 (2710–3630)	1360 (1063–1463)	1145 (788–1413)
Sex (male, %)	87 (57.6)	25 (48.1)	52 (53.1)
Brain damage (%)	1 (0.7)	10 (19.2)	12 (12.2)
O_2 duration (days)	0	4 (1–15.8)	2.38 (0.05–22.8)
pH umbilical artery	7.30 (7.26–7.33)	7.32 (7.29–7.37)	7.32 (7.28–7.36)
NEC (%)	0	0	3 (3.1)
Sepsis (%)	0	11 (21.2)	13 (13.3)
5-min Apgar	9 (9–9)	7 (5.25–8)	7 (6–8)
Death (%)	0	6 (11.5)	2 (2.0)
MATERNAL CHARACTERISTICS			
Age (years)	32 (29–36)	33 (28.3–36.0)	33 (29–36)
Amniotic infection (%)	5 (3.3)	13(25)	20 (20.4)
Preeclampsia (%)	16 (10.6)	20 (38.5)	16 (16.3)
PROM (%)	14 (9.3)	14 (26.9)	28 (28.6)
DM (%):			
Primary CS	76 (50.3)	26 (50)	27 (27.6)
Secondary CS	29 (19.2)	21 (40.4)	59 (60.2)
VD	46 (30.5)	5 (9.6)	12 (12.2)

GA, gestational age; BW, birth weight; BD, brain damage (PIVH and/or PVL); NEC, necrotizing enterocolitis; PROM, premature rupture of membranes; DM, delivery mode; CS, cesarean section; VD, vaginal delivery. GA, BW, O_2 duration, Apgar, pH and maternal age are presented as median and interquartile range.

NfL and Perinatal Characteristics in Cohort 1

Venous cord blood at birth correlated negatively with BW ($R = -0.403$, $p < 0.001$, **Figure 1**), GA ($R = -0.271$, $p < 0.001$), 5-min Apgar ($R = -0.295$, $p < 0.001$), and 10-min Apgar ($R = -0.363$, $p < 0.001$). In contrast, levels correlated positively with oxygen duration ($R = 0.333$, $p < 0.001$) and delivery mode ($R = 0.156$, p = 0.034).

Presence of preeclampsia (31.0 pg/mL vs. 16.2, $p < 0.001$) and sepsis (32.6 pg/mL vs. 17.85, $p = 0.033$) were associated with higher NfL levels.

In the MPT group NfL levels at birth were significantly higher in infants delivered vaginally than by primary or secondary cesarean section (21.8 vs. 13.9 and 14.4 pg/mL; $p = 0.002$) (**Figure 2**). This was not the case in the very preterm group, presumably due to the few vaginal deliveries ($n = 5$ vs. $n = 47$ cesarean sections). At DOL 3 there was no significant difference ($p = 0.07$) in NfL levels between birth modalities except for vaginal delivery vs. cesarean section (110 pg/mL, $n = 8$ vs. 48.7 pg/mL, $n = 31$; $p = 0.031$).

MLR testing for the best independent predictors of NfL levels at birth used BW, 5-min Apgar, delivery mode, preeclampsia, sepsis and oxygen duration as explanatory variables. The model was significant ($F_{(6, 176)} = 8.655$, $p < 0.001$), explaining around 23% of NfL variance ($R^2 = 0.228$). The predictors were BW (beta = −0.297, $p = 0.003$), delivery mode (beta = 0.237, $p = 0.001$), and preeclampsia (beta = 0.183, $p = 0.022$).

NfL and Perinatal Characteristics in Cohort 2

NfL at DOL 7 correlated negatively with the main neonatal characteristics such as BW ($R = -0.525$, $p < 0.001$, **Figure 1**), GA ($R = -0.487$, $p < 0.001$), and 5- and 10-min Apgar ($R = -0.247$, $p = 0.014$; $R = -0.228$, $p = 0.024$). Correlation was positive with oxygen duration ($R = 0.358$, $p < 0.001$) and maternal age ($R = 0.353$, $p < 0.001$).

Brain damage (211.5 pg/mL vs. 123, $p = 0.002$) and sepsis (184 pg/mL vs. 124.5, $p = 0.020$) were associated with higher NfL levels. Delivery mode had no significant impact ($p = 0.624$).

MLR analysis of cohort 2 used BW, 5-min Apgar, sex, brain damage, sepsis, amniotic infection, and oxygen duration as explanatory variables. The regression model explained around 37% of NfL variance ($R^2 = 0.366$, $F_{(7, 89)} = 7.331$, $p < 0.001$). Only BW (beta = −0.385, $p = 0.001$) and brain damage (beta = 0.222, $p = 0.015$) contributed significantly to predicting NfL (**Figure 2**).

DISCUSSION

Neuronal injury marker NfL has proved a sensitive and specific biomarker in adult peripheral blood, serving as a promising adjunct to monitoring and decision-making in acute and chronic neurologic disease (16, 17). Our study provides a first insight into neonatal NfL levels in term and preterm infants. The major findings are that NfL levels increase over the first few days of life, relate inversely to prematurity and BW, and identify BW, delivery mode, preeclampsia and brain damage as independent predictors.

NfL levels at birth in MPT infants resemble those in healthy adults (15). By DOL 3 they rise to the levels seen in adults with neurodegenerative disease such as multiple sclerosis (15). At DOL 7 in very preterm infants NfL levels are in the range of asphyxiated neonates at DOL 4 (11).

The main influencers of NfL in both cohorts were BW and maturity: birth and neonatal levels were both higher in low BW infants (**Figure 1**), perhaps because brain vulnerability to neuronal injury increases with prematurity. Alternatively, high NfL levels in preterm infants might be due to high neuronal turnover in general, with the much higher postnatal levels at DOL 3 and DOL 7 (**Figure 2**) simply reflecting a neuronal stress reaction to birth, as in healthy term neonates.

Preterm infants are at risk for perinatal brain damage, in particular PIVH and PVL (18). In our sample those with evidence of brain damage had significantly higher NfL levels than those without (**Figure 2**). Brain damage leads directly to neuronal injury, to a degree objectifiable by NfL: levels are higher in asphyxiated neonates with unfavorable brain MRI outcome (11). As in adults, cerebrovascular accident results in immediately higher NfL levels (19), compared to the more gradual neuronal damage seen in neurodegenerative disease (20).

In addition to a direct effect of brain damage, we identified two other stressors that increase NfL, namely delivery mode and preeclampsia. Levels were higher in infants delivered vaginally than by cesarean section (**Figure 2**), suggesting greater neuronal injury and confirming vaginal delivery as one of life's strongest stressors, causing incommensurable release of various fetal stress hormones (21). Preeclampsia, a pregnancy-specific syndrome defined by high blood pressure and other morbidities (22), was the additional stressor, raising NfL levels at birth even after adjustment for BW and GA. Our finding is consistent with the recent report of raised NfL levels in women with preeclampsia (23). Maternal hypertension is closely linked to placental insufficiency which compromises fetal perfusion and may cause cardiovascular disease later in life (24). Our data indicate that preeclampsia involves a risk of neuronal damage in the unborn child.

While the main source of NfL is considered to be the central nervous system, peripheral damage may contribute to increased NfL values as well, as recently revealed by studies on peripheral neuropathies (25, 26). Increased blood levels of the muscle enzyme creatine kinase in newborn infants after vaginal deliveries compared to cesarean sections have been reported (27). They support the notion that increased NfL in these babies may result, at least in part, from peripheral neuronal damage. However, data on the central nervous system biomarker S100 B measured in the maternal serum and cord blood show clearly increased S100B values after vaginal delivery compared to cesarean section (28). It has been shown previously that

TABLE 2 | Cohort neurofilament light chain concentrations at birth and at days of life (DOL) 3 and 7.

Cohort	Neurofilament light chain concentrations (pg/mL)			
	Birth (arterial)	Birth (venous)	DOL 3 (venous)	DOL 7 (venous)
1: Very preterm group (GA < 32 weeks) $n = 52$		32.5 (17.6–52.5) $n = 47$	48.5 (37.6–138) $n = 16$	
1: Moderate Preterm and Term group (GA ≥32 weeks) $n = 151$	17.7 (12.4–25.4) $n = 68$	15.3 (12.2–23.9) $n = 138$	51.4 (41.4–86.4) $n = 23$	
2: Very preterm group (GA < 32 weeks) $n = 98$				126 (78.8–225) $n = 98$

Median and interquartile range. GA, gestational age.

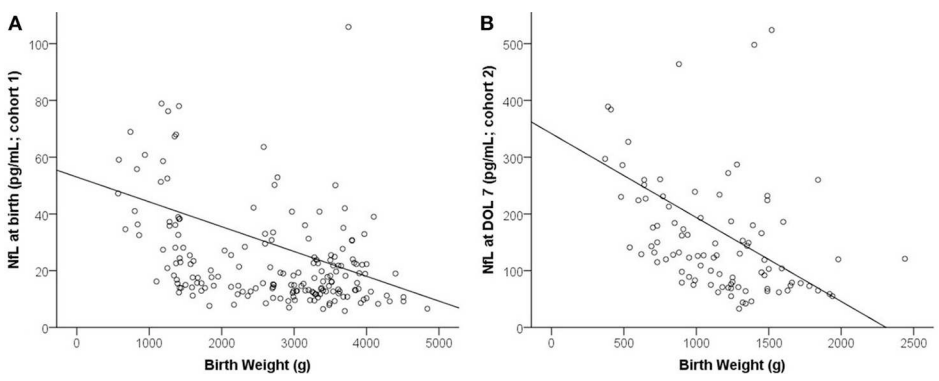

FIGURE 1 | Correlation between birth weight and NfL. **(A)** Birth (cohort 1). **(B)** DOL 7 (cohort 2).

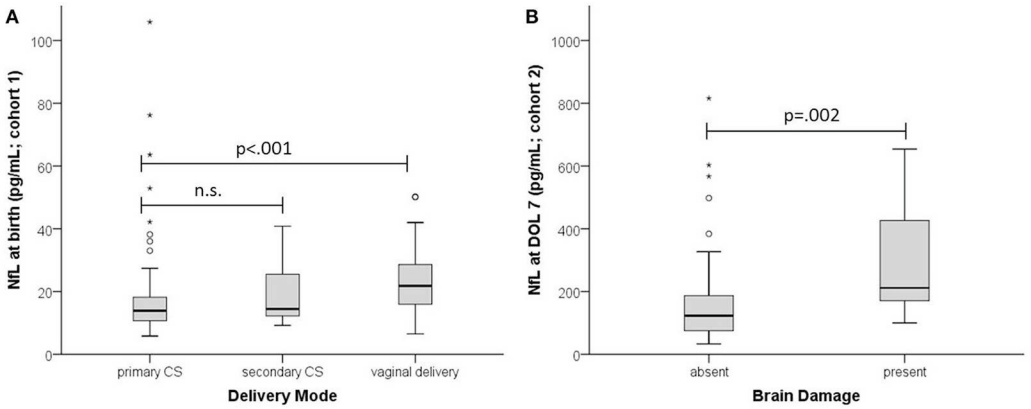

FIGURE 2 | Effect of delivery mode and brain damage on NfL. **(A)** NfL at birth in infants with GA ≥ 32 weeks (cohort 1). **(B)** NfL at DOL 7 (cohort 2). Absent, no brain damage; present, PIVH and/or PVL. Boxplots are presented with median and IQR. The * are extreme outliers.

extracranial sources of S100B do not affect serum levels (29). Taken together, the findings of Schulpis KH et al. corroborate our data that increased levels of the neuronal injury markers S100B and NfL might be caused by the compression on the fetus' brain during delivery.

Further, S100B levels in neonates with HIE exceeded those in healthy controls, proportionately to disease severity and worse outcome (30). Although S100B levels decreased overall from DOL 1 through DOL 9 (31), levels in preterm and term neonatal saliva followed a pattern similar to NfL, being higher in preterm than in term infants and correlating negatively with GA (32). Nerve growth factor (NGF) is a neurotrophic factor involved in brain development and neuroplasticity following brain damage. Unlike NfL, NGF levels in maternal and cord plasma are lower in preterm than in term deliveries (33).

To date the metabolism of NfL in cerebrospinal fluid (CSF) and blood is largely unknown, ways of elimination or protein degradation have not been described. One study examined the influence of blood brain barrier permeability and blood NfL levels. In this study there was no correlation between serum NfL concentration and CSF/serum albumin ratio (34).

Study limitations include the relatively few subjects sampled at DOL 3, which may account for the non-significant difference between very preterm and MPT infants at DOL 3. In the first week of life there is an apparent increase in NfL levels, but in the absence of data points post-DOL 7, the subsequent profile of NfL requires elucidation in further studies. Nor can we exclude other confounders that might influence and explain NfL. Cognitive outcome studies will need to confirm the use of NfL as a predictive biomarker of brain damage and eventual neurodevelopmental deficit. Such early biomarkers are sorely needed to complement ultrasound or MRI in conditions such as PVL (18). In addition, future studies may explore NfL together with other potentially promising biomarkers of brain damage (35). More generally, research is required to explore and disentangle the causes of the high degree of neuronal injury in the preterm brain.

CONCLUSION

This study provides an initial insight into neuronal injury marker NfL in term and preterm infants. Levels increase through the first week of life. They relate inversely to GA and BW and are higher in brain injury. Obstetric parameters such as delivery mode and preeclampsia also raise NfL levels. Our study supports the use of NfL in neonates to help us understand the factors leading to neuroaxonal injury and how we might monitor and prevent them.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

SW and UF designed the study. SW and RN collected the data. JK and CB assayed the serum samples. AD analyzed the data and wrote the manuscript together with SW and PW. All authors provided critical feedback and helped to improve the manuscript.

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Inverse Phosphatidylcholine/Phosphatidylinositol Levels as Peripheral Biomarkers and Phosphatidylcholine/Lysophosphatidylethanolamine-Phosphatidylserine as Hippocampal Indicator of Postischemic Cognitive Impairment in Rats

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Vascular dementia is a transversal phenomenon in different kinds of neurodegenerative diseases involving acute and chronic brain alterations. Specifically, the role of phospholipids in the pathogenesis of dementia remains unknown. In the present study, we explored phospholipid profiles a month postischemia in cognitively impaired rats. The two-vessel occlusion (2-VO) model was used to generate brain parenchyma ischemia in adult male rats confirmed by alterations in myelin, endothelium, astrocytes and inflammation mediator. A lipidomic analysis was performed via mass spectrometry in the hippocampus and serum a month postischemia. We found decreases in phospholipids (PLs) associated with neurotransmission, such as phosphatidylcholine (PC 32:0, PC 34:2, PC 36:3, PC 36:4, and PC 42:1), and increases in PLs implied in membrane structure and signaling, such as lysophosphatidylethanolamine (LPE 18:1, 20:3, and 22:6) and phosphatidylserine (PS 38:4, 36:2, and 40:4), in the hippocampus. Complementarily, PC (PC 34:2, PC 34:3, PC 38:5, and PC 36:5) and ether-PC (ePC 34:1, 34:2, 36:2, 38:2, and 38:3) decreased, while Lyso-PC (LPC 18:0, 18:1, 20:4, 20:5, and LPC 22:6) and phosphatidylinositol (PI 36:2, 38:4, 38:5, and 40:5), as neurovascular state sensors, increased in the serum. Taken together, these data suggest inverse PC/LPC-PI levels as peripheral biomarkers and inverse PC/LPE-PS as a central indicator of postischemic cognitive impairment in rats.

Keywords: global ischemia, cognitive impairment, phospholipid profile, biomarkers, serum, hippocampus

Abbreviations: ePC ether phosphatidylcholine; ePE, ether phosphatidylethanolamine; ePS, ether phosphatidylserine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PCA, principal component analyses; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLS-DA, partial least squares analysis; PS, phosphatidylserine; SM, sphingomyelin.

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INTRODUCTION

Cognitive impairment and dementia are common phenomena induced by acute and chronic brain injury, including Alzheimer's disease, stroke, and traumatic brain injury (TBI) (Nucera and Hachinski, 2018). Additionally, these conditions are predisposed to or worsened by poor lifestyles, including obesity, metabolic disorders, sedentary habits, smoking, and cardiocerebrovascular diseases, among others (Moskowitz et al., 2010). Long-term deprivation of oxygen and glucose via hypoperfusion or vasoconstriction of small vessels generates neurovascular unit injury, which is associated with vascular dementia after focal or global cerebral ischemic injury in response to cardiac arrest, coronary artery bypass surgery, cardiorespiratory failure, and other conditions due to drastic reductions in blood flow to the brain (Llinas et al., 2000; Vijayan et al., 2017).

In particular, the incidence of cerebrovascular disease, the third leading cause of death and the first leading cause of physical and mental disability worldwide, is increasing in developing countries (Cardona-Gómez and Lopera, 2016). Particularly, brain ischemia is caused by the occlusion of blood vessels, depriving of oxygen and glucose, resulting in an energy failure that alters mitochondrial ATP synthesis and upregulates the production of oxidative stress, free radicals and lipid peroxidation. Also„ the activation of dopamine and glutamate, as excitatory neurotransmitters, induces intracellular calcium overload, metabolic dysfunction and acidosis (Moskowitz et al., 2010). The increase in intracellular calcium causes activation of enzymes involved in lipid metabolism, such as "sphingomyelinases and phospholipases A2, C, and D, that, in turn, promote the release of second messengers, such as diacylglycerol (DAG), phosphatidic acid (PA), and arachidonic acid (AA), involved in inflammation, excitotoxicity and other cell death pathways" (Phillis and O'Regan, 2004; Tian et al., 2009). However, biomarkers supporting the clinical diagnosis of stroke are in development, and the development of biomarkers used to diagnosis the risk of dementia after stroke and its prevention is a true challenge.

In regard to lipids, these compounds are diverse, complex, and their functions depend on cellular distribution. Lipids are crucial in the homeostasis of cell membrane structure and act as signaling molecules and modulators in the central nervous system (CNS) (Martinez-Gardeazabal et al., 2017). Interestingly membrane lipids can be damaged by lipolysis under ischemia and by peroxidation of polyunsaturated fatty acids (PUFAs) during reperfusion (Schaller and Graf, 2004). Particularly, phospholipids, which are known for their high concentrations in the brain, play an important role both in normal neuronal activity and in pathological processes, even in those associated with memory impairment (Miyawaki et al., 2016). Therefore, we focused on elucidating the changes in lipid profiles of the hippocampus and serum of rats with cognitive impairment induced by global ischemia, as a continue of our previous studies (Marosi et al., 2006).

MATERIALS AND METHODS

Animal Procedures

"All of the animal procedures were performed in accordance with the ARRIVE guidelines, the Guide for the Care and Use of Laboratory Animals, 8th edition published by the National Institutes of Health (NIH) and the Colombian standards (law 84/1989 and resolution 8430/1993). These procedures were approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, Medellin, Colombia.

Male Wistar albino rats from our in-house, pathogen-free colony in the vivarium at SIU (Sede de Investigación Universitaria), University of Antioquia, Medellin, Colombia were kept on a 12:12 h dark/light cycle and received food and water *ad libitum*. Special care was taken to minimize animal suffering and to reduce the number of animals used. Three-month-old rats weighing 400–450 g were used. The rats were randomly divided into two groups, namely, the control and ischemic groups. Nine (9) rats were used per experimental group for behavioral, lipidomic and immunostaining evaluation (Marosi et al., 2006).

Global Cerebral Ischemia (2 VO)

"The animals were anesthetized using ketamine (60 mg/kg) and xylazine (5 mg/kg) and received a 2–4% isofiurane and 96% oxygen mixture via an inhalation anesthesia machine. A variation of the global cerebral ischemic model was implemented, involving a 2-vessel occlusion (2-VO; (Marosi et al., 2006). The right common carotid artery (CCA) was permanently occluded using a 6.0-gauge nylon suture (Corpaul, Bogota, Colombia), and the left CCA was obstructed for 20 min using a vascular clip. After the 20 min, the vascular clip was removed to allow reperfusion. Sham control rats underwent the same procedure without the CCA occlusion. The animals were sacrificed a month postischemia for lipid analyses" (Becerra-calixto and Cardona-gómez, 2017).

Immunochemistry and Immunofluorescence

Twenty four hours after the last behavioral test, animals were perfused intracardially with paraformaldehyde at 4%. Brains were removed and postfixed 48 h. Coronal sections (50 mm) obtained from vibratome were permeabilized, with 0.3% Triton X-100 and blocked with 1% BSA in PBS, using a previously described protocols for immunohistochemistry and immunofluorescence (Marosi et al., 2006), for the following evaluated primary antibodies: anti-NeuN (mouse monoclonal, Millipore, 1:500) anti-GFAP (monoclonal anti-glial fibrillary acidic protein, Sigma, 1:1000), anti-PECAM-1 (rabbit Platelet Endothelial Cell Adhesion Molecule 1, Abcam, 1:500), anti-myelin PLP (rabbit, myelin Proteolipid protein, Abcam, 1:200), COX-2 (rabbit, Cyclooxygenase 2, Abcam, 1:500). For immunofluorescence tissue, we incubated for 90 min at room temperature with mouse Alexa Fluor 488- or Alexa Fluor 594- conjugated anti-rabbit secondary antibodies (1:1000; Molecular Probes, Eugene, OR, United States). The tissues incubated in the absence of primary antibody did not display immunoreactivity.

Morris Water Maze Test

"Nineteen days after ischemia, the animals were evaluated in the Morris water maze (MWM) teste during 10 days (n = 9 per group). The test was performed using a previously described method (Becerra-calixto and Cardona-gómez, 2017). Briefly, a black plastic tank was filled with water (22 ± 2°C), and visual cues around the room remained in a fixed position throughout the experiment. The hidden platform (12 cm in diameter) was submerged 3 cm below the water level during spatial learning and 1.5 cm above the surface of the water during the visible session. Six sessions or trials were performed. Each session consisted of four successive subtrials (30 s intertrial interval), and each subtrial began with the rat being placed pseudorandomly in one of four starting locations. Then, the animals were provided with a 48 h retention period, followed by a probe trial of spatial reference memory, in which the animals were placed in the tank without the platform for 90 s. The latency to reach the exact former location of the platform was recorded during the probe trial. Later, the platform was moved to a new location and the ability of the animals to learn the new location was measured by determining the latency in 4 sessions conducted in the same manner as the learning phase. The latency to reach the platform was evaluated using a visible platform to control for any differences in visual-motor abilities or motivation between the experimental groups; the animals that could not perform the task were excluded. An automated system (Viewpoint, Lyon, France) recorded the behavior of the animals" (Marosi et al., 2006).

Tissue Preparation and Lipid Extraction

A month postischemia, four (4) "animals were sacrificed via decapitation, and the hippocampus of each rat was dissected, immediately frozen in liquid nitrogen and stored at 80°C until analysis. We performed the same procedure to obtain serum samples. The total lipids from the hippocampus and serum were extracted according to the FOLCH technique (Jordi Folch, 1957) using a mixture of 2 mL of chloroform (CHCl3) and 1 mL of methanol (MeOH) in a 2:1 (v/v) ratio. Then, 0.005% butylated hydroxytoluene (BHT) was added, and this mixture was used to homogenize the hippocampus. Subsequently, 1 mL of 0.9% NaCl was added, and the mixture was centrifuged at 3000 rpm for 3 min. The organic layer (lower layer) was removed and transferred to a new glass tube. The solvents were evaporated, and the extract was lyophilized to remove excess humidity. Finally, the lipid composition was analyzed via mass spectrometry" (Villamil-Ortiz et al., 2016).

Mass Spectrometry

"An automated ESI-MS/MS approach was used, and data acquisition and analysis were carried out at the Kansas Lipidomics Research Center using an API 4000 TM and Q-TRAP (4000Qtrap) detection system as described previously (Villamil-Ortiz et al., 2016). This protocol allowed the detection and quantification of low concentrations of the polar lipid compounds. The molecules were determined by the mass/charge ratios, which were compared with the respective internal standard to determine which species of lipids were present in the evaluated extract: 0.30 nmol 14:0 lysoPG, 0.30 nmol 18:0 lysoPG, 0.30 nmol di 14:0 PG, 0.30 nmol 14:0-lysoPE, 0.30 nmol 18:0-lysoPE, 0.60 nmol 13:0-lysoPC, 0.60 nmol 19:0-lysoPC, 0.60 nmol di 12:0-PC, 0.60 nmol di 24:1-PC, 0.30 nmol 14:0 lysoPA, 0.30 nmol 18:0 lysoPA, 0.30 nmol di14:0-PA, 0.30 nmol di20:0 (phytanoyl)-PA, 0.20 nmol di 14:0-PS, 0.20 nmol di Phy PS, 0.28 nmol 16:0-18:0 PI, and 0.10 nmol di 18:0-PI. The system detected a total of 12 different lipid species and their respective subspecies, which were identified by the number of carbons and the degree of unsaturation in the chain. The lipid concentration was normalized according to the molar concentration across all species for each sample, and the final data are presented as the mean mol%" (Villamil-Ortiz et al., 2016).

Profile of Other Lipid Fractions

Lipids were extracted from the hippocampus and serum using the Folch method. The solid-phase extraction (SPE) as described Bermudez-Cardona et al (Bermúdez-cardona and Velásquez-rodríguez, 2016), was used to separate cholesterol esters (CE), triglycerides (TG), and free fatty acids (FFA).

Statistical Analysis

The behavioral test comparisons between two groups were performed using Student's t-tests for parametric data or Mann–Whitney tests for nonparametric data. "The lipid levels of each sample were calculated by summing the total number of moles of all lipid species measured and then normalizing that total to mol%. Comparisons between groups were assessed either by one-way ANOVA, followed by the Tukey *post hoc* test, or the Kruskal–Wallis test, depending on the homoscedasticity and normality of the experimental data. Multivariate statistics were performed using principal component analysis (PCA) and a partial least squares discriminant analysis (PLS-DA)" (Barker and Rayens, 2003). "The PLS-DA was included because this analysis is particularly suitable for the analysis of datasets with a small number of samples and a large number of variables. The PLS-DA was carried out using the protocols described previously by our laboratory" (Villamil-Ortiz et al., 2016). The data are expressed as the mean the standard error of the mean. The statistical significance is indicated in the figures and tables.

RESULTS

Morphological Changes, Cognitive Impairment, and Hippocampal Phospholipid Profile Changes a Month Postischemia

The abilities of spatial learning and memory were examined by using the Morris water maze test. We found that, even a month postischemia, ischemic rats significantly increased their latencies to locate the platform in the MWM from trial 3 to trial 6 (**Figure 1A**). In the memory test, it was found that rats with global ischemia showed significant deficits in locating the submerged escape platform compared with sham rats (**Figure 1A**). In addition, during the relearning test, ischemic rats

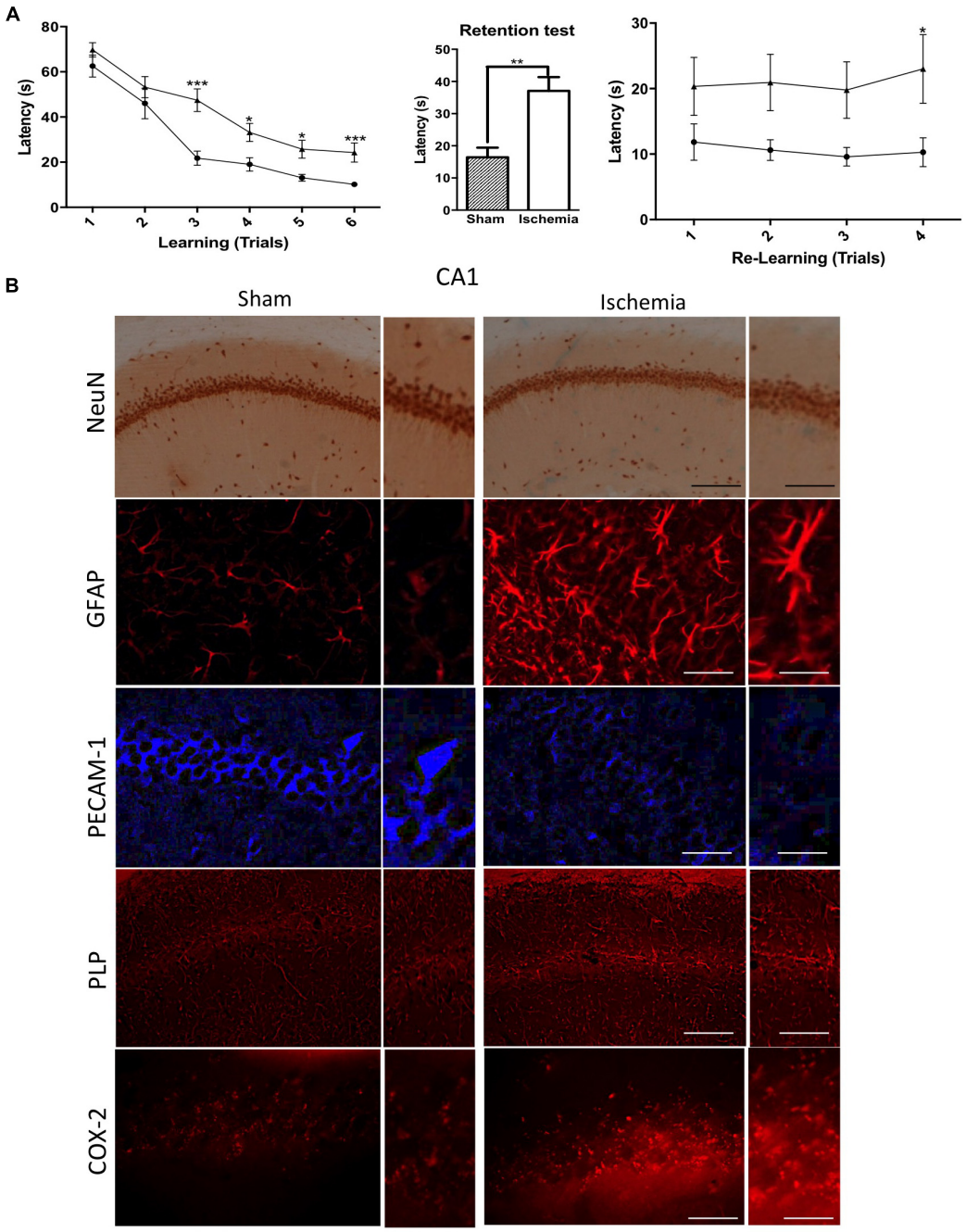


FIGURE 1 | Morphological alterations and cognitive impairment in the rat hippocampus after transient global ischemia. **(A)** The learning and memory task performance was evaluated with the Morris water maze on day 19, starting with the learning test and the first position of the platform. The retention test was conducted after 48 h without the platform. The transference test included the second position of the platform. Data are expressed as group means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; $n = 9$ animals/group. **(B)** Immunostaining in pyramidal cell layer in the hippocampus with Neuronal nuclei (NeuN) immunohistochemistry; glial fibrillary acidic protein (GFAP), Platelet Endothelial Cell Adhesion Molecule 1 (PECAM-1), myelin Proteolipid protein (PLP), Cyclooxygenase 2 (COX-2) immunofluorescences. $n = 5$. NeuN and PLP, Magnification: 10×, scale bar: 100 μm; Insert: Magnification 40×; scale bar = 50 μm; GFAP, PECAM, and COX-2. Magnification 20×, scale bar: scale bar = 50 μm; Insert: 60×, scale bar: 15 μm.

exhibited cognitive impairment a month postischemia, and the escape latencies were significantly lower compared with those of the sham group (**Figure 1A**). Which was supported by the morphological alterations of the hippocampus, although there was not a clear neuronal loss, we detected hypertrophic astrocytes with thickened processes and enlarged cell bodies (**Figure 1B**). PECAM-1, a cell adhesion marker, suggested disruption of the parenchima, PLP, a protein involved in the production of

myelin, presented aggregation at the CA1, in addition to the increased presence of COX-2, inflammatory marker, suggesting the structural damage in the hippocampus at 1 month post global ischemia respect to healthy rats (**Figure 1B**).

On the other hand, 311 species of phospholipids were evaluated via mass spectrometry in the hippocampus a month postglobal ischemia. At a glance, the analyses indicate that the changes seem to be related to the pathological condition. The lipid profiles of both groups show that they were primarily composed of high-abundance glycerophospholipids, such as PC (48.4 and 47%), PE (23.2 and 23.4%), PS (8.8 and 9.7%), and PI (3.8 and 3.9%); sphingolipids, such as SM-DSM (7.5 and 7.2%); low-abundance ether phospholipids, such as ePC (2.5 and 2.48%), ePE (2.48 and 2.5%), and ePS (0.02 and 0.03%); lysophospholipids, such as LPE (1.68 and 2.35%) and LPC (0.77 and 0.83%); and glycerophospholipids, such as PA (0.74 and 0.76%) and PG (0.11 and 0.12%) (**Figures 2A,B**).

The results of the PCA of the detected lipids indicated that nearly 77% of the total variance might be explained by the first two principal components (PC1 and PC2) (**Figure 2A**). The most relevant variables for these two components were related to reduced PC and increased PS subclasses (**Figure 2B**). The main Rho indices were PC 32:0, 34:1, and 36:1. PC 34:1 was composed of saturated palmitic acid (16:0) and oleic acid (18:1), while PC 32:0 was composed of two palmitic acids. In addition, PS 40:6, conformed by stearic acid (18:0) and docosahexaenoic acid (DHA) (22:6), was represented in the PCA. Complementarily, the PLS-DA showed ellipsoids that were completely different in terms of location; this could explain the reason that the control groups had a more diverse lipid composition compared with the ischemic group. The main changes were shown in LPE 18:1, 20:3, PE 30:0, PC 34:2, and ePC 36:5. Together these findings, based on the abundance by PCA and separability by PLS-DA, suggest decreased PC and increased PS and LPE composed of imbalanced

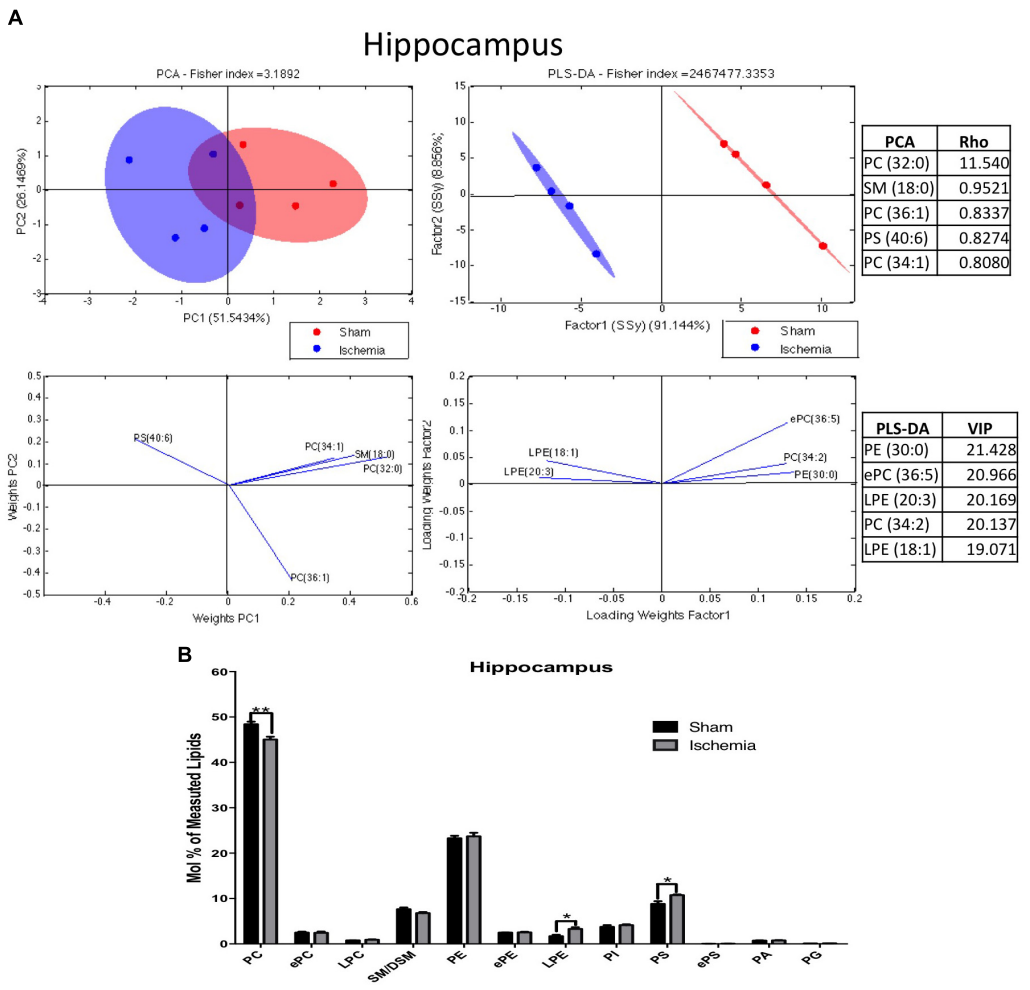


FIGURE 2 | Phospholipid composition in the hippocampus after 1 month postischemia. **(A)** Multivariate analyses of the lipid profiles in the hippocampus. PCA and PLS-DA were performed to discriminate between the lipid classes. The left panel illustrates the factor loadings for PC1 and PC2 with the indices of variance explained for each component. The right panels show the factor score plots for the PLS-DA. **(B)** The lipid class profiles are expressed as % mol composition. All lipid species are represented as the means ± SEM. Data for ischemic group were significantly different from those of the control group (*$p < 0.05$, **$p < 0.01$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

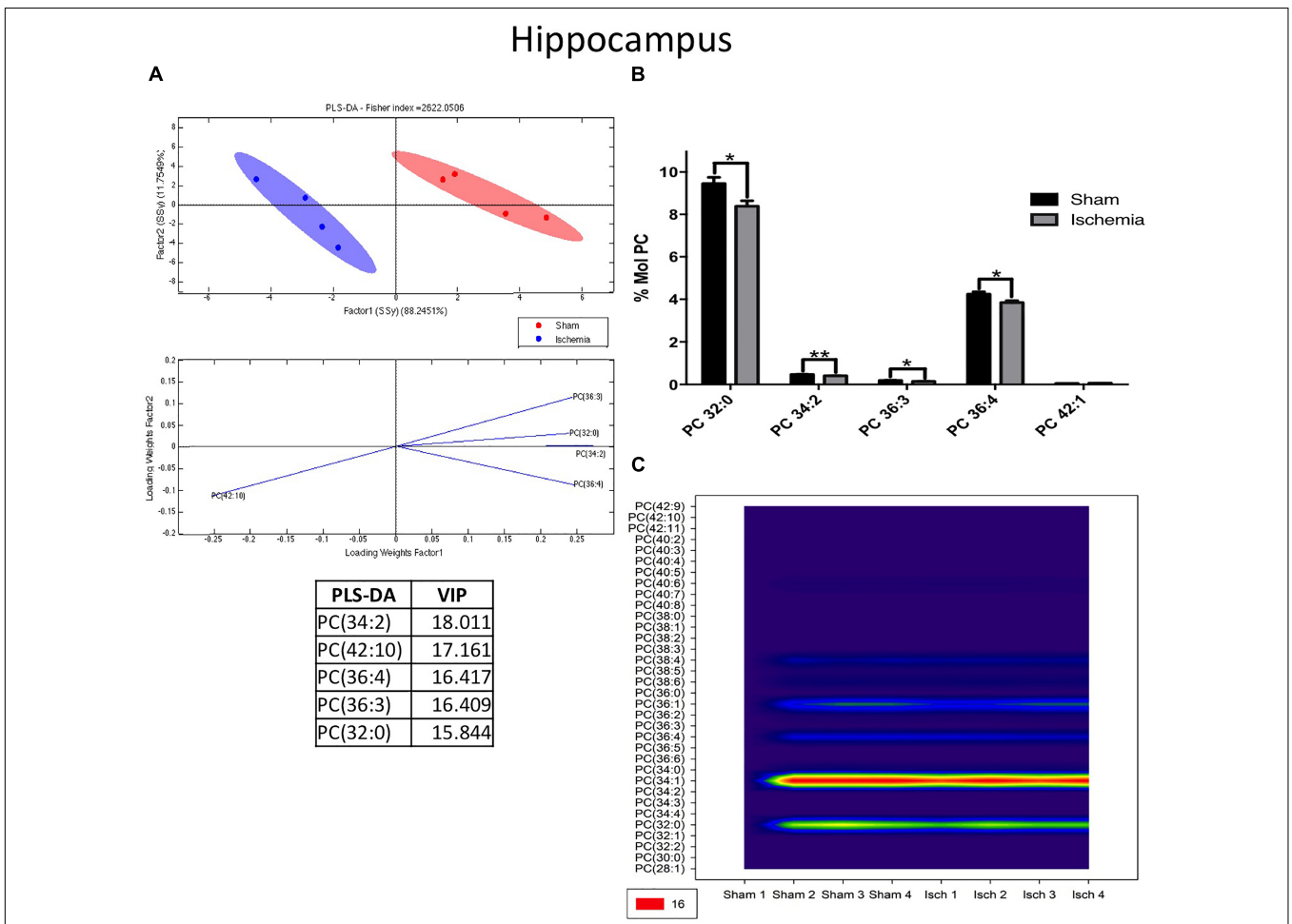
saturated fatty acid (SFA), monounsaturated FA (MFA) and in polyunsaturated FA (PUFA) after ischemia.

Inverse PC and LPE/PS Levels in Postischemic Hippocampus of Rats

PCs are a class of 1,2-diacylglycerophospholipids that are essential components of cell membranes and have structural roles defined primarily by chain length (Billah and Anthes, 1990; Whiley et al., 2014). In previous studies using tMCAO (transient middle cerebral artery occlusion), it has been described that several PC species and sphingomyelin (SM) were significantly decreased after infarction in the cerebral cortex; in the same manner, LPCs were elevated in the tissue (Wang et al., 2010).

We detected that global ischemia had a regulatory effect on the lipid profiles of PC and LPE from the hippocampus. The PLS-DA showed that the ischemic group had ellipsoids in different spaces, explained by the first component in 88% and the second one in 11% approximately (**Figure 3A**). In general, the PC levels decreased ($^*p < 0.05$) in the ischemic groups compared with their counterpart in the sham control group (**Figure 2B**). Four PC subspecies showed significant reductions in global ischemia compared with those in the controls: 32:0 (16:0/16:0 - $p < 0.05$), 34:2 (16:1/18:1 ($p < 0.01$), 36:3 (18:1/18:2 - $p < 0.05$), and PC 36:4 (18:2/18:2) (**Figures 3B,C**). In general, considering that PC as the more abundant PL detected in the profile, those results were supported by the tendency and significant reduction of C.16:0 and C 18:0 at the total Free fatty acid (FFA) detected at the hippocampus and inversely C16:0 and C 18:0 were increased in the content of total triglycerides by the global ischemia. However, the CE did not change, lignoceric acid (24:0) FFA increased and a generalized reduction of oleic acid (18:1) was observed in the three analyzed fractions in the ischemic hippocampus (**Supplementary Figures S1A–C**).

On the other hand, LPE, a plasmalogen derived from phosphatidylethanolamine (PE), had significant higher levels ($p < 0.05$) in the ischemia group than in the sham group. The



PLS-DA	VIP
PC(34:2)	18.011
PC(42:10)	17.161
PC(36:4)	16.417
PC(36:3)	16.409
PC(32:0)	15.844

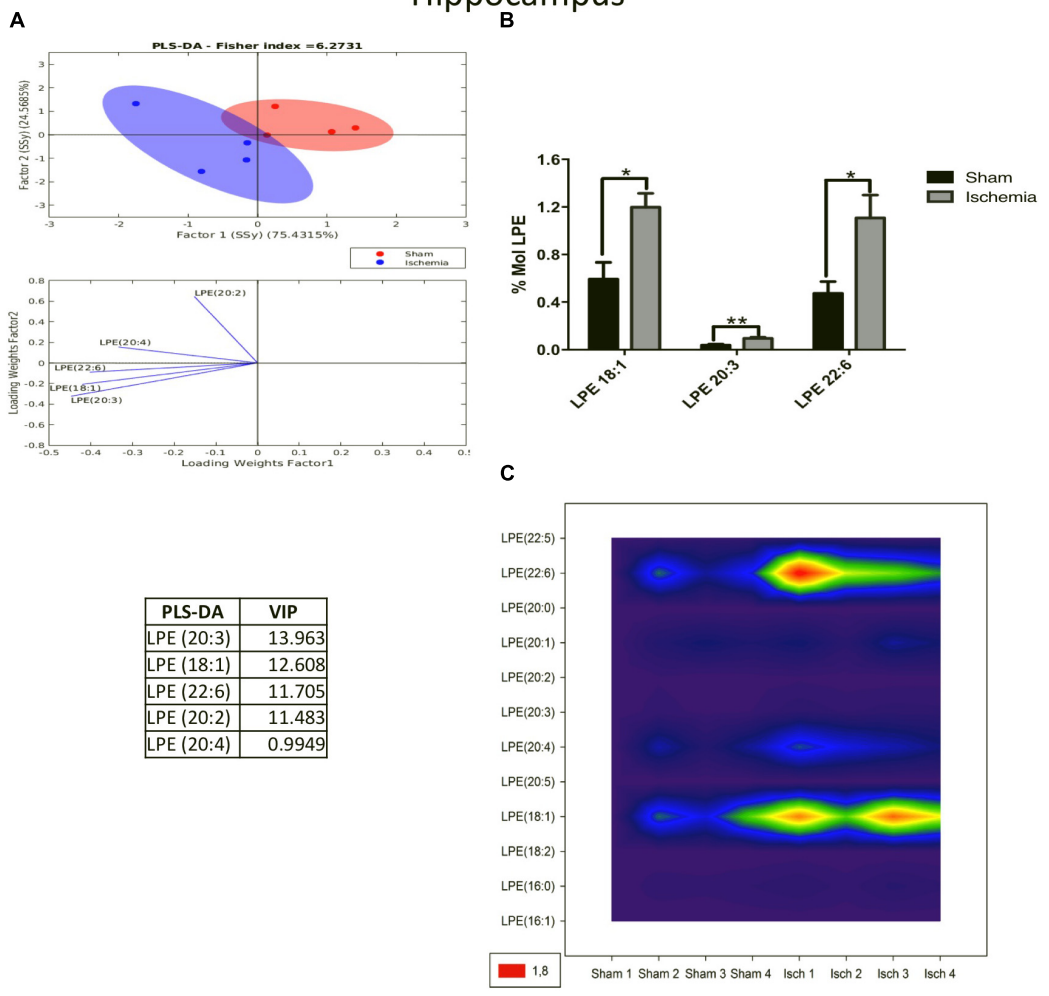
FIGURE 3 | Changes in phosphatidylcholine in the hippocampus after global ischemia. **(A)** Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. **(B)** The PC profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of PC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those of the control group and are represented as the means ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

PLS-DA demonstrated that the ischemic group occupied a close area with respect to that of the sham group, having a small overlapping area, however, the separability was explained by the first component at a rate of 75.43% (**Figure 4A**). When the most differential and relevant phospholipidic species was LPE 18:1, 20:3, and 22:6 increased in the ischemic group (**Figures 4A,B**). These results were supported by the contour graphic, which showed increasing levels in these PL subclasses in the global ischemia group, mainly LPE 22:6 and 18:1 (**Figure 4C**).

Complementarily, PS modulates the binding properties of glutamate receptors involved in neurotransmission and long-term potentiation in the brain (Farooqui and Horrocks, 2007). The PLS-DA showed a different distribution of the ischemic group relative to that of the sham group in the PCA graph. These data showed that the main changes occurred in the subspecies of PC 36:4, 38:4, 40:4, 38:5, and 44:11, as was indicated by the VIP index (**Figure 5A**). Most of these subspecies are composed of arachidonic acid (20:4), a proinflammatory molecule. Given that their levels significantly increased in the ischemic group ($^*p < 0.05$) (**Figure 5B**), this finding was in agreement with the high abundance shown in the counter plot graphic mainly by PS 40:4, 38:4, and 36:2 (**Figure 5C**).

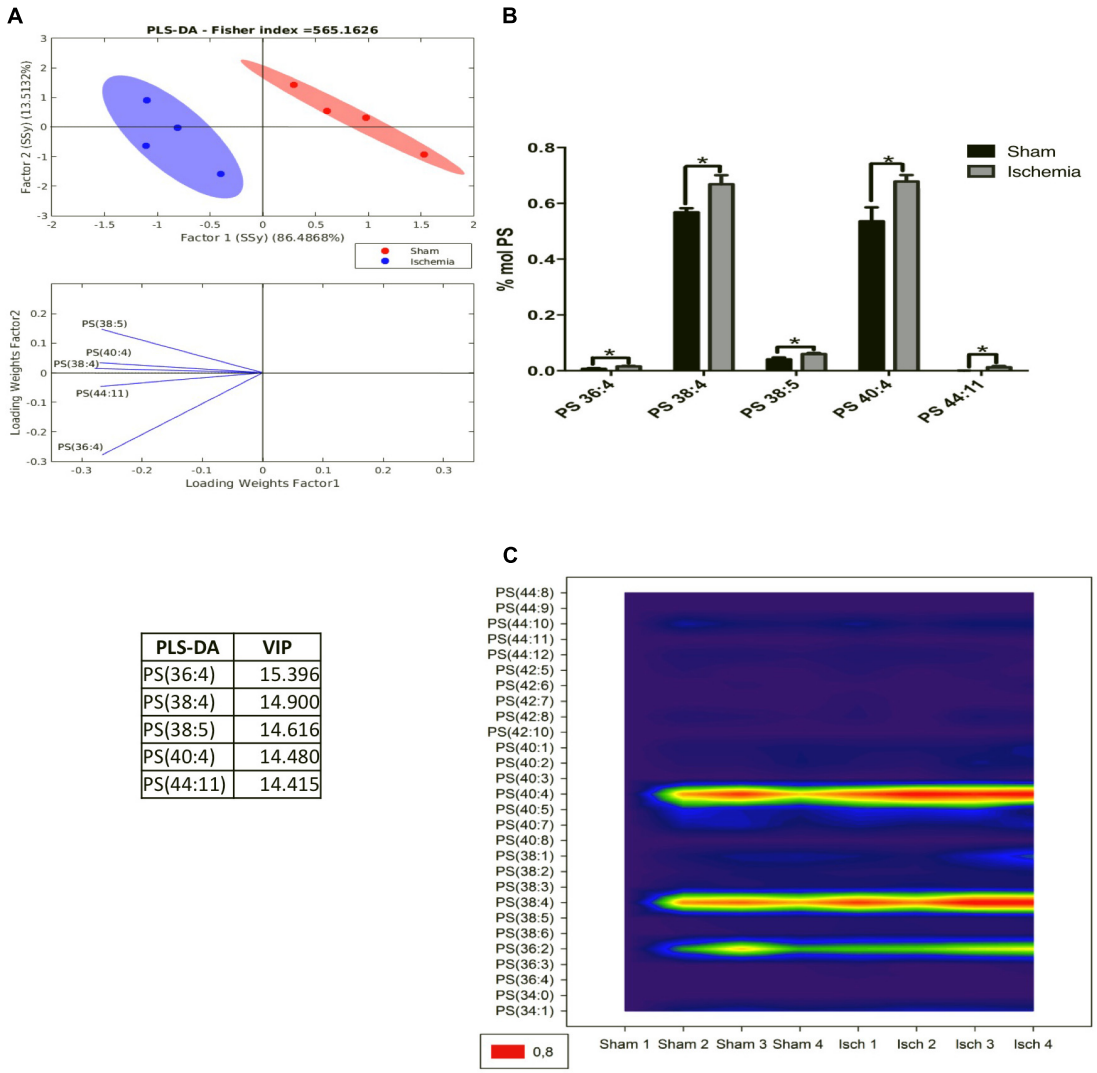
Phospholipidic Profile Changes in the Serum a Month Postischemia in Rats

Evaluation of the lipid profiles of serum in ischemic rats with cognitive impairment a month postischemia could suggest potential biomarkers. The results of the PCA of the detected lipids indicated that nearly 92% of the total variance might be explained by the first two principal components (PC1 and PC2). The



PLS-DA	VIP
LPE (20:3)	13.963
LPE (18:1)	12.608
LPE (22:6)	11.705
LPE (20:2)	11.483
LPE (20:4)	0.9949

FIGURE 4 | Increase in LPE species in the hippocampus after global cerebral ischemia. **(A)** Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. **(B)** The LPE profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of LPE (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different than those from the control group and are represented as the means ± SEM ($^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

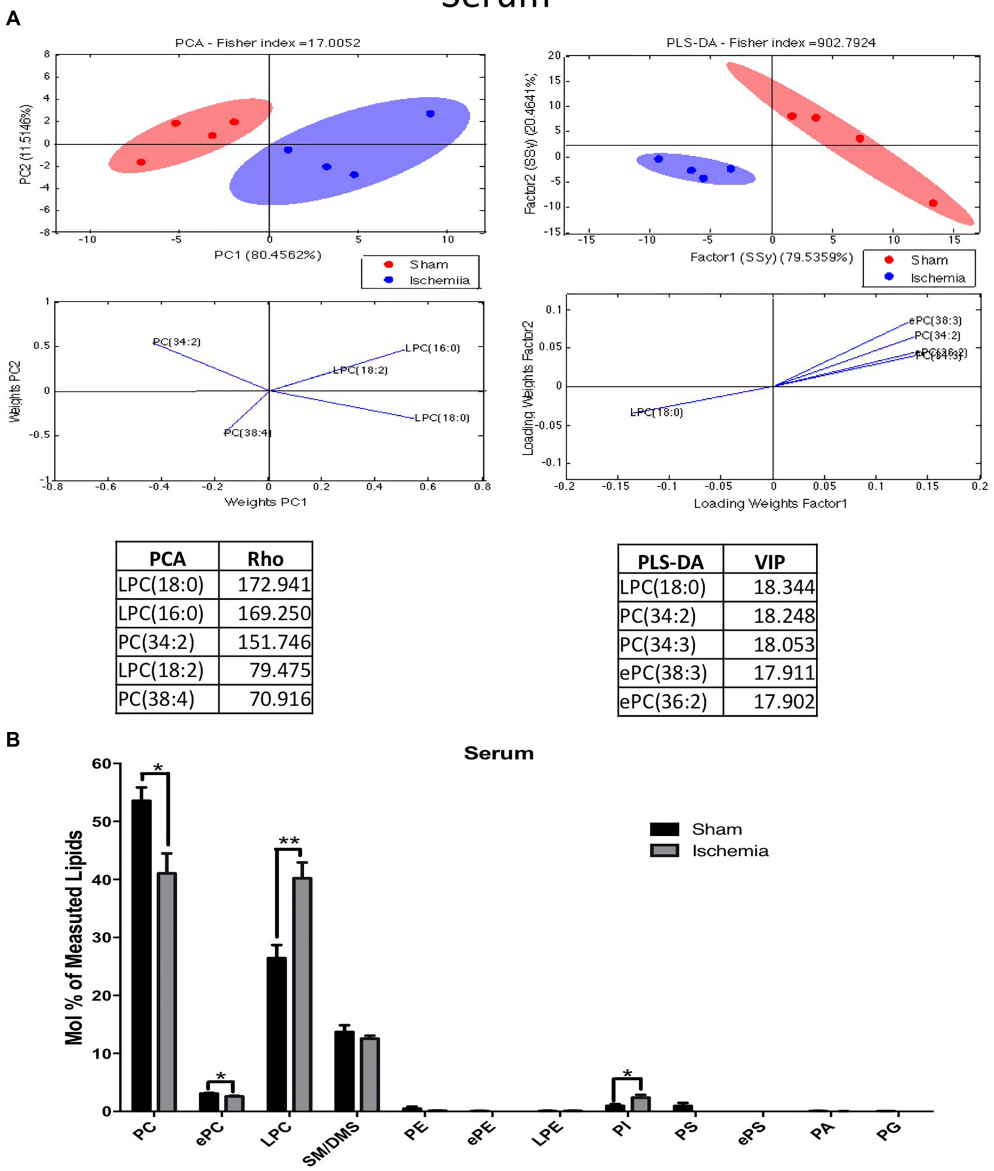


PLS-DA	VIP
PS(36:4)	15.396
PS(38:4)	14.900
PS(38:5)	14.616
PS(40:4)	14.480
PS(44:11)	14.415

FIGURE 5 | PS species increased in the ischemic hippocampus. **(A)** Multivariate analyses: PLS-DA used to discriminate between the lipid classes in the hippocampus showing the factor score plots. **(B)** The PS profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of PS (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (*$p < 0.05$ Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

distribution pattern in the plane showed divergent distributions between the ischemic and sham groups in the lipid profiles of the serum (**Figure 6A**). Complementarily, the PLS-DA confirmed a displacement in the left quadrant of the ischemic group, while the sham group occupied a different region on the right side. PCA showed abundant and differential locations of LPC and PC between the control and ischemic groups, with the main divergent phospholipid species being LPC 18:0, 16:0 and PC 34:2 with a Rho of 172.9, 169.25, and 151.74, respectively. While PLS-DA also identified LPC 18:0 and PC 34:2 as the more discriminant species, with a VIP of 18.34 and 18.24, respectively, between others explained by the first component in approximately 80% (**Figure 6A**).

The lipid profiles of both groups showed that they were primarily composed of high-abundance glycerophospholipids, such as PC (53.3 and 47.9%) and LPC (26.43 and 23.55%); sphingolipids, such as SM-DSM (13.7 and 13.2%); low-abundance ether phospholipids, such as ePC (3.07 and 2.85%), ePE (0.05 and 0.03%), and ePS (0.008 and 0.004%); PE (0.48 and 0.32%); PS (0.92 and 0.51%); PI (0.94 and 1.58%); LPE (0.068 and 0.074%); PA (0.05 and 0.04%) and PG (0.03 and 0.02%) (**Figure 6B**). In addition, these analyses showed that the main changes in serum were due to inverse levels of phosphatidylcholine and its plasmalogen, lysophosphatidylcholine, and the intermediate ePC species; together, a significant increase



PCA	Rho
LPC(18:0)	172.941
LPC(16:0)	169.250
PC(34:2)	151.746
LPC(18:2)	79.475
PC(38:4)	70.916

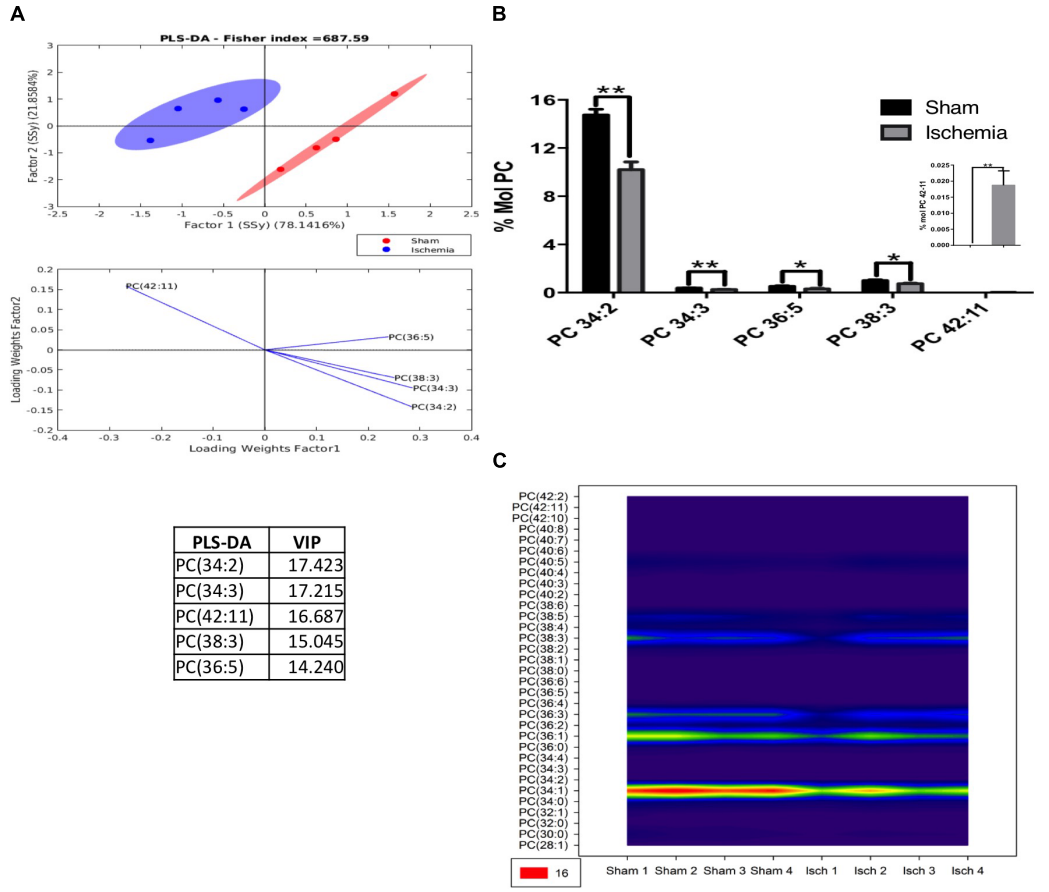
PLS-DA	VIP
LPC(18:0)	18.344
PC(34:2)	18.248
PC(34:3)	18.053
ePC(38:3)	17.911
ePC(36:2)	17.902

FIGURE 6 | Altered phospholipid composition in the serum 1 month postischemia. **(A)** Multivariate analyses of the lipid profiles in the serum. PCA and PLS-DA were used to discriminate between the lipid classes. The left panel illustrates the factor loadings for PC1 and PC2 with the indices of variance explained for each component. The right panels show the factor score plots for the PLS-DA. **(B)** The lipid class profiles are expressed as % mol composition. All lipid species are represented as the means ± SEM. Data from the ischemic group were significantly different from those from the control group (*$p < 0.05$, **$p < 0.01$, Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

in PI subspecies occurred in the serum of the ischemic group.

Inverse PC and LPC-PI Serum Levels in Postischemic and Cognitively Impaired Rats

Our results indicated that lipid phosphatidylcholine molecule subspecies, such as PC 34:2, PC 34:3, PC 36:5, and PC 38:3, significantly decreased in the serum of the ischemic group, showing a notable separability (**Figures 7A,B**) and reduced abundance (**Figure 7C**) relative to those in the sham group (**Figure 7**). Similarly, we observed a reduction in ePC, as shown in **Figure 8A**, and the PLS-DA showed that the sham and ischemic rats had different patterns of distribution, and these changes were reflected by the following subspecies: ePC 36:2, ePC 38:3, ePC 34:2, ePC 34:1, and ePC 38:2 (**Figure 8A**), all of which were significantly reduced with respect to the levels observed in the control group (**Figure 8B**) and supported by the counter graph (**Figure 8C**). Those results could be supported by a general



PLS-DA	VIP
PC(34:2)	17.423
PC(34:3)	17.215
PC(42:11)	16.687
PC(38:3)	15.045
PC(36:5)	14.240

FIGURE 7 | The majority of the species of PC decreased in the serum of postischemic rats. **(A)** Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. **(B)** The PC profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of PC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control groups and are represented as the means ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

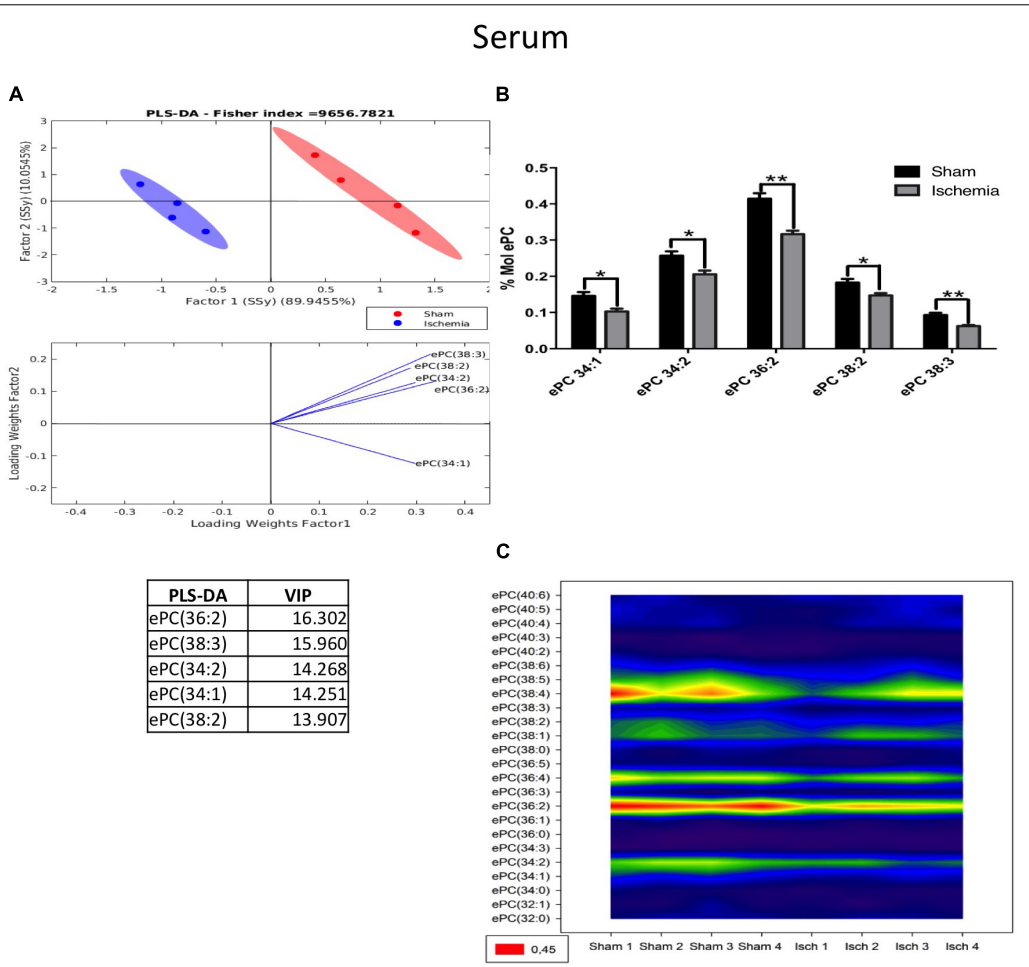
reduction of C 18 in the total FFA in serum (**Supplementary Figures S1D–F**), and no changes were detected in CE.

Inversely, LPC was shown to have increased in ischemic rats 1 month postischemia. The PLS-DA showed that ischemic and sham ellipsoids occupied different locations, supporting their different profiles (**Figure 9A**). The main subspecies that increased were LPC 18:0, LPC 22:6, LPC 20:5, LPC 18:1, and LPC 20:4 (**Figures 9A,B**) in cases when LPC 18:0, which is a fatty acid involved in inflammatory processes in cerebrovascular disease, detected at a high level also by the counter graph (**Figure 9C**).

For its part, the following PI subspecies demonstrated increased levels in the ischemic group: PI 36: 2 (18: 1/18: 1); PI 38: 4 (18: 0/20: 4), and PI 38: 5 (18: 1 and 20: 4), with PI 38:4 being the most abundant according to the histogram and counter plot analyses (**Figures 10A–C**). Interestingly, PLs composed of fatty acids with long carbon chains, such as 18: 0 (stearic acid) 18: 1 (oleic acid) and 20: 4 (AA), are involved in proinflammatory processes and were detected in the serum of ischemic rats a month postinjury; maybe these results could be in relationship with the general increase of detected C 18:2 in TG and FFA fractions (**Supplementary Figures S1E,F**), future analysis should be done.

DISCUSSION

Novelty, this study described hippocampal and peripheral phospholipid profile changes in long-term postischemia associated with cognitive impairment in rats. The main changes on PLs were associated to hippocampal dysfunction, represented by a downregulation of PC, as precursor of acetylcholine and inverse levels of LPE and PS associated with peroxisome damage, a proinflammatory environment and cell death in the hippocampal parenchyma after 1 month of anoxia, glutamate excitotoxicity and Brain Blood Barrier (BBB) disruption generated by global ischemia in rats (Becerra-calixto and



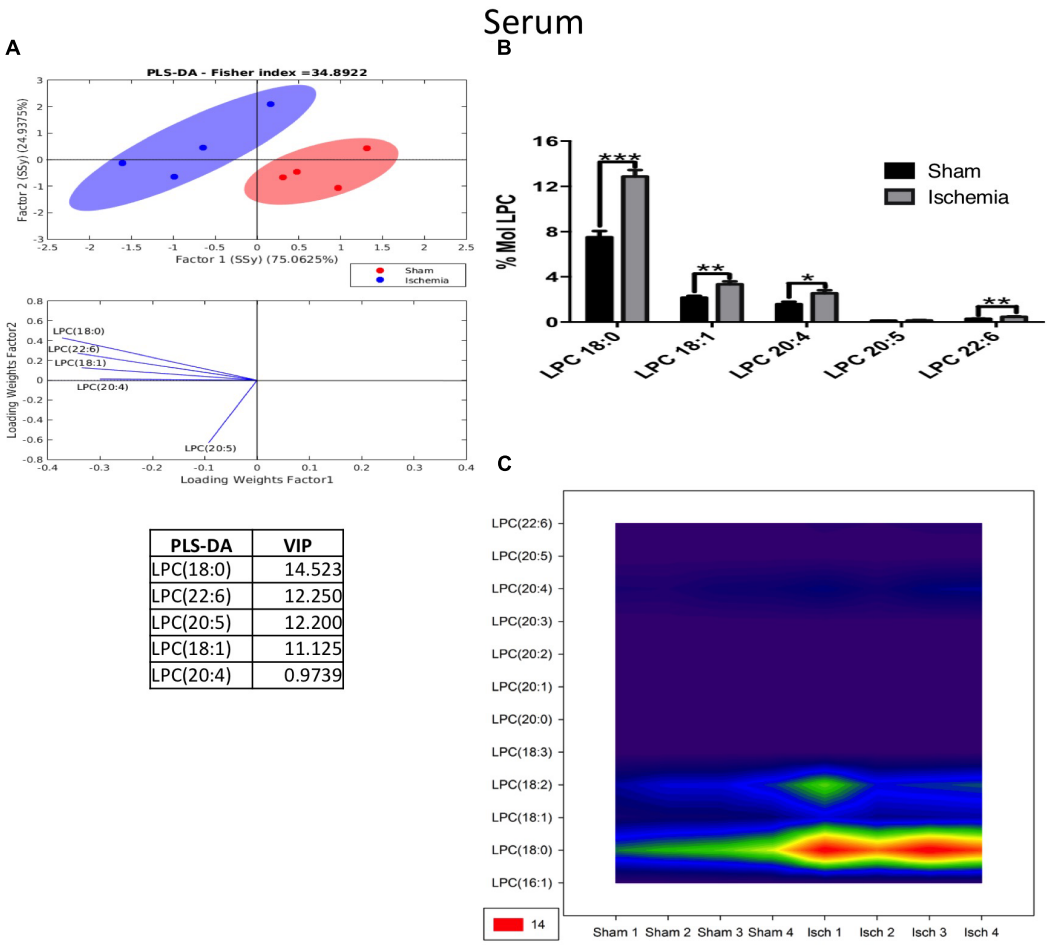
PLS-DA	VIP
ePC(36:2)	16.302
ePC(38:3)	15.960
ePC(34:2)	14.268
ePC(34:1)	14.251
ePC(38:2)	13.907

FIGURE 8 | Changes in ePC in the serum of postischemic rats. **(A)** Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. **(B)** The ePC profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of ePC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

Cardona-gómez, 2017). Also, these findings are supported by our recent study were proinflammatory phospholipid profile was associated to neurodegeneration and neurological dysfunction (Marosi et al., 2006).

Our data suggested that spatial learning and reference memory were significantly impaired in global ischemic rats, a finding that was in line with those of previous studies (Deng et al., 2017). These results demonstrated that the damage in ischemic rats is large, and the functional outcome can become worse over time. Recently, the high incidence of cognitive impairment after an ischemic stroke event has been described (Sun et al., 2014) and the comorbidity factors, such as atherosclerosis (Knopman et al., 2016). Additionally, plasma phospholipid changes have been suggested in cognitive impairment associated with brain stroke patients but have not been clearly identified by the variability in humans (Li et al., 2016). However, in subcortical ischemic vascular dementia and mixed dementia, an adaptative increase in polyunsaturated fatty acids and elevated membrane degradation (Lam et al., 2014) have been observed. With respect to mild cognitive impairment and its progression to dementia, this condition has been addressed in Alzheimer-type dementia, with lipidomic brain changes in serum PLs being observed, mainly via reductions in PC (Wood et al., 2016). The findings of these studies are in accordance with our current data from an experimental model of global ischemia, however, we showed a specific fatty acid composition imbalance constituted of 18:0, 18:1, 20:4, and 22:6 in the parenchyma and peripheral PLs profile changes in long-term postischemia associated with cognitive impairment. The fatty acid composition of imbalanced PLs in familiar and sporadic Alzheimer's disease in human brains has been commonly found, and in old triple transgenic AD mice with cognitive impairment, the imbalance has mainly been observed in PC and/or LPC, PE, and LPE (Villamil-Ortiz et al., 2016; Villamil-Ortiz et al., 2017).

Until now, few studies have focused on the impact of global ischemia on lipid signaling. Though some of these studies have investigated the first hours to 1 week of the acute phase of



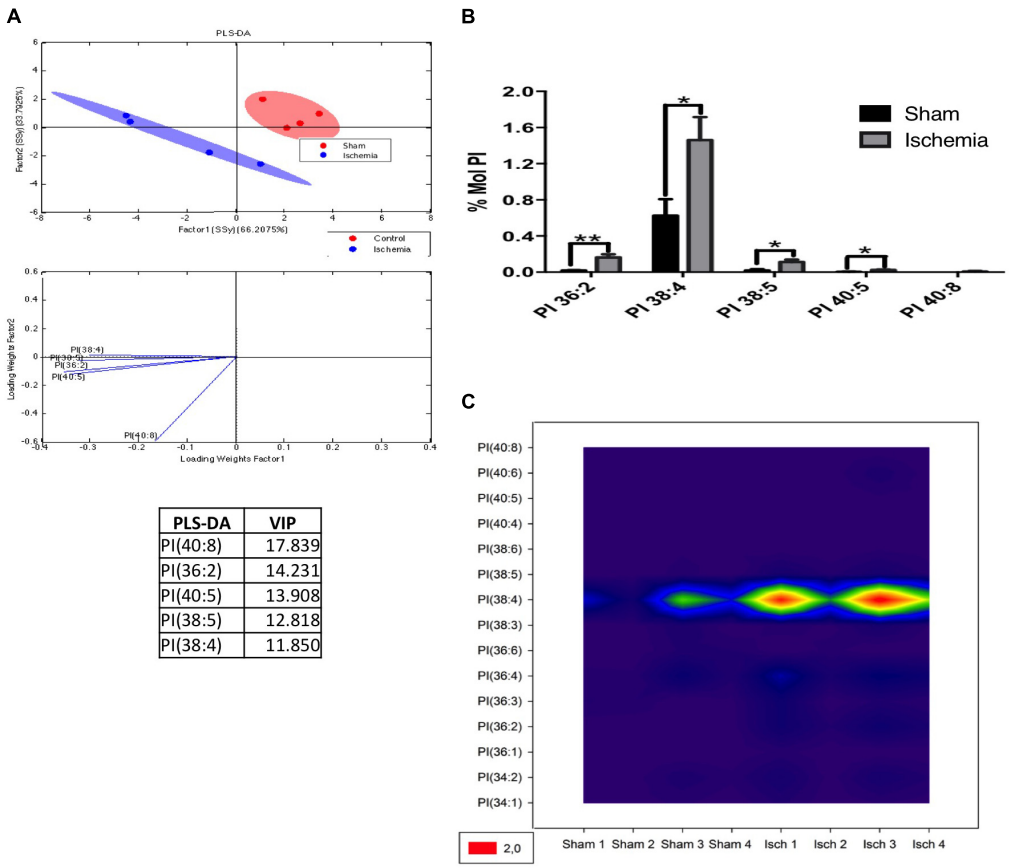
PLS-DA	VIP
LPC(18:0)	14.523
LPC(22:6)	12.250
LPC(20:5)	12.200
LPC(18:1)	11.125
LPC(20:4)	0.9739

FIGURE 9 | Altered LPC in the serum after global ischemia. **(A)** Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. **(B)** The LPC profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of LPC (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

postischemia (Adibhatla and Hatcher, 2007), few have evaluated lipid profiles in the brain or serum, and none have focused on the potential relationship of these profiles with cognitive impairment. Therefore, our data are valuable for the detection of 12 lipid species, six of which had significant concentration changes in the hippocampus and serum, specifically PC, LPE, and PS in the hippocampus and PC, LPC, ePC and PI in the serum of ischemic and cognitively impaired rats during long-term postischemia, possibly suggesting differential lipid signatures under pathological conditions due to ischemia, which has been suggested by some related neurological studies (Lin and Perez-Pinzon, 2013; Shen et al., 2014; Hamazaki et al., 2016).

Glycerophospholipids are multifunctional molecules, are the major constituents of membranes and are responsible for the membrane bilayer, "mainly via choline or ethanolamine and to a lesser extent, inositol, serine or rarely, threonine. Further diversity is introduced by the components at the sn-1 and sn-2 positions, composing subclasses of diacyl and ether GP. Although plasmalogens represent up to 20% of the total phospholipid mass in humans, their physiological roles have been challenging to identify and are likely to vary in different tissues, metabolic processes and developmental stages" (Braverman and Moser, 2012). As plasmalogens serve as storage depots for second messengers and their precursors, membrane activity and ion channels, the study of plasmalogens may also provide insight into neural membrane pathology (Farooqui and Horrocks, 2007).

In particular, PC are composed of fatty acids with polyunsaturated chains, such as 34:2, 34:3, 36:4, 36:5, and 38:3, which are composed of linoleic acid (18:2), a precursor of the biosynthesis of AA (20:4) or AA composition *per se* (Choque et al., 2014), and may be supported by the increased LPC (18:0) catabolism to PC after ischemia. Additionally, elevated LPCs have been related to pathological lipid breakdown and the state of parenchymal inflammation after ischemia as an important source of reactive oxygen species (ROS) (Adibhatla and Hatcher, 2007; Wang et al., 2010), as well as being correlated with macrophage/microglia responses and neuronal death (Nielsen et al., 2016), spatial memory dysfunction (Köfeler et al., 2010)



PLS-DA	VIP
PI(40:8)	17.839
PI(36:2)	14.231
PI(40:5)	13.908
PI(38:5)	12.818
PI(38:4)	11.850

FIGURE 10 | PI subspecies increased in the serum in postischemic rats. **(A)** Multivariate analyses: PLS-DA analysis used to discriminate between the lipid classes in the serum showing the factor score plots. **(B)** The PI profile is expressed as % mol composition. **(C)** Contour plots of the more influential subclasses of PI (variables) in the discriminant analysis for each evaluated variable. Data from the ischemic group were significantly different from those from the control group and are represented as the means ± SEM (*$p < 0.05$ Student's *t*-test for parametric data or the Mann–Whitney test for nonparametric data, $n = 4$ per group).

and its efflux and transport by ABCA7 in dementia by AD (Tomioka et al., 2017), also serving as a strategy in the forecast of ischemic stroke (Jickling and Montaner, 2015).

Furthermore, the lipid alterations found in our study suggested that phospholipid- modulating enzymes could be dysregulated in the brain, either due to increased or decreased phospholipase activity catalyzing the hydrolysis of LPC into PC. "The enzymes that catalyze the breakdown of PC to phosphatide (the phospholipase D or PLD enzymes) or to glycerophosphocholine and FFA (phospholipase A2 or PLA2 enzymes) have been directly associated with cerebral ischemia. In addition, alterations in the reaction cascades of PLD enzymes, leading to aberrant phosphatidic acid (PA) signaling, have been linked to neurodegenerative processes, with the activation of PLA2-family enzymes by β-amyloid peptide in neurons, in turn releasing secondary lipid messengers, such as AA. PLA2s also play a role in the modification of physical properties, such as the fluidity of the cellular membrane" (Whiley et al., 2014). It is accepted that, during ischemia and reperfusion, free fatty acid concentrations increase, particularly those of polyunsaturated fatty acids released from membrane PLs through activation of PLA2 (Hamazaki and Kim, 2013).

Another phospholipid that is highly involved is PS is the major acidic phospholipid in human membranes and one that constitutes 2–20% of the total phospholipid mass of adult human plasma and intracellular membranes (Van Meer et al., 2008). Hence, the presence of PS is essential for maintaining cell homeostasis, however, the fatty acid composition of PS is important, with studies of healthy human brains reporting that approximately 20–30% of the PS in human gray matter is composed of DHA (22:6), which is widely reported to be a pro-cell-survival fatty acid in the brain (Tanaka et al., 2012). Therefore, a reduction in the DHA content of PS is associated with the progression of mild cognitive impairment to dementia (Cunnane et al., 2012), possibly because the DHA in the PS conformation is essential for neuroprotection (Zhang et al., 2015). Additionally, PS synthesis may be inhibited by metabotropic glutamate receptor agonists, indicating that metabotropic glutamate receptor stimulation decreases not only the incorporation of

serine in PS but also modulates the generation of excitatory postsynaptic currents in rat cerebellar slices. In addition, in neural membrane, PS modulates long-lasting changes in learning and memory according to the membrane composition (Farooqui and Horrocks, 2007). Interestingly, in our study, the increased PS 1 month postischemia was mainly composed of polyunsaturated fatty acids, such as AA 20: 4 (36:4, 38:4, 40:4), possibly suggesting an imbalance between the DHA and AA concentrations in PS. In the context of cerebral ischemia, excess intracellular calcium (Ca_i^{2+}) activates various lipases, including (PLA2) and PLC, which breakdown both intracellular and membrane phospholipids and release AA, thereby enhancing the proinflammatory response (Wang et al., 2007).

Interestingly, we also observed an increase in different species of LPE in the hippocampus of ischemic rats. LPE can be generated from PE via a phospholipase A-type reaction (Farooqui et al., 2000). Currently, the physiological significance of LPE in the brain after global ischemia is unknown. However, increased LPE has been demonstrated in major depressive disorder (MDD) and chronic stress (Liu et al., 2016; Oliveira et al., 2016). It has been reported that LPE has a direct relationship with calcium influx, which is closely related to cell death in neurodegenerative diseases and contributes to the cognitive impairment in transgenic mice with AD (Villamil-Ortiz et al., 2017).

For its part, phosphatidylinositol (PI) is characterized by the phosphorylation of the inositol head group of phosphoinositide, with a rapid and reversible phosphorylation rate, which critically participates in signal cascades and intracellular membrane trafficking (Hammond and Balla, 2015). PI is produced in the ER where its synthesizing enzymes, namely PI synthase (PIS) and CDP-DG synthase (CDS), are located (Kim et al., 2011). Based on the fact that PI is converted to PI3P in early endosomes and PI4P in the Golgi, plasma membrane (PM), and early and late endosomes, it is assumed that PI must be present in all of these membranes, as it has an important role in signaling pathways (Hammond and Balla, 2015). However, one past article mentioned that the four-vessel occlusion model-stimulated [3H]Inositol monophosphate formation via excitatory amino acids was greatly enhanced in hippocampal slices 24 h or 7 days after reperfusion (Seren et al., 1989). Additionally, PI has been recently reported to be a predictive marker of ischemic stroke (Tu et al., 2014).

In summary, our data showed that cognitive impairment in long-term postischemia is associated with a hippocampal phospholipid signature that indicates imbalance, proinflammatory environment, excitotoxicity and cell death, as evidenced in the peripheral PL profile related to cerebrovascular disruption and proinflammatory signaling and concomitantly supporting biomarkers of neurogenerative and cognitive impairment state and progression to long-term postischemia. Finally, this study provided a frame of reference for phospholipids that could be targeted for therapeutic exploration either through pharmacological intervention or enzymatic control, as phospholipases, for example, require further evaluation. Additionally, our findings are potentially useful for improving prediction and intervention after cerebral ischemia and form the basis for a future understanding of phospholipid dysfunction in neurological pathologies and targeting for prevention.

AUTHOR CONTRIBUTIONS

AS-G designed and realized the experiments, analyzed the data, wrote the paper. JV-O analyzed the data and wrote the paper. JA-L analyzed the data and reviewed the manuscript preparation. GC-G designed, analyzed and interpreted the data, prepared the manuscript, and critical revision. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | Total changes in lipid fractions from hippocampus and serum after global ischemia. Cholesterol esters **(A,D)** triglycerides **(B,E),** free fatty, acid **(C,F)** from hippocampus and serum, respectively, from ischemic and control rats are shown. Individual concentrations of lipids are expressed as molar percentage. Myristic acid (14:0), palmitic acid (16:0), margaric acid (17:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidic acid (20:0), arachidonic acid (20:4), Erucic acid (22:1), Lignoceric acid (24:0), nervonic acid (24:1), docosahexaenoic, acid (22:6). Data represent means SEM of 4 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$; for differences between sham and control groups.

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Identification of Blood Biomarkers for Alzheimer's Disease Through Computational Prediction and Experimental Validation

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Background: Alzheimer's disease (AD) is the major cause of dementia in population aged over 65 years, accounting up to 70% dementia cases. However, validated peripheral biomarkers for AD diagnosis are not available up to present. In this study, we adopted a new strategy of combination of computational prediction and experimental validation to identify blood protein biomarkers for AD.

Methods: First, we collected tissue-based gene expression data of AD patients and healthy controls from GEO database. Second, we analyzed these data and identified differentially expressed genes for AD. Third, we applied a blood-secretory protein prediction program on these genes and predicted AD-related proteins in blood. Finally, we collected blood samples of AD patients and healthy controls to validate the potential AD biomarkers by using ELISA experiments and Western blot analyses.

Results: A total of 2754 genes were identified to express differentially in brain tissues of AD, among which 296 genes were predicted to encode AD-related blood-secretory proteins. After careful analysis and literature survey on these predicted blood-secretory proteins, ten proteins were considered as potential AD biomarkers, five of which were experimentally verified with significant change in blood samples of AD vs. controls by ELISA, including GSN, BDNF, TIMP1, VLDLR, and APLP2. ROC analyses showed that VLDLR and TIMP1 had excellent performance in distinguishing AD patients from controls (area under the curve, AUC = 0.932 and 0.903, respectively). Further validation of VLDLR and TIMP1 by Western blot analyses has confirmed the results obtained in ELISA experiments.

Conclusion: VLDLR and TIMP1 had better discriminative abilities between ADs and controls, and might serve as potential blood biomarkers for AD. To our knowledge, this is the first time to identify blood protein biomarkers for AD through combination of computational prediction and experimental validation. In addition, VLDLR was first reported here as potential blood protein biomarker for AD. Thus, our findings might provide important information for AD diagnosis and therapies.

Keywords: Alzheimer's disease, blood, protein, biomarker, computation

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INTRODUCTION

Alzheimer's disease (AD) is the major cause of dementia in population aged over 65 years, accounting up to 70% dementia cases (1). This disease is pathologically characterized with extracellular senile plaques (amyloid-β, Aβ) and intraneuronal neurofibrillary tangles (NFTs), which are the prime suspects in damaging and killing nerve cells (2). AD has become a major health problem in the world due to the lack of effective treatment. It was reported that there were approximate 48 million people worldwide affected by AD in 2015, and the number was estimated to reach 86 million by the year 2050 (3). Clearly, the increasing AD cases would load great burden on families and society, urging the physicians and scientists to find precise and effective ways to diagnose and treat this disease.

Currently, the clinical diagnosis of AD requires a series of examinations including medical history, neuropsychological assessment, and various radiological investigations (4). However, those diagnosis processes could not be used as routine examinations for AD, because they are time-consuming and largely depend on physician's experience. In order to diagnose AD objectively and accurately, researchers have used biotechnologies and bioinformatics methods to search for disease biomarkers. As cerebrospinal fluid (CSF) is affinity with brain, it is considered to contain potential biomarkers of AD pathologies. Several studies have indicated that the decreased concentration of $A\beta_{42}$ peptide and increased concentration of tau proteins in CSF of AD patients compared to controls might work as diagnostic biomarkers for AD (5, 6). While CSF collection by lumbar puncture is invasive and may lead to some side effects such as headache (7), which limits the application of these biomarkers for large-scale AD screening. Blood contains large number of disease-associated proteins and its obtaining is non-invasive, thus it becomes a good source for discovery of AD biomarkers.

Extensive researches have been done to discover plasma or serum biomarkers for AD. For example, Ray and colleagues used antibody arrays to identify an 18-panel protein signature from 120 cell-signaling proteins, which could differentiate ADs from non-demented controls and could also distinguish mild cognition impairment (MCI) patients who later progressed to AD from those unchanged or converted to other dementia (8). Liao and colleagues recognized 6 possible plasma biomarkers for AD patients by combining 2D-PAGE and LC-MS/MS methods (9). Pratico' et al disclosed that the F2-IsoPs, resulting from peroxidation of poly-unsaturated fatty acid (10), have high levels in plasma of AD and MCI patients by using GC-MS technology (11, 12). However, the identified AD biomarkers are discrepant dramatically due to the variations in research methods. Generally, discovery of blood biomarkers for disease was conducted through comparing the proteome of blood samples from disease and control. But this no-targeted method is very challenging because there are lots of proteins with relatively low abundance or with a wide range of orders of magnitude in blood, which could not all be covered by one mass spectrometer (13). As of today, there are no valid biomarkers for AD diagnosis in blood.

In this study, we conducted a combination of computational prediction and experimental validation to identify potential blood protein biomarkers for AD. We firstly analyzed previously published gene expression data of brain tissues from AD patients to identify differentially expressed genes for AD. Furthermore, we applied a blood-secretory protein prediction program on these genes to predict AD-related proteins in blood. Finally, several potential blood protein biomarkers for AD were selected and verified by enzyme-linked immunosorbent assay (ELISA) experiments and Western blot analyses on blood samples from AD patients and healthy controls. This work provides a more specific and effective way to investigate blood protein biomarkers for AD.

Abbreviations: AD, Alzheimer's disease; ROC, receiver operating characteristic; Aβ, amyloid-β; NFT, neurofibrillary tangles; CSF, cerebrospinal fluid; MCI, mild cognition impairment; ELISA, enzyme-linked immunosorbent assay; DEPs, differentially expressed probes; FDR, false discovery rate; FC, fold change; SVM, support vector machines; DSM-IV, Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition; BBB, blood-brain barrier; AUC, area under the curve; ROS, reactive oxygen species; SEM, standard errors of the means.

MATERIALS AND METHODS

The schematic diagram of the workflow in this study was given as **Figure S1**.

Gene Expression Data of Brain Tissues From AD Patients

Brain tissue-based gene expression data of AD patients were collected from GEO database (14). Two series of datasets, GSE48350 (15, 16) and GSE5281 (17), were selected for data analyses according to the criteria described as follows: first, the datasets we used for analysis are gene expression data of brain tissues from AD patients and healthy controls; second, each dataset must contain both samples of AD patients and healthy controls; third, the number of AD samples and healthy controls are no less than 10 respectively in each dataset. After analysis, we found that these two datasets meet our screening criteria, and have a relatively large number of samples for data analysis. The two datasets are all generated from the platform of Affymetrix Human Genome U133 Plus 2.0 Array, which includes 43285 probes corresponding to 21246 genes. There are 253 samples (80 ADs and 173 controls) in GSE48350, and 161 samples (87 ADs and 74 controls) in GSE5281. All CEL files of each dataset were downloaded from the database, and normalized by using Robust Multi-array Averaging (RMA) method (18) for further analysis. Detailed information about these samples can be accessed from GEO database.

Identification of Differentially Expressed Genes for AD

We first identified differentially expressed probes (DEPs), and then mapped these probes to their genes. The following procedure was used to identify DEPs for each dataset. Kolmogorov–Smirnov test (19) was used to examine whether the data come from a normal distribution. If they were from normal distribution, Student's *t*-test would be used to detect DEPs.

However, our results showed that the values of many examined probes did not fit normal distribution, Wilcoxon rank sum test (20) was applied to identify DEPs for AD with p-value < 0.05 as cutoff for significance. Additionally, Benjamini and Hochberg (21) method was used to control the false discovery rate (FDR) of the selected DEPs with q-value < 0.05 as cutoff. In order to further determine which probes were up-regulated and down-regulated in ADs, fold change (FC) was computed across samples for each probe. As a whole, probes with q-value < 0.05 and FC > 1.2 were considered up-regulated, and those with q-value < 0.05 and FC < 0.833 were down-regulated. Finally, we chose the differentially expressed probes with consistent change trend in these two datasets to map to their corresponding genes, which were considered to be differentially expressed genes for AD.

Prediction of AD-Related Blood Proteins Based on Differentially Expressed Genes

All differentially expressed genes were analyzed for prediction whether their protein products could be secreted into blood through a program developed by Juan Cui et al (22). The basic idea of this program was summarized as follows. First, human proteins that are known to be secretory proteins and can be detected in plasma/serum due to various pathological conditions were collected to form positive dataset. Second, non-blood-secretory proteins, which include proteins unrelated to secretory pathway and secreted proteins not involved in the circulatory system, were selected as negative dataset. Third, these proteins' physical and chemical properties, amino acid sequence and structural features were collected to identify what these blood-secretory proteins have in common. Fourth, a list of protein features such as signal peptides, glycosylation sites, secondary structural content, hydrophobicity and polarity measures etc. was identified due to their great power in distinguishing blood-secretory proteins from those that were deemed not. Finally, a classifier based on support vector machines (SVM) (23) was constructed to predict the blood-secretory proteins by using the positive and negative datasets and the identified protein features.

Validation of Potential Blood Protein Biomarkers of AD by ELISA Experiments

In this work, ELISA experiments were carried out on blood samples from AD patients and healthy individuals to validate the predicted blood protein biomarkers for AD. The research protocol of this study was approved by the Human Research Ethics Committee of Shenzhen University and had been performed in accordance with the ethical standards. A total of 123 subjects were enrolled in experiment from Shenzhen People's Hospital and the Eighth Affiliated Hospital of Sun Yat-sen University, including 54 AD patients and 69 healthy subjects. Informed consents were obtained from all participants in accordance with the Declaration of Helsinki prior to their inclusion in this study. All the patients were diagnosed by neuropsychiatrists in the hospital according to the criteria of Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV). The average age of the patients and controls were 74.3 (ranged from 52 to 93) and 73.9 (ranged from 53 to 94), respectively. The ratio of male to female was about 2:3. In each ELISA experiment, blood samples were selected from AD patients and age- and gender-matched healthy controls. Blood samples (5 ml) were collected using glass tubes. Serums were separated by centrifugation at 3000 g for 10 min, and then subdivided into aliquots and stored at -80°C for further use.

For ELISA experiments, commercial ELISA kits for proteins gelsolin (GSN), brain-derived neurotrophic factor (BDNF), metalloproteinase inhibitor 1 (TIMP1), pigment epithelium-derived factor (SERPINF1) and amyloid-like protein 2 (APLP2) were bought from Uscn Life Science Inc. (Wuhan, China). The catalog numbers of these ELISA kits were SEA372Hu, SEA011Hu, SEA552Hu, SEB972Hu, and SEG122Hu, respectively. Additionally, ELISA kits of inositol 1,4,5-trisphosphate receptor-interacting protein (ITPRIP), transmembrane emp24 domain-containing protein 10 (TMED10), very low-density lipoprotein receptor (VLDLR), mitogen-activated protein kinase 8 (MAPK8) and mitogen-activated protein kinase 1 (MAPK1) were bought from Sbj Biological technology Co., Ltd. (Nanjing, China) with catalog numbers of SBJ-H2157, SBJ-H2158, SBJ-H1100, SBJ-H2160, and SBJ-H2161, respectively. The concentrations of these proteins were measured under the manufacturer's instructions. The total protein concentrations of samples were determined using bicinchoninic acid (BCA) protein assay kit with product No. 23227 (Beyotime, Jiangsu, China).

Statistical Analyses for ELISA Experiments

Protein concentration of each sample detected by ELISA was normalized with its total protein concentration. For the normalized protein concentrations, G-test (24) was applied to detect the outliers for each group. Software GraphPad Prism 5 was used to visualize the normalized protein concentrations of AD samples and healthy controls. T-test was applied to make differential analysis on normalized protein concentrations of AD samples vs. controls, and then FDR (21) was employed to adjust the p-values obtained from T-test, using 0.05 as significant cutoff. Furthermore, receiver operating characteristic (ROC) curve analysis was carried out to evaluate the power of these proteins in distinguishing AD samples from healthy controls, which was generated by using package pROC on R (25, 26).

Further Validation of the Potential Protein Biomarkers of AD by Western Blot Analyses

To further validate the potential protein biomarkers of AD in blood, Western blot analyses were carried out on un-depleted serum samples of AD patients and healthy controls by specific antibodies. Total protein concentrations of these samples were measured by the BCA assay. Proteins (10 μg) were separated by SDS-PAGE on 12% polyacrylamide gels. After electrophoresis, the proteins were transferred onto 0.2 μm polyvinylidene fluoride (PVDF) membranes (Millipore, Massachusetts, USA), and the membranes were blocked with 5% nonfat-dried milk in Tris-buffered saline (TBS: 100 mM Tris, and 1.5 M NaCl, pH 7.6) for 1 h and then washed with TBS containing 0.4% (v/v) tween

20 (TBST), followed by incubation with primary antibodies (Bioss Biotechnology, Beijing, China) against VLDLR and TIMP1 overnight at 4°C and horseradish peroxidase (HRP)-conjugated secondary antibody (1:8000, Abmart Inc, Shanghai, China) for 2 h at room temperature. The membranes were washed three times each for 10 min in TBST and developed with enhanced chemiluminescence (ECL) kit (FDbio-Femto ECL kit, FDbio Science Biotech co., Ltd, Hangzhou, China). Immunoreactive signals were detected using a Kodak Image Station 4000M imaging system (Carestream Health Inc., Rochester, NY, USA). Quantitative analysis was performed on the protein bands by ImageJ analysis software (National Institutes of Health, USA). Equal amount of proteins were separated by SDS-PAGE and stained with Coomassie blue, which was used as the loading control.

Statistical Analysis for Western Blot

The data of Western blot were analyzed using the two-tailed Student's *t*-test to examine any significant differences between ADs and controls by GraphPad Prism 7 software (GraphPad Software, USA) and presented as the means ± the standard errors of the means (SEM). Differences were considered significant with p-value < 0.05.

RESULTS

Identification of Differentially Expressed Genes in the Brain Tissues of AD Patients

Two brain tissue-based gene expression datasets of AD patients were downloaded from GEO database. There were 5481 DEPs (2511 up-regulated and 2970 down-regulated) identified in GSE48350 and 12115 DEPs (4675 up-regulated and 7440 down-regulated) in GSE5281. Further comparing analysis was made on these two groups of DEPs, and 1545 probes (corresponding to 1186 genes) and 1981 probes (corresponding to 1568 genes) were found consistently up- and down-regulated in these two datasets, respectively (27). In addition, pathway enrichment analysis was conducted on these genes and showed that focal adhesion, TGF-β signaling pathway, and MAPK signaling pathway were significantly enriched by up-regulated genes, and synapse transmission, neuronal system, and calcium signaling pathway were significantly enriched by down-regulated genes [complete list shown in our previous study (27)]. These pathways are consistent with previous observations that AD is associated with neuronal damage and apoptosis, synaptic dysfunction, neuronal activity alteration, blood brain barrier dysfunction, neuro inflammation, oxidative stress, mitochondrial function and aberrant lipid metabolism (28). Therefore, these differentially expressed genes are speculated to be associated with AD pathogenesis.

Prediction of AD-Related Protein in Blood

It is well known that blood-brain barrier (BBB) controls substances exchange strictly between brain and blood. However, some evidence indicates that breakdown of BBB may account for AD occurrence or aggravation and could enhance the movement of proteins between brain and blood in either direction (29, 30). Thereby, there might be some protein biomarkers reflecting AD pathology in blood. Based on the information described above, we applied a program developed by Juan Cui et al (22) on the differentially expressed genes of AD to predict whether the corresponding proteins could be secreted into blood. Consequently, a total of 296 proteins encoded by 115 up-regulated and 181 down-regulated genes were predicted to be blood-secretory proteins, suggesting that they might be AD-related proteins in blood (**Table S1**). Some of these proteins have been previously reported as AD biomarkers, such as gelsolin (31), serotransferrin (32, 33), metalloproteinase inhibitor 1 (34), mitogen-activated protein kinase 1 (35), pigment epithelium-derived factor (36) and brain-derived neurotrophic factor (37, 38).

To gain a comprehensive understanding of these predicted AD-related blood-secretory proteins, we carried out GO enrichment analysis using DAVID (39). A variety of GO terms were enriched, including 66 biological processes, 30 cellular components and 30 molecular functions (**Table S2**). We found that the biological processes such as protein phosphorylation and microtubule-based process, cellular components like mitochondrion and neuronal cell body, and molecular functions like ATP binding and MAP kinase activity were enriched, which are all known to be involved in the development of AD. The top 10 GO terms of biological processes, cellular components and molecular functions are shown in **Figure 1**.

To further choose precise and important candidate biomarkers for AD, we manually checked the relationship between these proteins and AD through database and literature studies. First, we collected a total of 1493 AD-related genes from three databases, 1291 from GAD (40), 169 from KEGG (41), and 197 from MALACARDS (42). Generally, if genes were related with AD, their corresponding protein products were considered to be AD-related as well. Thus, 1493 proteins encoded by these AD-related genes were AD-related proteins. Second, we made literature searches and compiled 167 proteins that have been reported as potential blood biomarkers of AD. Third, we combined the AD-related proteins collected from database and literature, and obtained a total of 1590 AD-related proteins. Finally, we made a comparison analysis between these reported AD-related proteins with 296 predicted blood-secretory proteins, and found that 35 proteins were consistent in these two groups (**Table 1**).

In order to explore the relationship between these 35 proteins and AD pathology, we made a protein-protein interaction analysis through the online sever LENS (43). A network was generated, which contains the 35 AD-related proteins presented by red nodes, 4 key AD pathology related proteins (APP, APOE, PSEN1, and PSEN2) presented by blue nodes and other proteins presented by gray nodes, which connect the 35 proteins with the 4 key proteins (**Figure 2**). In the network, most proteins are connected to these 4 key proteins except PFKFB3, HMGCS1, ATAD1, and PADI2, suggesting that almost all these proteins were associated with AD pathogenesis.

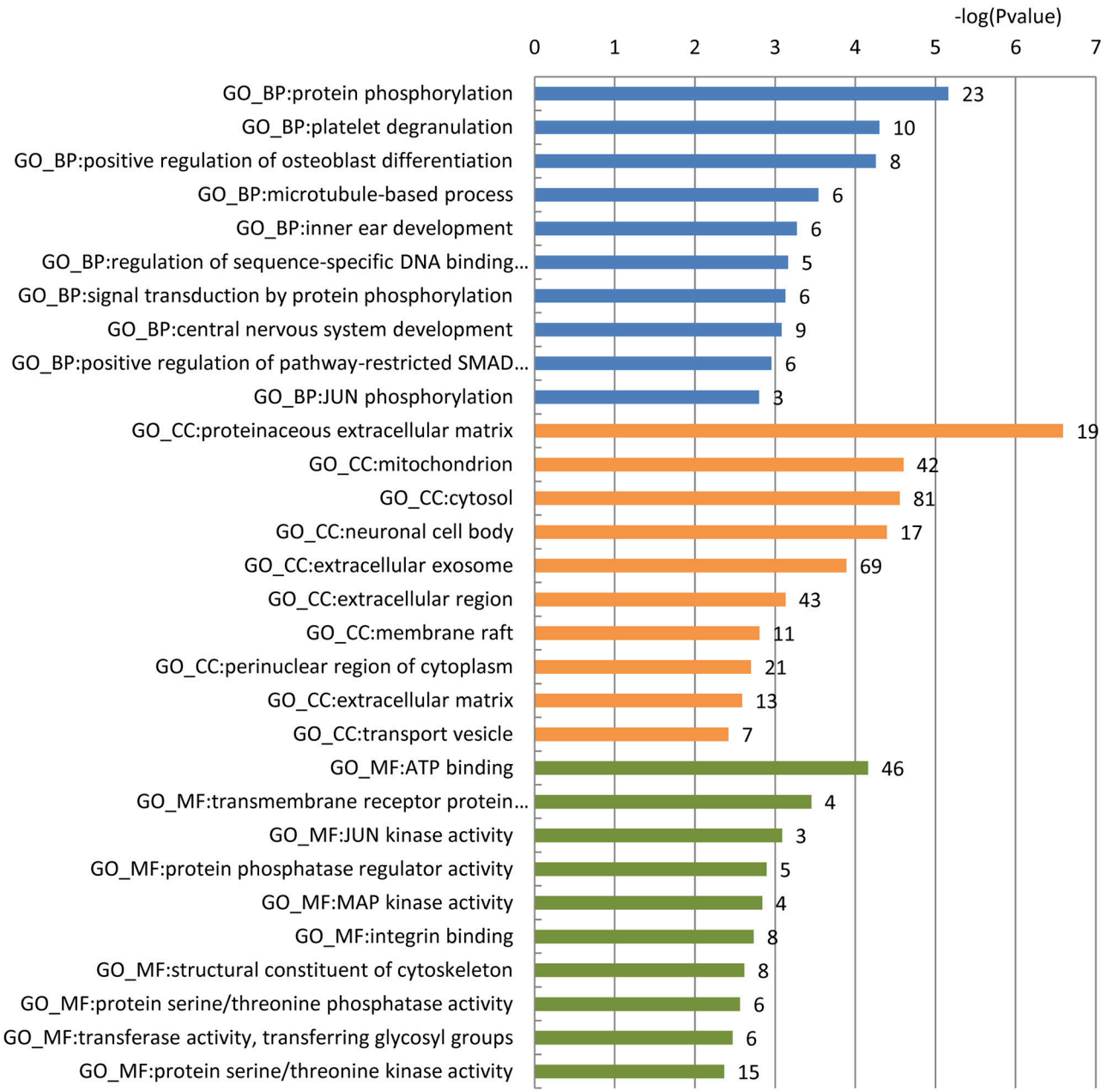

FIGURE 1 | GO enrichment analysis of the 296 predicted blood-secretory proteins. Blue, orange and green bars represent enriched biological processes, cellular components and molecular functions, respectively. The number of proteins enriched in each GO term is shown along with each bar.

Validation of Potential Protein Biomarkers of AD in Blood by ELISA Experiments

Based on the gene expression levels of these 35 proteins in AD samples and their functional annotations, 10 proteins were chosen for experimental verification. They are GSN, BDNF, TIMP1, SERPINF1, ITPRIP, TMED10, VLDLR, MAPK8, APLP2, and MAPK1.

ELISA experiments were performed to examine the protein levels in blood samples from AD patients and healthy controls. **Figure 3** shows that the expression levels of five proteins were significantly changed in AD samples vs. controls, among which GSN and TIMP1were increased in AD samples, while BDNF, VLDLR and APLP2 were decreased. Furthermore, comparison analyses were carried out on the results of computational prediction and experimental validation (**Table 2**). We found that these five proteins were consistent in their change trend among prediction and validation. In order to investigate whether age and gender would affect our validation results, further statistical

TABLE 1 | The list of 35 AD-related blood-secretory proteins.

Uniprot ID	Protein name	Gene name
P17655	Calpain-2 catalytic subunit	CAPN2
P19438	Tumor necrosis factor receptor superfamily member 1A	TNFRSF1A
P02654	Apolipoprotein C-I	APOC1
P01033	Metalloproteinase inhibitor 1	TIMP1
P02787	Serotransferrin	TF
Q15165	Serum paraoxonase/arylesterase 2	PON2
Q16875	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3	PFKFB3
Q8IWB1	inositol 1,4,5-trisphosphate receptor interacting protein	ITPRIP
Q9UQE7	Structural maintenance of chromosomes protein 3	SMC3
P25774	Cathepsin S	CTSS
P49716	CCAAT/enhancer-binding protein delta	CEBPD
Q9Y2G2	Caspase recruitment domain-containing protein 8	CARD8
P36894	Bone morphogenetic protein receptor type-1A	BMPR1A
P49755	Transmembrane emp24 domain-containing protein 10	TMED10
Q9Y2J8	Protein-arginine deiminase type-2	PADI2
P28482	Mitogen-activated protein kinase 1	MAPK1
P16298	Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform	PPP3CB
P98155	Very low-density lipoprotein receptor	VLDLR
P23560	Brain-derived neurotrophic factor	BDNF
Q00005	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	PPP2R2B
P29120	Neuroendocrine convertase 1	PCSK1
O76003	Glutaredoxin-3	GLRX3
P05019	Insulin-like growth factor I	IGF1
Q01581	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	HMGCS1
Q8NBU5	ATPase family AAA domain-containing protein 1	ATAD1
Q96FJ0	AMSH-like protease	STAMBPL1
O14975	Very long-chain acyl-CoA synthetase	SLC27A2
P02753	Retinol-binding protein 4	RBP4
P40938	Replication factor C subunit 3	RFC3
O00451	GDNF family receptor alpha-2	GFRA2
Q06481	Amyloid-like protein 2	APLP2
P45983	Mitogen-activated protein kinase 8	MAPK8
P53779	Mitogen-activated protein kinase 10	MAPK10
P06396	Gelsolin	GSN
P36955	Pigment epithelium-derived factor	SERPINF1

analyses were made on the concentrations of these five proteins according to the different age stages and genders of samples with AD and healthy controls (**Figures S2**, **S3**). We found that almost all these five proteins were significantly changed in samples of AD vs. control at different age stages and genders. Even though APLP2 is not changed with statistical significance in samples of AD vs. control at age stage 70–89, and BDNF and APLP2 are not significantly changed in male samples of AD vs. control, they still have downward trend in AD samples compared to controls, indicating that age and gender do not affect our experimental validation results.

ROC curve analyses were used to evaluate the performance of the five significantly changed proteins in distinguishing AD samples from controls (**Figure 4**). We found that VLDLR had the most discriminative ability with the area under the curve (AUC) of 0.932 (sensitivity 80.8%, specificity 96.7%), the AUC of TIMP1 was 0.903 (sensitivity 80.0%, specificity 100%) and the AUCs of GSN, BDNF and APLP2 were 0.826, 0.714, and 0.682 respectively. Since VLDLR and TIMP1 were with AUCs larger than 0.85, suggesting that they are more powerful in identifying ADs from controls, and might serve as potential protein biomarkers for AD in blood. Even though the AUCs of GSN, BDNF, and APLP2 were less than 0.85, they could also provide important information for AD diagnosis and therapies.

Further Validation of Potential Protein Biomarkers for AD by Western Blot Analyses

Based on the ELISA analyses, VLDLR and TIMP1 were chosen for further validation of their abilities in identifying the samples of AD patients by Western blot analyses. The serum samples

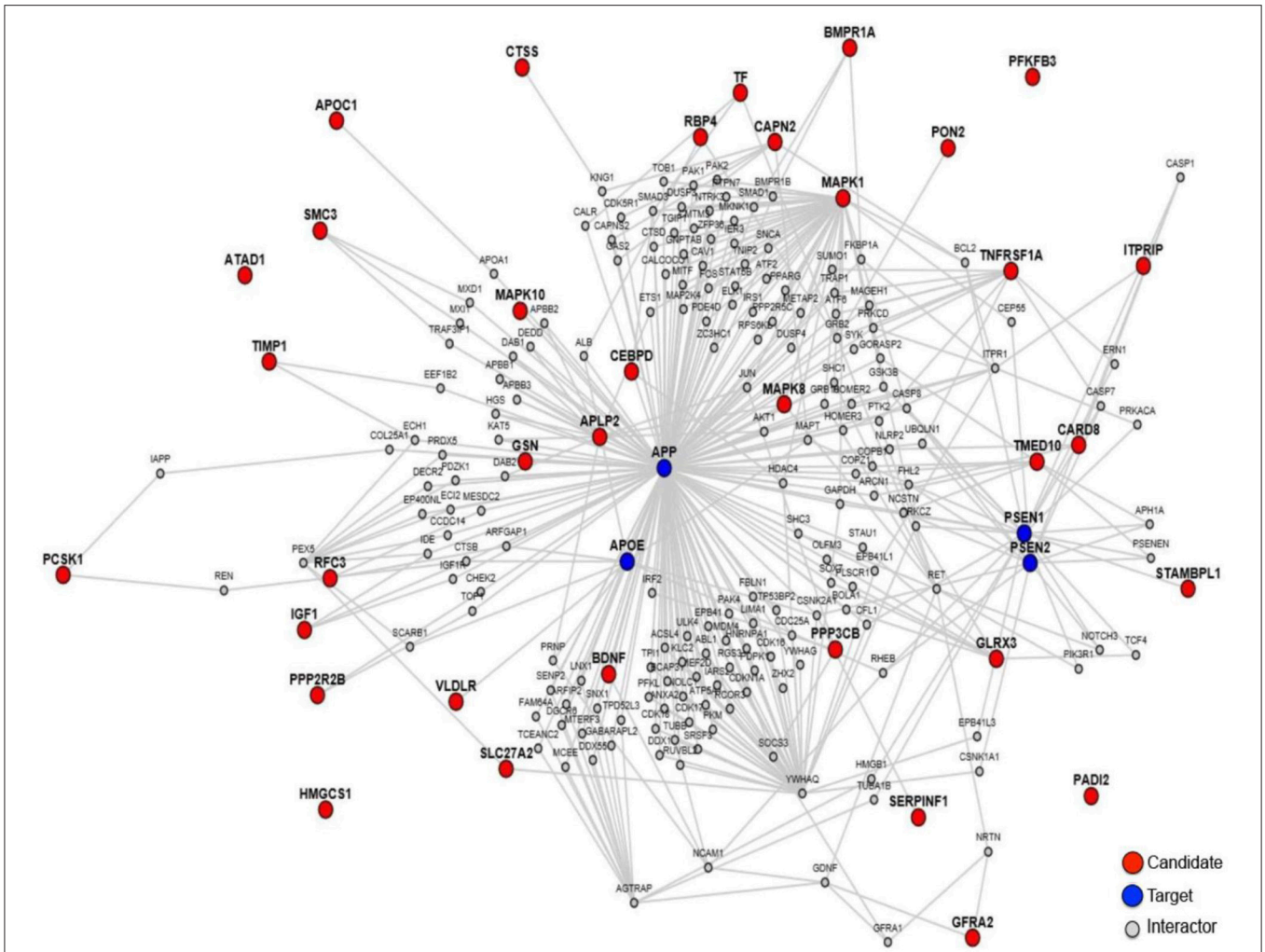

FIGURE 2 | The protein-protein interaction network of the 35 proteins. The red notes are 35 proteins worked as candidates, and the blue notes are AD pathology related proteins worked as targets.

of 5 AD patients and 5 age- and gender-matched healthy controls were used to detect the expression levels of these two proteins. After densitometry analysis on Western blots, VLDLR and TIMP1 were found down- and up-regulated in AD patients respectively as shown in **Figure 5**, which confirmed the results obtained in the ELISA experiments.

DISCUSSION

AD is the major cause of dementia. However, there are no valid biomarkers for AD diagnosis in blood so far. In this study, we searched for potential protein biomarkers of AD in blood through computational prediction combined with experimental verification. Based on this strategy, we predicted 296 AD-related blood-secretory proteins, which were predominant enriched in protein phosphorylation, microtubule-based process, mitochondria and MAP kinase activity. As widely known, AD is characterized by neurodegenerative plaques and neurofibrillary tangles in brain (44). Tau protein is microtubule-associated phosphoprotein, whose homeostasis plays a critical role in maintaining the microtubule stability. Hyperphosphorylation of tau has been confirmed to cause dynamic instability and disintegration of microtubule, and then formation of neurofibrillary tangles, which would result in neurodegeneration in the end (45). In addition, reactive oxygen species (ROS) have been reported to involve in the AD pathology mechanisms (46). Mitochondria are the most important places to generate ROS in AD. Some evidence indicated that mitochondria dysfunction in the patients of AD enhanced the oxidative stress and the cellular apoptosis (44). Since these predicted proteins were mainly involved in the processes related to AD pathogenesis (47), we considered that these proteins might be associated with AD pathology.

After careful analyses on these 296 proteins, 10 proteins were chosen for experimental validation by ELISA. Five proteins

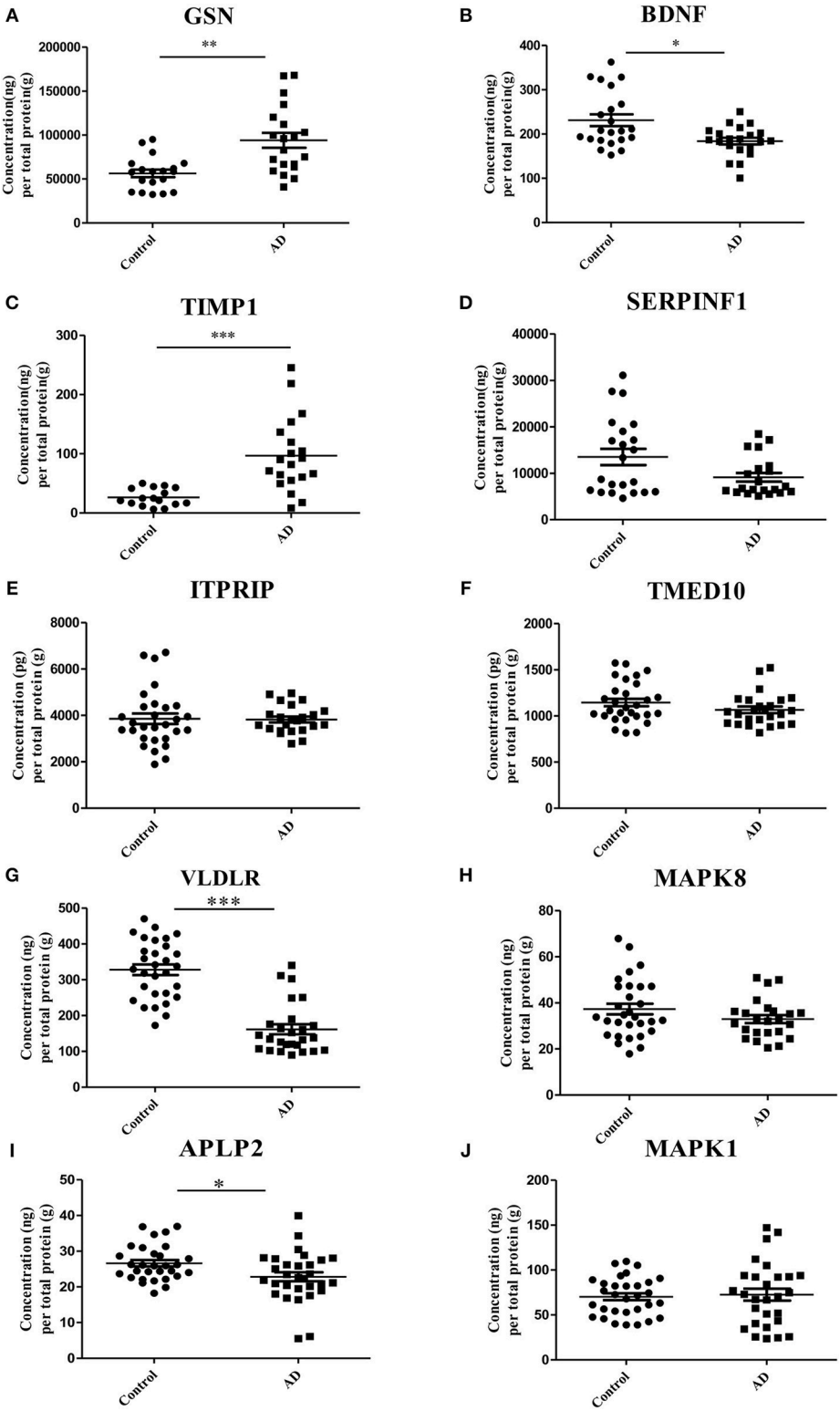

FIGURE 3 | Validation of the 10 selected proteins between AD samples and health controls by ELISA experiment. **(A)** The concentration of protein GSN in serum samples of AD and control. **(B)** The concentration of protein BDNF in serum samples of AD and control. **(C)** The concentration of protein TIMP1 in serum samples of AD and control. **(D)** The concentration of protein SERPINF1 in serum samples of AD and control. **(E)** The concentration of protein ITPRIP in serum samples of AD and control. **(F)** The concentration of protein TMED10 in serum samples of AD and control. **(G)** The concentration of protein VLDLR in serum samples of AD and control. **(H)** The concentration of protein MAPK8 in serum samples of AD and control. **(I)** The concentration of protein APLP2 in serum samples of AD and control. **(J)** The concentration of protein MAPK1 in serum samples of AD and control. $^{*}p < 0.05$ vs. controls; $^{**}p < 0.05$ vs. controls; $^{***}p < 0.0005$ vs. controls.

TABLE 2 | The results of 10 proteins in computational prediction and experimental validation.

Proteins	Computational result	Experimental result						
		Up/down	Means of protein concentrations		Means of relative protein concentrations		*P*-value	FDR
			Control	AD	Control	AD		
GSN	Up	Up	3512.06 (ng/ml)	5661.87 (ng/ml)	56470.35 (ng/g)	94026.19 (ng/g)	0.0004	0.0013
BDNF	Down	Down	15.55 (ng/ml)	12.04 (ng/ml)	231.09 (ng/g)	183.97 (ng/g)	0.0042	0.0105
TIMP1	Up	Up	1.65 (ng/ml)	5.18 (ng/ml)	26.49 (ng/g)	96.86 (ng/g)	0.0001	0.0005
SERPINF1	Down	Down	789.96 (ng/ml)	515.23 (ng/ml)	13508.58 (ng/g)	9117.03 (ng/g)	0.0345	0.0575
ITPRIP	Up	–	295.20 (pg/ml)	279.96 (pg/ml)	3855.06 (pg/g)	3825.37 (pg/g)	0.916	0.916
TMED10	Up	–	75.51 (pg/ml)	78.10 (pg/ml)	1145.62 (pg/g)	1066.62 (pg/g)	0.1542	0.1933
VLDLR	Down	Down	26.36 (ng/ml)	11.77 (ng/ml)	327.92 (ng/g)	161.63 (ng/g)	0.0001	0.0005
MAPK8	Down	–	2.48 (ng/ml)	2.41 (ng/ml)	37.33 (ng/g)	32.98 (ng/g)	0.1546	0.1933
APLP2	Down	Down	1.80 (ng/ml)	1.68 (ng/ml)	26.62 (ng/g)	22.83 (ng/g)	0.0184	0.0368
MAPK1	Down	–	5.45 (ng/ml)	5.30 (ng/ml)	70.31 (ng/g)	72.60 (ng/g)	0.7631	0.8479

In the table, up and down represent up-regulated and down-regulated proteins in the blood samples of AD patients when compared with those of controls.

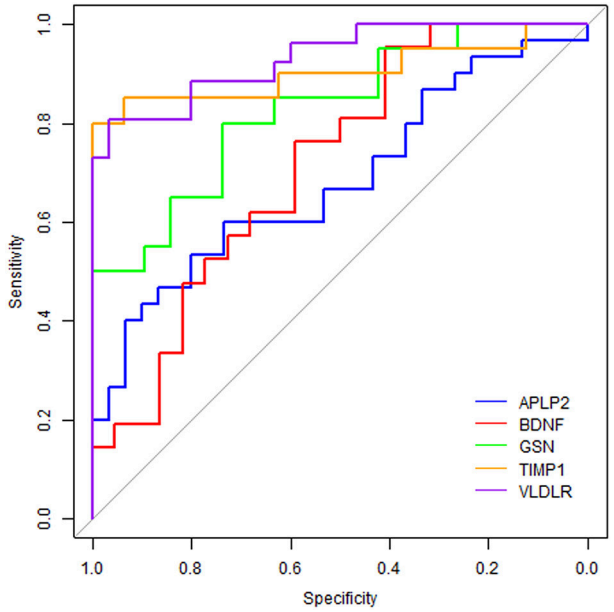

FIGURE 4 | Receiver operating characteristic curve analyses on the 5 proteins. The blue line represents protein APLP2, the red line is BDNF, the green line is GSN, the orange line is TIMP1 and the purple line is VLDLR.

(GSN, BDNF, TIMP1, VLDLR, and APLP2) were verified to be differentially expressed in AD patients vs. controls, suggesting that they might serve as potential biomarkers for AD in blood. Among them, GSN, BDNF, and TIMP1 have been reported to be potential blood protein biomarkers for AD in previous studies (34, 38, 48, 49), while VLDLR and APLP2 were first time reported here as potential protein biomarkers for AD in blood. To further understand the role of these proteins in the pathogenesis of AD, we present the relationship of these proteins with AD in details in the following parts.

GSN was reported to be implicated in AD due to its level changed with AD progression (50). GSN could bind amyloid beta (Aβ) peptide, inhibit its fibrillization, solubilize reformed Aβ fibrils, and promote its clearance from brain (51). Some studies found that the expression level of GSN was increased in serums of AD compared to controls (49), but others found the decreased expression level of GSN in plasm of AD vs. controls (48). In this study, we predicted and verified that the level of GSN was significantly higher in serums of AD comparing with controls, which was inferred that high expression level of GSN might attribute to the neuroprotective response in AD subjects through immune compensatory system.

BDNF could support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapses (52, 53). Previous studies suggested that BDNF had protective effects on neurons by reducing amyloid beta toxicity (54). BDNF depletion led to an increase in the numbers and size of the cortical amyloid plaque through analyzing on transgenic mouse model of AD (55). It has been reported that BDNF is lower in brain tissue of AD patients (54), which is consistent with our analysis. Kim BY and colleagues made a comprehensive systematic review and meta-analysis on articles and found that BDNF was increased in early AD serum samples and decreased in AD with low MMSE scores respectively comparing with healthy individuals (38). In this study, lower BDNF expression was predicted and experimentally confirmed in blood of AD patients.

TIMP1 is a tissue inhibitor of MMP9 and plays an important role in the development of AD for its function of inflammatory mediation (56). MMP9 was reported to be associated with neurodegeneration processes including extracellular Aβ degradation, neurons degeneration and neurofibrillary tangles formation (57), thus TIMP1 interacting with MMP9 promoted cell proliferation of glial and enhanced the inflammatory response to eliminate amyloid deposition from AD (56). Meanwhile, neurotoxic Aβ fragment could induce the release of MMP9 and TIMP1, and cause their expression changes, which was correlated with the neurotoxicity process (58). The imbalance of levels between MMP9 and TIMP1 in AD patients was associated with senile plaque homoeostasis and tau oligomer formation in brain regions. James D. Doecke and colleagues

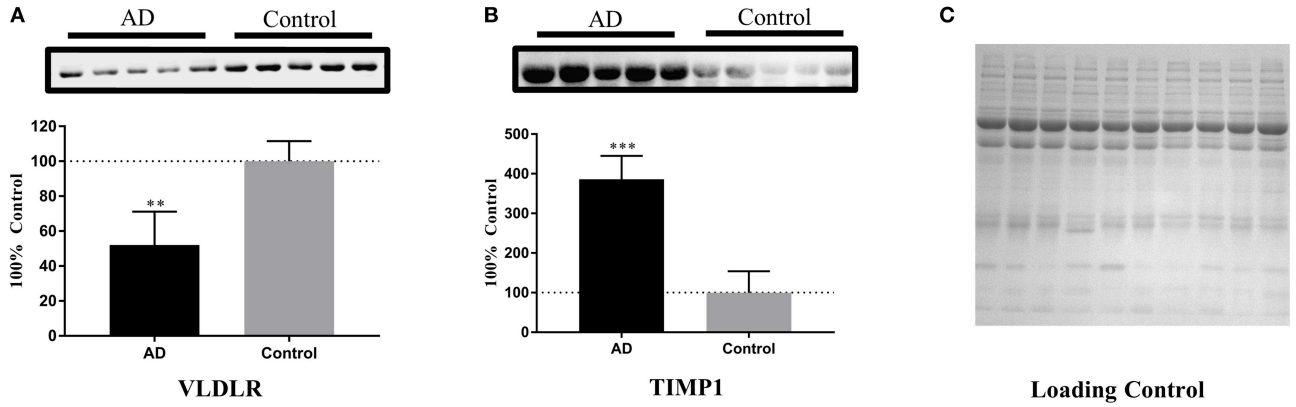

FIGURE 5 | Further validation of the potential protein biomarkers for AD by Western blot analyses. **(A)** The concentration of VLDLR was decreased significantly in AD samples, with $^{**}p < 0.01$ vs. control samples. **(B)** The concentration of TIMP1 was increased significantly in AD samples, with $^{***}p < 0.001$ vs. control samples. The expression level of protein was normalized by the mean of the controls ($n = 5$), with each bar representing SEM. The upper images of Western blot analysis correspond to the lower histograms of semi-quantification. The statistical results of the data were show as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(C)** A loading control is presented aiming to verify the normalization of protein amounts.

identified that the level of TIMP1 in plasma of AD was higher than that in healthy controls (34). However, Lorenzl S et al did not observe the level change of TIMP1 in plasma between AD patients and healthy subjects (59). Herein, we found that the level of TIMP1 was significantly up-regulated in AD serums.

VLDLR is an apolipoprotein E receptor involved in synaptic plasticity, learning, and memory (60). It was presented at synaptic compartments, and could alter presynaptic composition and postsynaptic dendritic spine formation through the Ras signaling pathway that is associated in neurodegeneration such as AD (60). Thus, it could be speculated that VLDLR might involve in AD pathogenesis through Ras signaling pathway. Additionally, VLDLR was reported to be one of receptors for AD-related risk factor ApoE (61). ApoE4 was shown to mediate its effects in AD pathogenesis by interfering with Reelin signaling in the brain (62). While Reelin is the major ligand for VLDLR, so it could be speculated that VLDLR might be involved in AD pathogenesis through the ApoE4-Reelin pathway as well. In our study, we found that VLDLR was down-regulated in the brain of AD patients and its encoded protein was predicted and validated with a lower concentration level in blood of AD patients relative to controls.

APLP2, an APP like protein, could bind to synaptic signaling molecules exhibiting synaptogenic activity (63). Furthermore, APLP2 shares essential functions with APP, as it could also interact with proteins Stub1 and CRL4 (CRBN) to facilitate ubiquitination of proteins involved in presynaptic functions and neurodegeneration (64). Herein, we predicted and validated that the encoded protein of APLP2 was down-regulated in the blood of AD patients.

As a whole, this novel biomarker discovery strategy, namely computational prediction combined with experimental verification, provides some potential blood biomarkers for AD. To our knowledge, this is the first report to use such a strategy for AD blood biomarker discovery. Meanwhile, VLDLR is the first time reported here as potential protein biomarker for AD in blood. In addition, this strategy for biomarker discovery could also be used for discovering biomarkers of other nervous system diseases such as Parkinson's disease. Worth noting, this method provides an effective way to find pathology-associated biomarkers in blood, but there are still some shortages in this strategy that could affect our results. For example, there might be some false positive blood-secretory proteins coming from the computational prediction, so the sensitivity of the blood-secretory protein predictor need to be improved in the future. Additionally, gene expression changes in ADs vs. controls could not accurately reflect their proteins' expression changes, so the predicted proteins need to be validated on large scale blood samples further.

CONCLUSION

A total of 2754 genes were identified differentially expressed in brain tissues of AD, among which 296 genes were predicted to encode blood-secretory proteins. GO enrichment analysis on the predicted blood-secretory proteins suggested that they were associated with AD and might act as candidate protein biomarkers of AD in blood. Furthermore, ten proteins were chosen for validation by ELISA and five proteins (GSN, BDNF, TIMP1, VLDLR, and APLP2) were validated changed significantly in serum samples of AD vs. controls. ROC curves analyses on these five proteins showed that VLDLR and TIMP1 were with more power in distinguishing AD samples from controls. Western blot analyses on VLDLR and TIMP1 were further revealed that they might serve as potential blood biomarkers for AD. Obviously, further studies are required to confirm these findings.

AUTHOR CONTRIBUTIONS

FY, QL, and JN conceived and designed this study. YZ collected data from database and literature. KZ and SX

designed and performed the experimental work. FY and LS processed the data and carried out the statistical analysis. YG and AL recruited and diagnosed the patients and provided the blood samples. FY and LS wrote the manuscript. All authors have read and proved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.01158/full#supplementary-material

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REVIEW
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Biomarkers in Spinal Cord Injury: Prognostic Insights and Future Potentials

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Spinal Cord Injury (SCI) is a major challenge in Neurotrauma research. Complex pathophysiological processes take place immediately after the injury and later on as the chronic injury develops. Moreover, SCI is usually accompanied by traumatic injuries because the most common modality of injury is road traffic accidents and falls. Patients develop significant permanent neurological deficits that depend on the extent and the location of the injury itself and in time they develop further neurological and body changes that may risk their mere survival. In our review, we explored the recent updates with regards to SCI biomarkers. We observed two methods that may lead to the appearance of biomarkers for SCI. First, during the first few weeks following the injury the Blood Spinal Cord Barrier (BSCB) disruption that releases several neurologic structure components from the injured tissue. These components find their way to Cerebrospinal Fluid (CSF) and the systemic circulation. Also, as the injury develops several components of the pathological process are expressed or released such as in neuroinflammation, apoptosis, reactive oxygen species, and excitotoxicity sequences. Therefore, there is a growing interest in examining any correlations between these components and the degrees or the outcomes of the injury. Additionally, some of the candidate biomarkers are theorized to track the progressive changes of SCI which offers an insight on the patients' prognoses, potential-treatments-outcomes assessment, and monitoring the progression of the complications of chronic SCI such as Pressure Ulcers and urinary dysfunction. An extensive literature review was performed covering literature, published in English, until February 2018 using the Medline/PubMed database. Experimental and human studies were included and titles, PMID, publication year, authors, biomarkers studies, the method of validation, relationship to SCI pathophysiology, and concluded correlation were reported. Potential SCI biomarkers need further validation using clinical studies. The selection of the appropriate biomarker group should be made based on the stage of the injuries, the accompanying trauma and with regards to any surgical, or medical interference that might have been done. Additionally, we suggest testing multiple biomarkers related to the several pathological changes coinciding to offer a more precise prediction of the outcome.

Keywords: spinal cord injury, biomarkers, central nervous system injury, neuroinflammation, proteomics, spinal cord injury pathophysiology

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INTRODUCTION

Spinal Cord Injury (SCI) remains one of the most devastating and difficult to manage medical pathologies despite the tremendous progress in neuroscience and neurosurgery. The National SCI Statistical Center (NSCISC) in 2016 reported more than 17,000 new cases and a total of 282,000 patients living with SCI in the USA alone. This type of injury most commonly occurs in young to middle-aged populations due to road traffic accidents, violence, and contact sports (1). The survivors of SCI are left with immediate neurological losses and a state of spinal shock that jeopardizes continued their survival (2). Unfortunately, survivors of SCI often experience a severe decrease in quality of life, as they struggle with the burden of continuous medical care to manage SCI-related complications and comorbidities.

SCI is a broad term that includes variable grades of neurological deficits. Only about 1% of SCI patients experience injuries that fully resolve. Almost 45% of cases experience severe neurological loss, in some cases with complete or incomplete tetraplegia, with or without respiratory compromise (1). However, the available tools to assess the severity of spinal cord tissue destruction and to predict recovery for SCI patients are still limited, particularly in developing countries, because they often require lavish resources to perform various imaging tests to decide the best neurosurgical intervention for each case. It is also essential to address the lack of reliable treatment interventions for such groups of patients, as most of the medical decisions are targeting the stabilization of the patients and preventing further injury, but no definitive treatment for the present state of the Central Nervous System (CNS) trauma exists (3, 4).

The progression of SCI is divided into different stages/pathophysiological phases experienced by the patient. In the first stage, termed the acute stage or primary injury, the patient not only develops neurologic deficits directly related to the injury but also suffers from spinal shock in the form of respiratory/circulatory disruption and urinary incontinence. The primary attention in this stage is dedicated to protecting patient's airways, ensuring proper respiratory function, and providing hemodynamic support. Additionally, until recently became not recommended, administering high-dose steroids was also employed in the first 8 h management postinjury in order to minimize further neurological inflammation and deficits (5, 6). Within a few days to postinjury, the patient progresses to the subacute stage or the secondary phase, where they typically recover from the spinal shock. However, the neurologic deficits, as well as the accompanying complications of the original trauma, persist. Finally, the patient advances to the chronic phase of SCI, which is dependent upon the general state of the patient, and the extent of the injury (3). During this phase, the patients may show partial neurologic recovery, maturation of adaptive mechanisms, or the onset of more delayed neurologic symptoms such as neuropathic pain, dysautonomia, urinary bladder dysfunction, musculoskeletal atrophy, lipodystrophy, and abnormal skeletal postures (7). The clinical course of SCI is noticeably related to the pathophysiology of the injury. As the injury occurs, local ischemia and edema develop, resulting in further ionic dysregulation and mitochondrial dysfunction, ultimately leading to necrotic cell death of various cell populations in Spinal Cord (SC) tissue (8). Furthermore, damage to the Blood Spinal Cord Barrier (BSCB) allows neutrophils, in the first few hours postinjury, and macrophages, on the second- and third-days postinjury, to infiltrate the spinal cord at the injury site.

Consequently, a significant neuro-inflammatory process initiates the activation of the residing microglia. These inflammatory cells cooperate to remove the necrotic remains and the released myelin products from the damaged axons (9). However, the released cytokines, chemokines, and the activated complement cascade components trigger apoptosis in some of the surviving neurons and glial cells while recruiting and stimulating astrocytes to begin the process of scar formation (8). Over time, astrocytes secrete several types of proteoglycans such as Chondroitin Sulfate Proteoglycans, NG2 proteoglycan, Phosphacan, Brevican, Versican, and Neurocan (10). During the chronic phase of SCI, these substances form a physical and chemical barrier known as the glial scar, which inhibits axon regeneration. Another significant process that develops after injury is oxidative stress. The original traumatic insult initiates oxidative stress due to cellular releases of cytoplasmic components in addition to mitochondrial dysfunction. Oxidative stress continues to persist through the secondary phase, as the neuroinflammation is still prominent (11).

In the past two decades, studying the pathophysiology of SCI in the acute and subacute phases has become a major focus among practitioners in the field in order to understand the underlying mechanisms and provide targets for therapeutic strategies that would prevent further expansion of the injury and avoid the continued loss of neuronal functions (3). Moreover, the nature of these early phases determines many potential tools to predict not only the injury severity but also neurological recovery (12). Some of the major difficulties preventing the effective management of SCI are identifying non-invasive, practical, specific, and sensitive predictive measures to diagnose the severity and treatment of SCI. In the presence of effective diagnostic and predictive tests, different treatments such as surgical intervention would be more personalized to each patient, which ensures better evidence-based practices (13). In this comprehensive review of the literature, we searched the PubMed database with keywords "Biomarkers" and "Spinal Cord Injury" and screened the results. We prioritize discussing clinically significant and reliable biomarkers that have the potential to predict recovery after SCI, distinguish an array of severities, monitor complications, and estimate neurological and non-neurological prognoses. Informative biomarkers with these characteristics are of primary interest in SCI research. There are common biological phenomena that typically account for the appearance of biomarkers in SCI patients. The first biomarker origin is from the BSCB breach, where cellular components such as proteins, phospholipids, neurotransmitters, and metabolites leak from the injury site into the Cerebrospinal Fluid (CSF) and blood. These components are derived from the SC neurons, glial cells, or are factors in the glial scar formation process. The second important source of biomarkers are products of the neuroinflammatory processes or regenerative attempts that occur in the subacute or chronic phase. These include an increase

in expression of proteins such as cytokines and growth factors (14). Moreover, there are some efforts made to profile the CSF and blood components at different timepoints postinjury. These efforts are aiming to study the metabolomics and proteomics of the spinal cord utilizing broad array techniques to increase the probability of finding sensible correlations between some of the studied components and the clinical progression of the injury (15, 16). It is noteworthy to highlight the shortage of human studies for many of the suggested potential biomarkers (13). Although animal experiments may show some significance regarding the correlation between the biomarkers and recovery status, the translation of utilizing these biomarkers for making sensitive and specific predictions might still be troublesome. These challenges can be attributed to the differences in the nature of SCI between humans and experimental animal models, as human SCIs usually present in variable severities and often in the context of polytrauma. Also, the animal spinal cord tissue has a relatively more straightforward neuronal structure and tends to have a different recovery curve following moderate to severe injuries (17). Therefore, it is always preferred to test the presence of such biomarkers in the human SCI population.

Some of the determinants of quality for studying biomarkers are the clinical tools used to follow the neurological recovery state. The American Spinal Injury Association (ASIA) Impairment Scale (AIS) is used to dictate and categorize progression of the injury (18). In addition, the timing at which the biomarkers are tested is critical, as some biomarkers are specific to certain phases of SCI and some are present in varying quantities at different timepoints postinjury. Another contributing factor to the specificity of the biomarkers is the cellular source. For example, inflammatory markers might not serve as a specific predictive tool in the presence of general inflammatory reactions in patients suffering from multiple traumas in addition to SCI, therefore some markers that serve as neuronal cytoskeletal elements such as Tau or Glial Fibrillary Acidic Protein (GFAP) may be more appealing (19). In this review, we divided our discussion into segments. The first part narrates the studies of potential biomarkers in SCI that are directly related to the injury and neurological loss, subdivided into structural and inflammatory sections. An overview of the biomarkers' studies discussed in the review and their results is shown in a summary table (**Table 1**).

The second portion reviews studies utilizing proteomics techniques to profile CSF or serum components after SCI. Lastly, we discuss the current status of biomarkers of SCI-related complications and what needs to be addressed in chronic SCI patient populations.

BIOMARKERS DIRECTLY RELATED TO TRAUMATIC SPINAL CORD INJURY

Structural Biomarkers

Neurofilament Proteins (NF)

Neurofilaments (NFs) are cytoskeletal proteins that are expressed abundantly and uniquely in the cytoplasm of axonal fibers in the CNS. NF regulates axonal transport and signaling (12), and has been a focus in neurological disorders due to the extracellular accumulations of NF in multiple neurological pathologies (45). NF can be divided into three major subunits: Neurofilament-light (NF-L), medium (NF-M), and heavy (NF-H) chains and are thought to be released from the cytoplasm of damaged neurons in traumatic SCI. Moreover, in the progression of secondary injury, as apoptosis and neuroinflammation are peaking, NF is thought to leak extracellularly along with other cytoplasmic components (45). Therefore, they are hypothesized to potentially indicate the severity of the neuronal loss in SCI as well as the extent of damage in the secondary stages.

Phosphorylated NF-H (pNF-H) is the primary subunit studied as a possible biomarker for CNS injury because of its traceable leakage into the CSF and serum following trauma (22, 24). A clinical study by Hayakawa et al. consisting of 14 patients with acute cervical SCI showed pNF-H levels in plasma correlated to injury severity. Enzyme-Linked Immunosorbent Assay (ELISA) revealed increased pNF-H was associated with increased axonal/neuronal disruption. pNF-H also successfully differentiated severe (grade A), moderate (grade B), and mild (grades C + D) cases, indicating it has the potential to accurately reflect different forms of SCI as well as severity (23).

In another study, Shaw et al. demonstrated the use of serum pNF-H as a distinguishing factor between injured and control rats. The model consisted of two injury groups; one received a contusion and the other a spinal cord hemisection injury. Both were compared to a sham group, which received only laminectomies. ELISA revealed high pNF-H levels were present only in the injury groups, while the sham groups remained negative (24).

In addition to pNF-H, NF-H has also commonly been examined as a potential SCI marker. A prospective cohort study measured CSF levels of NF-H along with other structural proteins (GFAP, neuron-specific enolase, S-100Beta, and tau) and were tested in 16 acute SCI patients within 24 h postinjury to be correlated with baseline AIS classifications and again at 6 or 12 months for further analysis of their release into the CSF. Mean NF-H levels were significantly higher in AIS A and B patients when compared to the levels in AIS C and D (20).

NF-L has also been studied as a potential biomarker for SCI. In one of the earliest clinical studies of SCI biomarkers, CSF samples were drawn from 6 acute SCI patients and were tested for NF-L and GFAP. Patients with complete motor loss showed higher levels of both biomarkers than patients with incomplete injury. In the same study, out of 17 patients with severe whiplash injuries, 3 patients had increased CSF levels of NF-L, indicating neural damage (21).

In another clinical study of patients with central cord syndrome ($n = 4$) and patients with varying degrees of motor loss SCI ($n = 23$) tested against healthy controls ($n = 67$), an Electrochemiluminescence (ECL) technique was used to quantify NF-L concentrations in serum. They found elevated levels of NF-L was associated with more severe SCI and poorer neurological prognosis (22). These results demonstrate the potential use of NFs as SCI biomarkers, despite the need for bigger sample sizes, more statistically significant correlations, and more specific clinical tools.

TABLE 1 | A summary of Spinal Cord Injury (SCI) biomarkers.

Biomarker	Paper	Sample Source	Results
NF-H	(20)	Human CSF	Mean levels were significantly higher in AIS A and B patients than in AIS C and D patients.
NF-L	(21)	Human CSF	Patients with complete motor loss showed higher levels compared to patients with incomplete motor loss.
NF-L	(22)	Human Serum	Elevated levels were associated with more severe SCI and poorer neurological prognosis.
pNF-H	(23)	Human Plasma	Increased levels were associated with increased axonal/neuronal disruption and differentiated severe, moderate, and mild SCI cases based on pNF-H concentration.
pNF-H	(24)	Rat Serum	Presence of pNF-H distinguished injured rats from non-injured rats.
pNF-H	(25)	Human Serum	Higher levels were associated with more severe SCI.
GFAP	(26)	Human CSF	At 24 h, levels of GFAP predicted future AIS graded injury severities as well as 6-month post injury segmental motor improvements.
GFAP	(21)	Human CSF	Patients with complete motor loss showed higher levels compared to patients with incomplete motor loss.
GFAP	(27)	Human CSF	Higher levels correlated with higher injury severities and predicted future neurological outcome.
GFAP	(20)	Human CSF	No significant correlations between AIS grade and injury severity.
GFAP	(25)	Human Serum	Higher levels were associated with more severe SCI.
NSE	(25)	Human Serum	Higher levels were associated with more severe SCI.
NSE	(20)	Human CSF	Mean levels were significantly higher in AIS A and B patients than in AIS C and D patients.
NSE	(28)	Rat Serum	Higher levels were associated with more severe SCI.
NSE	(29)	Rat CSF and Serum	Increased levels correlated with higher neurological defects and injury severity as well as increased in concentration in a stepwise manner to peak at 2 h postinjury.
S100-β	(29)	Rat CSF and Serum	Increased levels correlated with higher neurological defects and injury severity as well as increased in concentration in a stepwise manner to peak at 2 h postinjury.
S100-β	(20)	Human CSF	Mean levels were significantly higher in AIS A and B patients than in AIS C and D patients.
S100-β	(27)	Human CSF	Higher levels correlated with higher injury severities and predicted future neurological outcome.
S100-β	(28)	Rat Serum	No significant difference in concentrations of injured and non-injured rats.
Tau	(20)	Human CSF	No significant correlations between AIS grade and injury severity.
C-Tau	(27)	Human CSF	Higher levels correlated with higher injury severities and predicted future neurological outcome.
MAP2	(30)	Rat Dendrites	Presence of MAP2-immunoreactive dendrites extending into white matter with extensive beading patterns indicated worse behavioral recovery.
MBP	(31)	Mouse and Rat oligodendrocytes	Possible development of new myelin.
MBP	(32)	Swine CSF	Concentrations in injured swine were significantly higher than healthy controls. Injured swine MBP levels steadily increased over a 3-h period, possible indicating remyelination efforts.
MMPs	(33)	Human Serum	MMP-8 and 9 were upregulated post injury.
MMPs	(34)	Mouse and Rat CSF	Increased concentrations of MMP-8 correlated with poorer neurological recovery.
MMPs	(14)	Mouse and Rat CSF	Significant correlation between elevated MMP-9 levels and impaired neurological recovery.
MMPs	(35)	Canine CSF	MMP-9 levels 7 days postinjury were elevated in dogs that had the more severe IVDH injuries.
TGF-B	(36)	Human post-mortem spinal cord tissue	Injured tissue showed high levels of TGF-B1 two days postinjury, and TGF-B2 24 h postinjury.
TGF-B	(37)	Human Serum	An initial decrease in the concentrations of these cytokines was followed by a significant increase. 12 weeks postinjury, the observed elevated levels were correlated with the absence of neurological recovery.
IGF-1	(37)	Human Serum	An initial decrease in the concentrations of these cytokines was followed by a significant increase. 12 weeks postinjury, the observed elevated levels were correlated with the absence of neurological recovery.

(Continued)

TABLE 1 | Continued

Biomarker	Paper	Sample Source	Results
IGF-1	(38)	Human Serum	Higher concentrations were correlated with greater neurological recovery.
sCD95L	(37)	Human Serum	An initial decrease in the concentrations of these cytokines was followed by a significant increase. 12 weeks postinjury, the observed elevated levels were correlated with the absence of neurological recovery.
sCD95L	(39)	Human Serum	Concentrations during the first week significantly decreased, followed by an increase in the second week, and reached peak expression during the 4th week post injury, possibly indicating the apoptotic effect to the spinal cord tissue.
sCD95L	(40)	Human Serum	Levels dropped at 4, 9, 12, and 24 h postinjury, and increased at 8 and 12 weeks.
sCD95L	(37)	Human Blood	High concentrations proved to be a marker for poor neurological improvement based on ASIA classification.
TNF-alpha	(41)	Human Serum	Patients who experienced an improvement in AIS grade also had a significant decrease of TNF-Alpha over 12 weeks.
TNF-alpha	(27)	Human CSF and Serum	Concentration predicted the AIS grade of the patient and the neurological recovery 6 months later.
TNF-alpha	(42)	Human CSF	No significant correlations were found between serum concentrations and injury severity and ASIA classification.
TNF-alpha	(43)	Human Serum	Increased concentrations were associated with an increased risk of NP
TNF-alpha	(44)	Rat and Mouse Serum	Elevated levels of IL-1B and IL-6 were found in injured rats when compared to non-injured controls.
ILs	(44)	Rat and Mouse Serum	Elevated levels of IL-1B and IL-6 were found in injured rats when compared to non-injured controls.
ILs	(27)	Human CSF	Higher levels of IL-6 and IL-8 correlated with higher injury severities and predicted future neurological outcome.
ILs	(42)	Human Serum	No associations between elevated serum concentration and injury degree were found. Alternatively, increased levels were correlated with injury complications i.e., NP, UTI, or pressure ulcers.
ILs	(41)	Human Serum	Concentrations between 4 h and 1-week postinjury did not correlate to any improvements or declines in neurological recovery. However, between 1 and 4 weeks it showed a significant drop exclusively in patients who experienced less improvement.
MCPs	(26)	Human CSF	24 h postinjury MCP-1 levels could predict patients' ASIA grade.
MCPs	(27)	Human CSF	Significantly lower MCP-1 concentrations in the patient groups that achieved improvement vs. those who did not.

Glial Fibrillary Acidic Protein (GFAP)

GFAP is an intermediate filament protein found exclusively in the astroglia of the CNS. GFAP is responsible for the proper assembly and development of the cytoskeleton of astroglial cells, and upon injury or dysfunction, GFAP expression is upregulated by these cells (46). GFAP is an established biomarker for traumatic brain injury (TBI) and shows promise for similar applications in SCI (12, 47).

In the context of SCI, Kwon et al. studied GFAP and an array of cytokine concentrations in the CSF in a mix of 27 complete and incomplete SCI patients. The samples were taken within 72 h and analyzed using ELISA and multiplex cytokine array systems. At 24 h, levels of GFAP predicted, with 89% accuracy, future AIS graded injury severities as well as predicted 6-month postinjury segmental motor improvements (26). In a more recent study by Kwon et al. comprising of 50 acute SCI patients of varying severities scaled according to AIS, GFAP concentration in the CSF measured 24 h postinjury not only correlated with the severity of the injury but served as a predictor of future neurological outcome (27). GFAP concentrations differed significantly between patients that had improved recovery and those that did not (defined by a change in AIS grade and motor score) over the course of 6 months. An accuracy of 83% was achieved in predicting AIS outcome using linear discriminant analysis monitoring. These findings have important implications for identifying and predicting recovery in SCI patients. Another study corroborating these findings analyzed GFAP, pNF-H, and Neuron Specific Enolase (NSE) concentrations in 35 SCI patients of varying severities. GFAP serum levels were sampled at 24, 48, and 72-h timepoints postinjury and analyzed using ELISA. After 24 h, mean serum levels of GFAP in SCI patients were significantly higher than healthy control levels. Also, there was a significant variation of GFAP levels between grade A, grade B and grades C + D cases. Therefore, they concluded that GFAP at 24 h postinjury would be helpful to estimate the severity of SCI. They also attributed the drop in protein concentrations at 48 and 72 h postinjury to surgical decompression. However, if combined with neurological testing, they can offer more accurate estimates of SCI severity before spinal Computed Tomography (CT) or surgical interventions (25). In another study by Pouw et al. mentioned previously, 16 acute traumatic SCI patients' GFAP and other structural protein levels in the CSF were measured

using sandwich ELISAs within 24 h after injury and tested against admission, 6 m, and 12 m AIS classifications. All protein concentrations were significantly elevated in SCI patients when compared to healthy controls. Although other proteins such as NSE and NFH concentrations significantly correlated with motor complete or incomplete functionality, GFAP showed no statistically significant correlations in this fashion, nor with AIS grade differentiation. One possible explanation for this, besides the small sample size, could be that GFAP does not reach peak plasma levels until after 24 h postinjury, which is outside the window tested in this study (20).

Cleaved Tau (C-Tau)

Tau is a Microtubule Associated Protein (MAP) that maintains the stabilization of axons and plays a role in axonal transport (12). After the injury, C-Tau is present in high concentrations within disrupted axons, and detectable in the sera/CSF upon BSCB breach (46). Kwon et al. studied Tau in addition to GFAP in CSF of SCI patients (referenced above) and found the same correlations of higher protein concentrations in more severe cases of SCI (27). As previously mentioned, Pouw et al. performed a study on 16 acute SCI patients with ranging severities on AIS and found that although there was a tendency of tau CSF concentrations to increase with increasing severity, there were no statistically significant differences between protein concentrations and AIS grades (20). These results are in direct contrast to the study performed by Kwon et al. and doubt the reliability of Tau as a SCI biomarker, although it could be due to a small sample size possibly. However, the study also concluded patients categorized into AIS A that contained lower concentrations of tau demonstrated higher incidences of conversion to AIS B (20). These discrepancies in the literature necessitate further research efforts to properly identify tau's potential role as a SCI biomarker.

S100-β

S100-β is a calcium binding protein found in glial cells and has previously been established as a marker for brain injury (12). S100-β has a wide variety of homeostatic activities including regulation of calcium fluxes, cell proliferation and differentiation, enzymatic/metabolic activity, and stabilization of MAPs (12, 46). S100-β is another structural marker reported to potentially predict SCI recovery in an aforementioned clinical study (27). Another experiment by Low et al. using an ELISA revealed increased S100-β serum levels 6 h after injury in 30 rats that underwent a contusive SCI when compared to control rats receiving only a laminectomy (28). However, 24 h after injury there were no significant differences in S100-βconcentrations between sham and injured rats. The number of studies available on S100-βlevels in tissue, serum or CSF is insufficient, and is worthy of further investigation in both animal models and the clinical level.

Neuron Specific Enolase (NSE)

NSE is an enzyme found primarily in neuronal cytoplasm, belonging to the glycolytic enzyme enolase family. NSE is an established biomarker for ischemic brain injury and axonal deformation and is released in high concentrations following damage to axons to reestablish cellular homeostasis (48). In an experimental rat model of SCI, NSE was abundantly expressed in the spinal cord tissue at the injury site in both neurons and glial cells, especially replicating microglia and astrocytes. These results indicated a significant link between NSE and neuroinflammation and astrogliosis after SCI (49). Because of these atypically high concentrations in surrounding tissues postinjury, NSE may serve as a useful biomarker. Loy et al. (discussed above) studied NSE in addition to S100-β and found significantly increased serum levels of NSE at both 6 h and 24 h when compared to sham animals (28). In a similar experimental study, serum and CSF levels of NSE along with S100-βwere measured in a rat acute SCI model at multiple timepoints postinjury (30 min, 2, 6, 12, and 24 h). The severity of the injury models correlated with the neurological deficits found later on, and the levels of both proteins in CSF and serum significantly correlated with the severity of the injury. Interestingly, both proteins increased in CSF and serum in a timely stepwise manner immediately after the injury to peak at approximately 2 h postinjury (29). Although the data about NSE shows promising potential to serve as a biomarker for SCI severity and to predict some prognostic outcomes, according to our literature survey, clinical studies are still lacking and essential in order prove these correlations and extrapolate any predictive use of NSE.

Microtubule-Associated Protein (MAP) 2

Microtubule-Associated Protein 2 (MAP2) is a protein specific to dendrites and has been previously used as a marker for dendritic injury (46). MAP2 utilizes a tubulin-binding domain to interact with the acidic portion of the C-terminal region found on tubulin (50). Although MAP2 has been routinely used as a TBI marker, there is an overall paucity of studies testing MAP2 in SCI. One study by Zhang et al. utilized a rat contusion injury model and found a rapid loss of MAP2 within 1–6 h. On the contrary, there was some correlation between MAP2-immunoreactive dendrites extending into the white matter displaying an extensive beading pattern and the behavioral recovery in the animals (30). Similarly, there is a shortage of studies (animal and human) to test the use of MAP2 as a cytoskeletal dendritic injury marker in the early phase of the injury.

Myelin Basic Protein (MBP)

Myelin Basic Protein (MBP) is found abundantly in the white matter; forming and maintaining the structure of the compact myelin sheath (46). MBP is comprised of positively charged amino acid groups and contains 4 primary isoforms. This heterogeneity is due to separate mRNA translation events (51). MBP is primarily studied in the context of TBI and Multiple Sclerosis (MS), however, due to its critical involvement in the myelination of axons, it has promise as a SCI biomarker. In one study, Hesp et al. demonstrated in a spinal cord contusion rat and mouse injury model that 3 months postinjury, immunostaining showed localization of MBP in newly formed oligodendrocytes, suggesting the development of new myelin (31). In another study aiming to simulate behind the armor blunt trauma to the spinal cord, pigs, wearing protective body armors, were shot in the T8

vertebrae. Tissue, CSF and serum levels of MBP were measured using an ELISA, which revealed that postinjury levels of MBP in the CSF steadily increased over a 3-h period. Additionally, MBP concentrations in injured swine were significantly higher when compared to healthy controls (32). Therefore, MBP is thought to represent a potential indicator of remyelination efforts in the process of recovery after SCI.

Matrix Metalloproteinases (MMPs)

Matrix Metalloproteinases (MMPs) are 23 different isoforms, which work in tandem with neurons and glia to modulate cellular migration via the degradation of extracellular matrix (ECM) proteins. They are critical to the CNS's injury response regulation repair (52). Popular methods in the literature to quantify MMP expression are Polymerase Chain Reaction (PCR), western blots, ELISA, Immunohistochemistry (IHC), gelatin zymography, and enzyme activity assays (52). MMPs are expressed at the highest rates during the subacute phase of SCI, indicating a potential to serve as a possible biomarker for predicting the future neurological outcome (53). In a recent clinical study, Moghaddam et al. analyzed the presence of MMP-2, 8, 9, 10, and 12 in the serum of 115 patients with traumatic SCI over the course of 12 weeks. Using a High Sensitivity Cytokine Panel, they found multiple forms of MMP were upregulated, specifically MMP-8 and 9, which they concluded could be a useful indicator for recovery potential (33) . Light et al. utilized a rodent SCI model where MMP-8 levels were analyzed 12 days postinjury in the CSF using a 34-cytokine sandwich microarray. The results showed increased concentrations of MMP-8 in rodents that correlated with poor neurological recovery (34). In corroboration of this finding, Kwon et al. performed a similar rodent study evaluating CSF levels of MMP-9 and other possible biomarkers over a 7-day time course postinjury. They found a significant correlation between elevated MMP-9 levels and impaired neurological recovery (14). Another study analyzing CSF levels of MMP-9 in canines with an Intervertebral Disc Herniation (IVDH) injury model, 6 dogs with variable degrees of neurological deficits after IVDH were included. MMP-9 levels 7 days postinjury were markedly elevated in animals that had the most severe injuries and worst prognostic profile (35). These studies provide some evidence that MMPs are a good candidate for biomarkers in delayed phases after acute SCI, which may suit some clinical scenarios.

Inflammatory Biomarkers

Transforming Growth Factor Beta (TGF-B)

TGF-B is a polypeptide that regulates a wide variety of biological functions including stem cell differentiation, recovery processes, inflammation, and immune responses, and embryogenesis. The mechanism by which TGF-B acts is through transmembrane kinase receptors serine and threonine (54), as well as by astrocytic phosphorylation of Smad2. Ultimately, TGF-B hinders neurite extension, promotes astrogliosis, and accumulation of proteoglycans (55). Subunits of TGF-B, 2 and 3, are present ubiquitously in the CNS, while TGF-B1 is predominantly found in portions of the basal ganglia, cerebral cortex, choroid plexus, and meninges (56). To better understand TGF-B1 and 2 in SCI, Buss et al. analyzed the expression of these subunits post-mortem in the spinal cord of patients with SCI. When compared to healthy controls, injured tissue showed high levels of TGF-B1 2 days postinjury, and TGF-B2 24 h postinjury, which retained its abnormally high levels for 1 year. While it seems TGF-B1 and 2 play a critical role in the subacute inflammatory response, in order to verify these findings, a larger sample size is necessary (36). Additionally, a study by Schachtrup et al. identified that TGF-B is carried by blood protein fibrinogen, allowing for its easy accessibility upon upregulation (55). Ferbert et al. conducted a clinical study measuring TGF-B1, Insulin-like growth factor 1 (IGF-1), and sCD95L in the serum of 23 SCI patients. In the acute and subacute injury phase, an initial decrease in the concentrations of these cytokines was followed by a significant increase. Twelve weeks postinjury, the observed elevated levels were correlated with the absence of neurological recovery, implying the potential use of these cytokines to predict the progression of SCI (37).

Tumor Necrosis Factor (TNF)

TNF-Alpha is a proinflammatory cytokine expressed within a few hours after injury, suggesting it may be a useful indicator of SCI pathology (57). Upon injury, the BSCB is often breached, allowing for the migration of leukocytes and other cells into the spinal cord. After trauma, these cells, along with the resident microglia, secrete TNF-Alpha, which adds to the overall inflammatory stress of the injury (12). Biglari et al. measured TNF-Alpha and Interleukin 1 (IL-1) concentrations in the serum of 23 SCI patients over a 12-week period post-injury. Patients who experienced an improvement in AIS also had a significant decrease of TNF-Alpha (41). In another clinical study, Kwon et al. analyzed TNF-Alpha levels using a multiplex cytokine array system and standard ELISA in the CSF and serum of 27 patients with complete SCI. These measurements, taken at 24 h post-injury, predicted with 89% accuracy the AIS grade of the patient as well as the neurological recovery 6 months later (27).

On the contrary, Davies et al. measured TNF-Alpha in a clinical study consisting of 56 patients with SCI of different degrees of severity with matched controls. No significant correlations were found between serum concentrations and injury severity and ASIA classification (42). These results suggest that CSF samples are more reliable for predicting neurological recovery and injury severity (14, 58). However, serum concentrations were found to be useful in predicting the onset of specific complications such as neuropathic pain (NP), urinary tract infection (UTI), and pressure ulcers. TNF-alpha is one example that is suggested as a predictive tool for several long-term complications of SCI. Xu et al. examined the relationship between inflammatory markers such as TNF-Alpha and NP in 70 chronic SCI patients. They found increased concentrations of this cytokine in the serum was associated with an increased risk of NP, thereby concluding TNF-Alpha has the potential to predict chronic NP in SCI patients (43). These data need to be interpretted with caution. Cytokine levels in serum generally spike due to systemic inflammation in response to trauma, and may not be the most specific indicator of inflammation in SCI. As a result, it is recommended that they be used in

parallel with other diagnostic biomarkers to ensure accuracy (12). Interestingly, Neefkes-Zonneveld et al. performed a systematic review of the relationship between long-term physical therapy and the levels of inflammatory markers in several human studies. They found that improvement was correlated with a decrease in expression of these serum inflammatory markers (59). In another review, the levels of serum adipokines such as TNF-Alpha dropped with exercise, and was attributed to the fact that it is released by adipose tissue, concluding that exercise can help with decreasing the general systemic inflammation state in our patient population (60, 61). These results indicate the influence of factors like exercise on the serum levels of inflammatory cytokines. Therefore, CSF appears to be a better source for sampling inflammatory markers in a SCI-specific context when compared to their serum levels, as they were found much lower in comparison to their levels in the spinal cord tissue (14, 58).

Insulin-Like Growth Factor (IGF)

IGF−1, also known as Somatomedin C (SM-C), is a soluble growth factor primarily made in the liver that is expressed in myocytes, bone cells, chondrocytes, and other various tissues. It contains 70 amino acid peptide chains and has multiple traceable isoforms that can be found in blood serum (38, 62). In an *in vitro* model, IGF-1 demonstrated the ability to increase the survival of neuronal cells and impede excitotoxicity in motor neurons (62, 63). In a clinical study consisting of 45 traumatic SCI patients, IGF-1 was measured in the peripheral blood serum 1-week postinjury and revealed that higher concentrations of this growth factor were correlated with greater neurological recovery (38). In contrast, the study performed by Ferbert et al. IGF-1, TGF-Beta, and Soluble CD95 Ligand (sCD95L) levels showed a significant increase in their serum levels in patients with worse neurologic recovery 12 weeks postinjury (37). Because of the discrepancies found in the literature, there is a need for better designed human studies with bigger sample sizes to examine the fluctuation of its serum levels, possibly using its levels as an adjunct to other biomarkers to achieve the best predictive correlations with neurological recovery.

Interleukins (ILs)

Interleukins (ILs) are a family of cytokines produced by leukocytes that help regulate and stimulate immune function and growth. The proinflammatory role of ILs in SCI has been previously well-characterized in the literature. For example, Wang et al. identified Interleukin 1 beta (IL-1B) as a critical player in increasing inflammation and glial scar tissue formation in SCI (64). Hasturk et al. corroborated this finding in their study, which revealed elevated serum levels of IL-1B, IL-6, and TNF-Alpha in a rodent ischemia/reperfusion injury model when compared to controls (44).

In the study performed by Biglari et al. (previously mentioned) following the temporal changes in the serum levels of IL-1B and TNF-Alpha in 23 SCI patients, IL-1B concentrations fluctuated greatly between 4 h and 1-week post-injury. During this time period, the differences between these levels did not correlate to any improvements or declines in neurological recovery. However, between 1 and 4 weeks postinjury, IL-1B showed a significant drop exclusively in patients who experienced less improvement. Also in this study, patients received neither corticosteroids nor non-steroidal anti-inflammatory medications which offers a good chance to extrapolate their results (41). Additionally, Kwon et al. included reports on IL-6 and IL-8 in their cohort of 50 SCI patients. As previously discussed, the CSF levels of these cytokines were significantly different, and predicted with 89% accuracy patient improvements of an AIS grade over 6 months after injury compared to those that did not improve. Additionally, 6-month motor score improvement was correlated with these cytokines' levels 24 h postinjury. Specifically, IL-6 and S100B CSF levels 24 h postinjury correlated with the conversion from AIS A to B or C (27). These studies provide evidence that ILs are strong candidates for inflammation biomarkers in the context of SCI.

However, a study performed by Davies et al. analyzing IL-1B, 2, 4, and 6 levels in the serum of 56 SCI patients with varying severities demonstrated no associations between elevated ILs serum concentrations and injury degree. Alternatively, increased levels of ILs were correlated with subjects who presented complications of the injury, i.e., NP, UTI, or pressure ulcers. Although this aligns with the nature of inflammatory cytokines in the systemic circulation, the presence of these cytokines might not necessarily be indicative of injury severity, especially if the patient has progressed passed the acute/subacute phase and has entered chronic SCI stage. However, in general, these conclusions still suggest ILs as a predictor of inflammatory-related pathologies in SCI, which can result in improved treatments for these conditions (42).

Soluble CD95 Ligand (sCD95L)

sCD95L, also known as the Fas ligand, plays a critical role in the induction of the extrinsic apoptotic cascade, which is a vital portion of the pathophysiology in the subacute stages of SCI. Following cleavage of type II transmembrane protein CD95L, sCD95L is released, and binds to Fas to activate apoptotic pathways (65, 66). sCD95L regulates activation-induced cell death, and therefore plays an important role in maintaining multiple immune functions as well as in cancer stem cells survival (67). Studies have shown that after spinal cord injury, CD95 receptor production is upregulated on the oligodendrocytes and spinal cord neurons, leading to activation of the apoptotic cascades in these cells, and a further loss of spinal cord cell population after the primary trauma (68, 69). As CD95L is cleaved and released, a portion of it extravasates to the peripheral blood, facilitated by the breach in BSCB, which might explain the changes in its levels in the serum, especially at the subacute phases when apoptotic activity is more prominent (70). Also, several preclinical studies have demonstrated that CD95 deficient mice, targeting CD95 receptors, or using sCD95R intrathecally, to neutralize sCD95L in CSF, could reduce apoptosis, tissue destruction and achieve better functional recovery (71–73). These results provide a rationale for testing sCD95L serum/CSF levels as a potential biomarker in the context of SCI.

Biglari et al. conducted a pilot study in 8 SCI patients analyzing serum sCD95L levels using ELISA. Samples from the patients were collected on the 1st and 3rd days of admission and

in the 1st, 2nd, 4th, 8th, and 12th weeks after injury. The serum concentrations during the first week significantly decreased, followed by an increase in the second week, and reached peak expression during the 4th week in all 8 subjects. Due to the study's small sample size, it was difficult to draw meaningful conclusions, however, sCD95L appears to have potential to serve as a biomarker in the subacute stage of SCI, particularly, to reflect the destructive apoptotic effect to the spinal cord tissue and the subsequent neurological loss (39). As an extension, Biglari et al. confirmed serum levels of sCD95L in 23 SCI patients dropped at 4, 9, 12, and 24 h postinjury, while levels increased at 8 and 12 weeks (40). Although the study provided significant differences in the levels hours after injury vs. 8–12 weeks after injury, the study failed to present a healthy control group lacking SCI. In a following study by the same group, Ferbert et al. evaluated sCD95L levels in blood samples from 23 SCI patients and their relationship to neurological outcome based on ASIA classification. Significantly high sCD95L serum concentrations proved to be a marker for poor neurological improvement, demonstrating sCD95L's potential as a SCI biomarker (37). In summary, sCD95L has consistently shown CSF level patterns which can serve as a therapeutic target as well as a specific marker in the secondary phases of injury, particularly apoptosis (12).

Growth Factors

Although insufficient work has been invested in linking different types of CNS growth factors to SCI in the context of diagnostic measures, plenty of studies highlighting their regenerative potentials have been discussed. Some of these growth factors include Brain-derived Neurotrophic factor (BDNF), Glial cell-derived Neurotrophic Factor (GDNF), Neurotrophin-3, and Neurotrophin-4/5 (74). Moreover, Nerve Growth Factor (NGF) was investigated for its potential benefit in monitoring lower urinary tract dysfunction (mentioned later in complications section). We hypothesize that these growth factors can act as biomarkers for SCI in serum and/or CSF which should be studied in more depth, due to the therapeutic success of these growth factors in the previous literature (74).

Macrophage Inflammatory Proteins (MIPs) and Monocyte Chemoattractant Proteins (MCPs)

MCP-1 and MIP-1alpha are chemotactic cytokines (chemokines) that are expressed in the spinal cord following initiation of the secondary phase of SCI and during the start of axonal degenerative processes (75). Although the function of these chemokines in SCI is unclear, they are thought to play a vital role in apoptosis and inflammation, as well as regulate clearance of cellular debris and released myelin, which are considered some of the critical factors that affect regenerative efforts by neurons and glial cells later on McTigue et al. (76), Zhang et al. (77), Perrin et al. (78), and Ousman and David (79). In a study by Kwon et al. MCP-1 was tracked along with other cytokines in the CSF of 27 complete SCI patients with variable ASIA classifications over 72 h postinjury. Their MCP-1 levels showed severity-related elevations. Moreover, the 24 h post-injury levels could predict patients' ASIA grade with relatively high accuracy, suggesting these cytokines as a potential tool for severity prediction for acutely injured patients (26). In a subsequent study by the same group, a larger patient population of 50 SCI patients presenting with varying AIS grades was included. CSF samples at 24 h postinjury were tested for ILs, S100B, tau, GFAP, and MCP-1 and they collectively showed again a significant difference in the patient groups that achieved improvement vs. those who did not. This provides increasing evidence of the potential benefits of these proteins as biomarkers (27). However, more human studies are required that include healthy control subjects to rule out analytical errors.

SCI BIOMARKER DISCOVERY USING PROTEOMICS

With the recent emergence of new techniques to study proteome changes, proteomics is becoming an important avenue for biomarker discovery in SCI patients. Biomarker discovery using proteomics is generally done using a "shotgun" approach, where protein quantities are assessed using mass spectrometry or microarray-based techniques often combined with gel electrophoresis of tissue or CSF samples taken from SCI patients and compared to control groups (80, 81). This approach, therefore, removes bias and uncovers targets that describe the activity of various molecular pathways activated or suppressed after injury, effectively providing a temporal map of physiological response to injury that can be used to predict recovery and outcomes.

Recently, Moghieb A. and colleagues used reversed-phase liquid chromatography-tandem mass spectrometry was combined with immunoblotting and analyzed proteome changes within spinal cord segments caudal to the injury site at 24 h and 7 days following moderate or severe thoracic SCI in rats. Proteome analysis revealed upregulation of 22 proteins at both 24 h and 7 days post-SCI, as well as downregulation of 19 and 16 proteins at 24 h and 7 days, respectively. Further analysis identified 12 proteins as potential SCI markers: TF (Transferrin), FASN (Fatty acid synthase), NME1 (Nucleoside diphosphate kinase 1), STMN1 (Stathmin 1), EEF2 (Eukaryotic translation elongation factor 2), CTSD (Cathepsin D), ANXA1 (Annexin A1), ANXA2 (Annexin 2), PGM1 (Phosphoglucomutase 1), PEA15 (Phosphoprotein enriched in astrocytes 15), GOT2 (Glutamic-oxaloacetic transaminase 2), and TPI-1 (Triosephosphate isomerase 1). Out of these 12 potential biomarkers, TF, CTSD, TPI-1, and PEA15 were verified in both rat spinal cord tissue and CSF following SCI, as well as human CSF from SCI patients, therefore showing their potential use as biomarkers (82). Another study, led by Sengupta and colleagues, used difference gel electrophoresis, matrix-assisted laser desorption/ionization mass spectrometry, as well as immunoblotting, and analyzed 49 proteins from the CSF of SCI patients and identified a subset of 8 proteins of interest. Out of the 49 proteins analyzed in this study TF, Beta-2 glycoprotein I precursor, General transcription factor 2C polypeptide 5, Immunoglobulin gamma-4 chain C region, Immunoglobulin gamma-2 chain C region, and Zinc alpha 2 glycoprotein were abundant at 1–8 days post complete injury, while Haptoglobin, serum albumin precursor, and Transferrin

were abundant only following incomplete injury at 1–8 days. This group also reported reversal in Haptoglobin and Zinc alpha 2 glycoprotein expression at 15–60 days postinjury both in incomplete and complete injury (16). Effectively, this report provides an additional set of protein markers for SCI progression and recovery potential.

Finally, several reports focused on matrix molecules responsible for ECM remodeling after injury, which is a critical part of SCI response and lesion formation. Proteome analysis revealed upregulation of various danger-associated molecular patterns, or known as alarmins, involved in the inflammatory response (83). One of the upregulated proteins involved in the inflammatory response was matrix metalloproteinase 8 (MMP-8), a neutrophil collagenase, which has been shown to correlate with tissue damage and BSCB disruption at subacute timepoints after SCI (34). Using mass spectrometry Didangelos and colleagues described a set of 47 alarmins upregulated at 8 weeks following T10 contusion in rats, and Western blot analysis confirmed upregulation of Asporin, Col1a1, Dermatopontin, Mimecan, Fibromodulin, Periostin, Prolargin, Decorin, and Neurocan after injury, while NF200 and Aggrecan were downregulated in injured samples. These alarmins are responsible for the inflammatory response and were found to act via IL1 and The nuclear factor (NF)-κB transcription factor (NFκB) signaling as well as toll-like receptor-4 (TLR-4) and the receptor for advanced glycation end-products (RAGE) (83).

BIOMARKERS ROLE IN PREDICTING AND MANAGING SCI-RELATED COMPLICATIONS

In this section, we discuss biomarkers that are studied as potential predictive tools to help manage SCI-related complications such as pressure ulcers, NP, and urinary complications. The patient populations found in this section are mainly chronic SCI patients (estimated by 280,000 patients in the USA alone) with established spinal cord tissue pathology and neurological losses. Thus far, the primary attention is given to validating interventions to target neurological loss, preventing the progression of the SCI itself, stabilizing the patients, and preventing the consequent complications. We believe developing reliable biomarkers specific to certain complications is of high importance, as it will serve the chronic SCI patients and help them resume their social and daily activities, improve their quality of life, and reduce the utilization of healthcare resources.

Pressure Ulcers

Pressure ulcers are considered a severe and prevalent complication for patients with chronic SCI, as some reports estimate the lifetime risk for these individuals at approximately 85% and recurrence rate that reaches up to 91% (84, 85). Consequently, such problems form a heavy burden on healthcare resources by adding up to the cost of continuous care for these patients and worsening their quality of life (86). Pressure ulcers commonly occur in the body parts affected by SCI, most commonly ischium, due to the loss of the natural skin's vital protective properties. The risk of their occurrence increases with the duration of the injury, age, lack of care, urinary incontinence, smoking and associated medical conditions such as diabetes mellitus (87). To understand the predisposing factors of pressure ulcers, reports attribute the pathophysiology of pressure ulcers to poor wound healing and diminished expression of leukocytic adhesion molecules (88). In one study, samples were taken from chronic SCI patients, and immunostained for several adhesion molecules typically expressed on leukocytes in peripheral blood and compared to samples from healthy controls. The results demonstrated a marked reduction of fibronectin expression in the ulcers, which led to poor leukocytic adhesion and interaction at the sites of the ulcers (89). Because the goal is to find reliable and practical biomarkers to predict and help prevent ulcers, few trials have been designed to test proteins, mainly inflammation-related, as potential candidates. In one study, whole blood and serum of SCI patients with pressure ulcers were compared to samples of ulcer-free SCI patients. Patients who presented with pressure ulcers demonstrated a significant correlation between levels/counts of C-reactive Protein (CRP), Hematocrit (Hct), lymphocytes, Red Blood Cells (RBCs), White Blood Cells (WBCs), and serum proteins and the grade of the pressure ulcers (90). In another study, ulcer and plasma or urine samples were taken from 32 individuals with chronic SCI and matched to urine or plasma samples from SCI patients with no ulcers. There was a statistical correlation between Interferon (IFN)-Gamma-induced protein in plasma as well as the drop of IFN-Alpha in urine and the occurrence if the first ulcer after SCI. This suggests that the changes of IFNs might be of use to predict pressure ulcers (91). However, we think markers might not carry a significant specificity to pressure ulcers and can be altered by several other systemic inflammation factors such as the high incidence of pneumonia and urinary tract infection in our population of interest (92). Therefore, further and larger human studies are required in addition to a broader spectrum of screening for new candidates (not only the hypothesis-driven ones), with the inclusion of healthy controls and pressure ulcer-free SCI patients.

Lower Urinary Tract Dysfunction

Lastly, urinary tract dysfunction is considered one of the most impactful complications of SCI, as almost all patients suffer from some degree of urinary dysfunction postinjury. Some studies indicate about 80% of SCI patients reported urinary complaints as a major cause of reducing their quality of life (93). Such a persistent complication affects the patients' personal and social lives and is always one of the most distressing issues in health questionnaires. It also has negative psychological impacts, as it causes embarrassment, which can lead to a withdrawal from their community and the avoidance of physiotherapy and medical follow-up appointments (94). Briefly, immediately after SCI in the phase of the spinal shock, the urinary bladder becomes areflexic, causing patients to suffer from urinary retention. Following the resolution of spinal shock, lower urinary tract shows one of two main classic presentations based on the extent and the level of SCI. If the SCI is above the lumbosacral segment, it leads to loss of upper control on the sacral circuit

preserving some of the sensory fibers, and after a while, reorganization of the sacral center occurs, leading to involuntary bladder contractions known as neurogenic detrusor overactivity (NDO). In addition, when involuntary micturition reflex is initiated, the synchronization between the detrusor contraction and the sphincter relaxation is lost, leading to detrusor-sphincter dyssynergia (DSD). This presentation leads to persistent high pressure in the urinary bladder, hence long-term deterioration of renal functions (95, 96). The other presentation occurs when the SCI is in the sacral segment or below, damaging the sacral micturition center and leading to loss of control of the detrusor and sphincter muscles as well as bladder sensation. Consequently, the urinary bladder becomes atonic and patients suffer from low-pressure overflow urinary incontinence, which increases the risk of urinary tract infections, but not deterioration of renal function (97). Currently, the management of this problem is using intermittent self-catheterization to relieve the high pressure and decrease its effect on renal functions (95). Pharmacological treatments such as intravesical irrigation with antimuscarinic drugs or sphincter injections with botulinum toxin have been introduced as well as implantation for artificial urinary sphincter to control the overflow incontinence (98, 99). Such interventions could achieve a better prognosis for SCI patients, yet the follow up for lower urinary tract functions requires regular urodynamic testing, including electromyographic recording for the external sphincter and radiologic examinations. These extra examinations complicate regular health care for patients, especially when they suffer from significant functional loss, and requires high and persistent will to commit to their medical care, thus the need for an easier, more practical tool to monitor the morphological and functional changes in the lower urinary tract and make the follow-up process more bearable (93). An additional benefit of any potential biomarker for urinary dysfunction would enable researchers to evaluate the effectiveness of pharmacological and surgical interventions. One protein that has been suggested as a candidate is NGF which is primarily secreted by urinary bladder mesothelium and transported in a retrograde fashion to sacral micturition centers. A study tested urinary levels of NGF in SCI patients and compared it to control adults and found a significant elevation of NGF in the urine of SCI patients who had elevated intravesical pressure due to NDO. Also, urinary NGF/Creatinine ratio showed a difference between urinary retention states after catheterization and relief of increased pressure, which indicates the sensitivity of urinary NGF levels in regards to monitoring renal changes related to NDO (100). In another clinical study, tissue NGF from endoscopic biopsies was used as a marker for recovery of a sustained increase in the intravesical pressure and compared to urodynamic recordings in 23 patients with NDO who were treated with intravesical Botulinum-A toxin (BTX-A) injections. BTX-A induced a state of NGF deprivation in the bladder tissue that lasted for up to 3 months (101). NGF reduction was also correlated with the drop of pressure resulting from detrusor relaxation, which indicates the strong link between the prolonged high intravesical pressure states and levels of NGF, either in tissue or urine, thus suggesting its link with SCI-related lower urinary tract dysfunction and the feasibility to use it as a monitoring tool (100). Interestingly, in another study analyzing Never Growth Factor (NGF) concentrations in the urine of 37 chronic SCI patients suffering from lower urinary tract dysfunction and 10 controls, there was no correlation between NGF levels and injury-related neurogenic lower urinary dysfunction. Factors that could have affected the outcome of this study include sample size, and patient injury severities (102). Such controversy requires NGF need to be studied on a bigger scale in human cohorts at different time points in the chronic phase and to be compared to healthy control levels to prove its sensitivity as a biomarker.

CONCLUSION

For the past two decades, there has been a growing interest in developing novel, reliable, and practical tools to diagnose severity and predict the progression of SCI. The need for such tools is multidisciplinary. First, medical care decisions would become more personal and tailored to each case, which minimizes unnecessary interventions and makes patient follow-up easier. Secondly, such tools would be helpful in providing health care for SCI patients in developing countries that lack sophisticated medical resources. Third, it would substantially boost SCI research efforts at both preclinical and clinical levels. Possessing the tools to monitor specific changes related to the pathology of SCI would enable researchers to test more therapeutic strategies and increase the chances of improving the current medical practice. Although initial steps have been taken to research the use of biomarkers in SCI, additional efforts in this field are required before achieving approval from clinicians and surgeons to integrate them into everyday practice. Currently, there is a paucity of clinical studies showing high evidence for correlations between biomarkers and severity or improvement. One of the problems with studying biomarkers in CSF is the difficulty to obtain CSF sample especially from SCI patients as it is painful and is associated with a risk of epidural hematoma that may lead to further spinal cord compression.

Hypothesis-driven SCI biomarker studies are usually generated in an unintentionally biased process in which the researchers base their studies on proteins commonly reported to have major roles in the pathophysiology of SCI. This might lead to overlooking significant potential biomarkers if they are not given enough attention in the SCI literature. Thankfully, several research groups started to adopt broader approaches to quantify potential markers in CSF and serum samples using high throughput techniques to screen for the proteomic profiles at several time points after the injury. Although these studies are still sparse and often do not get translated to human trials, we think they offer great promise to uncover innovative and more specific biomarkers. We also suggest that biomarker studies put more focus on investigating postinjury correlations between the biomarkers of interest and imaging findings at later time points. Doing so may illuminate new links between structural or inflammatory proteins and MRI spinal cord tissue findings. Finally, one of the least researched areas in the field of SCI is identifying SCI-related complication biomarkers. Complications such as urinary bladder dysfunction and pressure

ulcers cause significant distress to chronic SCI patients and can negatively affect all aspects of their lifestyle. Additionally, they require consistent and exhausting measures to follow up treatments and management, yet very few studies are targeting these biomarkers that can ease the process of follow-up and prediction. Ironically, there are at least 10 times more chronic SCI patients than new SCI cases, so, we hope these large numbers of patients are incorporated when designing clinical studies for SCI-related complications, as it will benefit SCI universally.

In conclusion, the biomarkers mentioned in this review were characterized based on their purpose in the direct neurologic insult of SCI, including those related to degrees of severity, recovery trajectory, and the occurrence of complications as a side effect of the initial injury. We prioritized presenting studies that were recent, contained practical methods of biomarker level validation, comprised of human subjects, and had a large sample size. Despite the growing popularity of the SCI biomarker field, more studies are required before we can integrate these marking techniques into the universal SCI screening and diagnostic standard of care. SCI biomarkers have the potential to serve as predictive measures for injury severity and neurological progression, as well as identify and alleviate complications of SCI, ultimately resulting in betterment of quality of life for patients that suffer from SCI.

AUTHOR CONTRIBUTIONS

AA: study design, data collection, writing, and submission; AR and PS: data collection and writing; SA, EE, and NO: data collection; DS, AO, and BA: study outline, reviewing-proofreading, and formatting.

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REVIEW
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Peripheral Demyelinating Diseases: From Biology to Translational Medicine

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Demyelinating diseases represent a spectrum of disorders that impose significant burden on global economy and society. Generally, the prognosis of these diseases is poor and there is no available cure. In recent decades, research has shed some light on the biology and physiology of Schwann cells and its neuroprotective effects in the peripheral nervous system (PNS). Insults to the PNS by various infectious agents, genetic predisposition and immune-related mechanisms jeopardize Schwann cell functions and cause demyelination. To date, there are no effective and reliable biomarkers for PNS-related diseases. Here, we aim to review the following: pathogenesis of various types of peripheral demyelinating diseases such as Guillain-Barre syndrome, Chronic Inflammatory Demyelinating Polyradiculoneuropathy, Anti-Myelin Associated Glycoprotein Neuropathy, POEMS syndrome, and Charcot-Marie-Tooth disease; emerging novel biomarkers for peripheral demyelinating diseases, and Schwann cell associated markers for demyelination.

Keywords: peripheral demyelinating disease, schwann cell, biomarker, Guillain-Barre syndrome, chronic inflammatory demyelinating polyradiculoneuropathy, anti-MAG neuropathy, POEMS syndrome, Charcot-Marie-Tooth disease

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INTRODUCTION

Peripheral demyelinating diseases (PDD) refer to a spectrum of disorders that involves substantial damage to axons and glial cells, particularly Schwann cells (SC) in the peripheral nervous system (PNS) (1). The incidence of these diseases is variable (2–4). Disease states are manifestations of damage against the myelin sheath caused by various inciting factors, such as infectious agents, auto-immune processes or genetic mutations (1, 5–7). Oxidative stress, the primary risk factor in many diseases (8), has also been implicated in demyelination disorders (9).

Schwann cells are principal glial cells in peripheral nerves that originate from the neural crest, which is a multipotent embryonic structure that also differentiates into other main glial subtypes of the PNS (10). SC development occurs through a series of embryonic and postnatal phases, which are tightly regulated by a number of cellular signaling pathways. During the early embryonic phase, neural crest cells differentiate into SC precursors that represent the first transitional stage in the SC lineage, that subsequently further differentiate into immature SC (10). At time of birth, these immature SC differentiate into either myelinating or non- myelinating SC that populates the mature nerve trunks and wrap around axons through a process known as myelination (10).

Myelination is a process whereby SC develops a multi-layered membrane called the myelin sheath around the axonal membrane (11). Mostly, larger axons (>1 um) are selected specifically by SC to form multiple internodes of the myelin sheath (12). Myelination begins with the establishment of a 1:1 relationship with the axon. At this level, the production of myelin structural proteins such as myelin protein zero (P0), peripheral myelin protein (PMP22), myelin basic protein (MBP) are increased along with lipid biosynthesis (11).

The myelin sheath is made of multiple sleeves of whitish lipoprotein plasma membranes of SC wrapped around the axon of a neuron in a spiral fashion (13). It is constituted of water, lipid and proteins that exist as segmented internodal structure around the axons (13). These internodes create insulation that facilitates propagation of action potentials by mean of saltatory conduction (jumping) at the node of Ranvier. Myelin sheath not only facilitates the conduction velocity of nerve impulse but also confers protection and nutritional support to axons. However, exposure to various factors such as autoimmunological insult, trauma, and injury to the nerve could trigger demyelination, and eventually neurodegeneration (14).

PNS DEMYELINATING DISEASES

Demyelination describes the loss of the myelin sheath, where SC are being destroyed or unwrapped from axons (15). Demyelination causes neurological disability due to conduction block and axonal degeneration. Diagnosis of PDD depends on electrophysiological and cerebrospinal fluid (CSF) analysis. However, in some cases, no biomarkers are clinically available for diagnosis, disease monitoring and prognosis.

Acquired Demyelinating Disease

Guillain-Barre Syndrome

Guillain-Barre Syndrome (GBS) is an acute idiopathic autoimmune demyelinating disease of the PNS that is characterized by acute flaccid ascending neuromuscular paralysis (16). GBS is rare, with an incidence of 0.8–1.9/100,000 per annum across Europe and North America (17). Currently, the specific causative agent of GBS is unknown, but numerous theories have been proposed (18). Most cases of GBS are preceded by antecedent infections of several microbes of the gastrointestinal and upper respiratory tracts (19). Among those, 60% of GBS cases were related to autoantibodies, anti-monosialotetrahexosylganglioside- 1 (anti-GM1) and anti-ganglioside GD1a (anti-GD1a) associated with C. *jejuni* infection (20). Other microbes involved include M. *pneumoniae*, cytomegalovirus, Epstein-Barr virus, varicella zoster virus, and influenza virus (21–24). Apart from infections, some GBS cases are results of trauma, surgical interventions, treatment with monoclonal antibodies and vaccination (rare) (20).

The most frequent GBS variant is acute inflammatory demyelinating polyradiculopathy (AIDP); other axonal variants include acute motor axonal neuropathy (AMAN), acute motor sensory axonal neuropathy (AMSAN), Miller-Fisher syndrome and oropharyngeal weakness (25, 26). In AIDP, there are areas of segmental demyelination with inflammatory infiltrates such as lymphocytes and macrophages (27). Due to the observation of a high incidence of GBS after a preceding infection, it has been theorized that molecular mimicry plays a role in triggering an autoimmune response against peripheral nerve tissues. The presence of anti-GM1 and anti-GD1a antibodies in the serum suggest that ganglioside-like moieties carried by lipooligosaccharides found in the bacterial wall of C. *jejuni* has cross-reactivity against neural tissues of the PNS (5) (**Figure 1**). It has also been found that patients treated with gangliosides for pain and neuropathy in the early 1990s later developed GBS (28). Gangliosides, axo-glial junctional proteins, neurofascin and gliomedin at nodes of Ranvier could contribute toward the autoimmunity seen in GBS (29).

The clinical manifestations of GBS include acute ascending fairly symmetric paralysis and paresthesia, choking and difficulty in breathing over the course of hours to several days (2). Involvement of the respiratory muscles in GBS may require the need for artificial ventilation (30). Some patients also experienced autonomic dysfunctions such as cardiac arrhythmia, arterial hypotension, gastrointestinal dysmotility, urinary retention, and abnormal sweating (31). Management of GBS is mostly supportive (20). Affected patients would require comprehensive assisted respiratory ventilation with monitoring for cardiac arrhythmia and bed-bound complications such as ventilator-associated pneumonia, thromboembolism and infections (32). Plasma exchange and intravenous immunoglobulin (IVIG) have been shown in large randomized trials to be beneficial (33). Overall, most cases of GBS have good prognosis with functional recovery within 12 months after disease onset (34). However, some patients do suffer from residual deficits (35).

Chronic Inflammatory Demyelinating Polyradiculoneuropathy

Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is an acquired immune mediated demyelinating disease of the PNS characterized by progressive loss of motor and sensory functions (36). CIDP sometimes is quite similar to GBS, with the

Abbreviations: PDD, Peripheral Demyelinating Disease; SC, Schwann Cell; PNS, Peripheral Nervous System; CNS, Central Nervous System; P0, Protein Zero; PMP22, Peripheral Myelin Protein 22; MBP, Myelin basic protein; CSF, Cerebrospinal fluid; GBS, Guillain Barre Syndrome; GM-1, Monosialotetrahexosylganglioside-1; GD1a, Ganglioside GD1a; EBV, Epstein Barr virus. AIDP, Acute inflammatory demyelinating polyradiculopathy; AMAN, Acute motor axonal neuropathy; AMSAN, Acute motor sensory axonal neuropathy; LOS, Lipooligosaccharides; IVIG, Intravenous immunoglobulin; CIDP, Chronic inflammatory demyelinating polyradiculoneuropathy; NF155, Neurofascin-155; CNTN1, Contactin-1; MAG, Myelin Associated Glycoprotein; VEGF, Vascular endothelial growth factor; CMT, Charcot Marie Tooth; GDAP1, Ganglioside-induced differentiation-associated protein 1; MFN2, Mitofusin-2; GQ1b, Gangliosides Q1b; NLR, Neutrophil-lymphocyte ratio; PLR, Platelet-lymphocyte ratio; MRC, Medical Research Council; MLR, Monocyte-lymphocyte ratio; gAChR, Ganglionic nicotinic acetylcholine receptor; CNTN1/CASPR1, Contactin-1/contactin-associated protein-1; NCAM, Neuronal cell adhesion molecule; NavB1/2, Sodium channel at node of Ranvier ½; SGPG, Sulfoglucuronyl glycosphingolipid; ELISA, Enzyme-linked immunosorbent assay; P2, Protein 2; PC, phosphatidylcholine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; MDL, Major denseline; PDN, Peripheral Demyelinating neuropathy; PN, Polyneuropathy; GJB1, Gap junction beta 1.

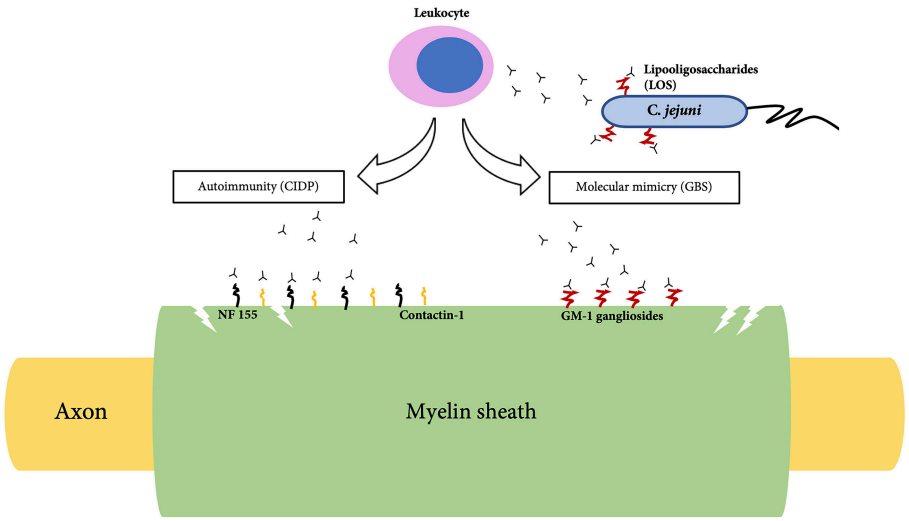

FIGURE 1 | Pathogenesis of Guillain-Barre syndrome (GBS) and Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).

distinction that its clinical course is chronic with relapses (37). The onset is insidious and occurs more commonly in older age individuals (38, 39).

The immune system primarily attacks and damages the myelin sheath of the PNS followed by segmental demyelination and axonal degeneration (6). Histological findings of CIDP demonstrate thin myelin sheath with short internodes described as onion bulbs. Demyelination is indicated by the slow nerve conduction velocity suggestive of conduction block (6). Recently evidence of autoimmunity toward neurofascin-155 (NF155) and contactin-1 (CNTN1) in some patients have been reported. (40, 41) (**Figure 1**). NF155 is an adhesion molecule that is expressed at paranodes of glial side which interacts with CNTN1, a key axonal adhesion molecule (42). This interaction is essential for the formation of paranodal septate-like junction and loss of this junction is associated with slow conduction (42).

Symptoms of CIDP develop slowly but progressive and neurological deficits peak after 8 weeks of disease onset (36). Typical symptoms are tingling/numbness of the extremities due to the association of large nerve fibers, symmetrical weakness and paresthesia of legs and arms, loss of reflex, fatigue, ataxia and limb incoordination (6). Treatment with oral glucocorticoids usually produce a favorable response (43). Apart from that, plasmapheresis and IVIG are also effective (36).

Anti-Myelin Associated Glycoprotein (MAG) Neuropathy

Anti-Myelin Associated Glycoprotein (MAG) neuropathy is a demyelinating polyneuropathy associated with IgM monoclonal gammopathy towards MAG in peripheral nerves (44). MAG is a type I transmembrane glycoprotein l presents in peri-axonal SC and oligodendroglial membranes of myelin sheaths that central in glial-axon interaction and maintenance of axonal function (45). Loss of MAG compromises the myelin sheath integrity and axonal function. MAG contains a carbohydrate epitope shared with other glycoconjugates that serve as primary antigenic targets for IgM paraproteins (44). Injection of serum containing IgM anti-MAG paraproteins into chickens causes segmental demyelination and conduction block (46).

The disease is also described as progressive mild to moderate distal muscle weakness; along with progressive sensory ataxia and frequent tremors (47). The clinical course is generally benign, with minimal functional deterioration manifested over time (47). As the symptoms of anti-MAG neuropathy usually are minimal and do not interfere with the patient's daily activities initially; management at this stage comprises of supportive care such as exercise and balance training. However, patients with sensorimotor weakness should be treated. Steroids, IVIG and plasmapheresis are rarely effective. Rituximab, a monoclonal antibody against CD20 surface antigen is promising (48).

POEMS Syndrome

POEMS syndrome is a rare paraneoplastic syndrome with demyelinating neuropathy (49). Emprical data on POEMS syndrome is deficient owing to the complexity and multisystemic nature of its clinical manifestations. It is usually associated with an underlying plasma cell neoplasm (50). POEMS syndrome commonly presents in the fifth to sixth decade (49). The pathogenesis of POEMS syndrome is not well understood, but several hypotheses have been proposed. High serum level of vascular endothelial growth factor (VEGF) is detected in POEMS patients, whereas low levels are often reported upon successful treatment (50). The pathological assessment does not reveal inflammatory infiltrates or immunoglobulin deposition within the nerves; instead, there is endothelial cell hypertrophy with extended process, reduced luminal diameter, and disrupted tight junction that could cause leakage (51). Excessive VEGF secreted by plasma cells is thought to cause endothelial proliferation and subsequent leaky vessels that compromise blood flow (51).

POEMS is an acronym of its multiorgan features: Polyneuropathy, Organomegaly, Endocrinopathy, M protein, and Skin changes. The polyneuropathy involves both sensory and motor systems (52). Patients usually begin to experience sensory abnormalities described as tingling, paresthesia and coldness in the feet, along with touch, pressure, and proprioception disturbances (52). Motor symptoms then develop, including symmetrical severe weakness on extremities progressing distally with gradual spreading to proximal (52). Nerve conduction studies, as well as nerve biopsies show evidence of demyelination and axonal loss (49). Hepatomegaly is commonly reported, but splenomegaly and lymphadenopathy are not frequent. Endocrinopathy, usually gonadal dysfunction is noted by testicular atrophy and gynecomastia and diabetes mellitus. The M-protein of IgG or IgA is commonly detected. Skin changes include hyperpigmentation and hypertrichosis. Other additional features that are not included in the acronym are sometimes present. These include peripheral edema, effusion in body cavities such as ascites, sclerotic bone lesions, Castleman's disease, elevated intracranial pressure, papilledema, fatigue, renal failure and clubbing (50). However, not all features are necessarily required for the diagnosis.

Treatment with high dose chemotherapy and autologous peripheral blood stem cell transplant are the first line therapy. Alternative therapeutics are including corticosteroids, low-dose alkylator therapy and radiation therapy. The median survival for patients with POEMS syndrome is about 13.8 years. Mortality usually results from cardiorespiratory failure, infection and renal failure. Supportive care such as physical and occupational therapy should be in line with the treatment to improve outcome and quality of life. Some patients might even require assisted ventilation due to respiratory muscle weakness (49).

Inherited Demyelinating Disease

Charcot Marie Tooth Disease

Charcot Marie Tooth disease (CMT) is a rare hereditary neurological disorder affecting the peripheral nerves (7). Although CMT is rare, it is the most commonly inherited form of neuropathy affecting approximately 1 in 2,500 people (53). The majority of CMT have an autosomal dominant inheritance but X-linked and autosomal recessive pattern also exist (54). The gene abnormalities in CMT disrupt the structure and functions of Schwann cell and peripheral nerve axons. Several subtypes of CMT have been identified: mutations of genes encoding myelin-related proteins such as PMP22, P0, and connexin 32 are classified as demyelinating subtype (54); mutations of proteins involved in axonal transport such as mitofusin-2(MFN2), ganglioside-induced differentiation- associated protein 1 (GDAP1), heat shock factor binding protein 1 are classified as axonal neuropathies subtype (54). Next-generation gene sequencing involving 17,000 samples with neuropathy identified the prevalence of specific mutations as such: 78.6% involve PMP22 mutations, 6.7% involve GJB1, 5.3% involve P0, and 4.3% involve MFN2 (55).

The most common form of CMT, CMT1A is a result of duplications of the PMP22 gene that increased expression of PMP22 structural protein (56). CMT1B on the other hand, involves mutations of P0 gene that result in misfolding and retention of a mutant P0 protein intracellularly (54). This condition triggers the activation of unfolded protein response which later lead to cell apoptosis (57). CMT2A involve mutations of MFN2 which aids in the fusion of mitochondria (54, 58). Typically, the progression of CMT is slow. The neuropathy of CMT could affect both motor and sensory nerves. Patients may experience distal muscle weakness, foot drop that formed pes cavus, scoliosis and hammer toes. Respiratory insufficiency is rare, but possible. Neuropathic pain and fatigue have been reported in several cases.

At present, the management of PDD is central upon synthetic drugs and natural products (59). However, this disease remains underdiagnosed owing to lack of reliable biomarkers and a disease specific-diagnostic criteria.

Table 1 summarized the pathogenesis, clinical features and management of PDD.

EMERGING BIOMARKERS OF PERIPHERAL DEMYELINATING DISEASE

According to the National Institute of Health Biomarkers Definitions Working Group, biological marker (biomarker) is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" (60). Biomarkers serve as an important clinical tool in disease diagnosis, therapeutic response and prognosis (60).

To identify the recent progress in biomarkers of PDD, we have conducted a literature search from SCOPUS search engine and database on biomarkers of distinct PDD from the year 2014–2018. Only original articles and in the English language were selected to be included in the review (**Table 2**).

Biomarkers of GBS

Traditionally, the diagnosis of GBS has relied on clinical features such as electrodiagnostic studies and CSF analysis. Testing for serum IgG antibodies to gangliosides Q1b (GQ1b) is available and useful for the diagnosis of GBS variants (Miller Fisher syndrome) with 85 to 90 percent sensitivity although it is not routinely indicated[1]. Biomarkers for GBS have negligible clinical value, low sensitivity/specificity and costly. Furthermore, the laboratory measurement standard for these biomarkers also has not been established and studies using different methods have been plagued by inconsistent findings (71).

At present, novel biomarkers are being explored for better prognosis of GBS patients. The likes of neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) has received much attention as novel prognostic biomarkers of inflammation. Analysis of NLR and PLR levels of 62 GBS patients prior to- and following intravenous immunoglobulin treatment (IVIG) revealed NLR as a better prediction tool for the acute period

[1] Francine J Vriesendorp, MD Guillain-Barré syndrome in adults: Clinical features and diagnosis Post TW, ed. UpToDate. Waltham, MA: UpToDate Inc. http://www.uptodate.com (Accessed on May 09, 2018).

TABLE 1 | Pathogenesis, clinical features and management of various types of Peripheral Demyelinating diseases.

	Etiology	Risk factors	Pathology	Clinical features	Management
ACQUIRED DEMYELINATING DISEASE					
Guillain-Barre syndrome	Unknown	Antecedent infections: C. jejuni, M. pneumoniae, cytomegalovirus, Epstein-Barr virus, varicella zoster virus Trauma Surgery Vaccination	Segmental demyelination Inflammatory (macrophage, lymphocyte) infiltrates	Acute ascending symmetric paralysis, paresthesia, choking, difficulty in breathing, autonomic dysfunctions (hours to several days)	Supportive therapy Plasma exchange IVIG
Chronic inflammatory demyelinating polyradiculoneuropathy	Unknown	Autoimmunity	Segmental demyelination Thin myelin sheath Onion bulb (short internodes) formation Perivascular inflammatory infiltrates	Slow, progressive neurological deficits such as tingling, numbness, symmetrical weakness of limbs, paresthesia of limbs, loss of reflex, ataxia, limb incoordination. (slow, progressive)	Glucocorticoids Plasmapheresis IVIG
Anti-Myelin Associated Glycoprotein (MAG) neuropathy	MAG IgM monoclonal gammopathy		Segmental demyelination, Immunoglobulin deposits	Benign, minimal distal muscle weakness, progressive sensory ataxia, tremors. (several years)	Supportive therapy (exercise, balance training) Corticosteroids, IVIG, Plasmapheresis (rarely effective)
POEMS syndrome	Unknown (paraneoplastic syndrome)	Plasma cell neoplasm	Endothelial cell hypertrophy with disrupted tight junction No inflammatory infiltrates No immunoglobulin deposition	Polyneuropathy (paresthesia, motor weakness, sensory disturbance), organomegaly (hepatomegaly, lymphadenopathy), endocrinopathy (testicular atrophy, gynecomastia), paraproteinemia (M-protein), skin changes (hyperpigmentation, hypertrchosis), peripheral edema	High dose chemotherapy Stem cell transplant Corticosteroids Alkylator therapy Radiation therapy Supportive therapy
INHERITED DEMYELINATING DISEASE					
Charcot Marie Tooth disease	Mutations of genes (PMP22, P0, connexin 32, mitofusin-2, etc.)		Segmental demyelination Onion bulb formation	Distal muscle weakness, foot drop, scoliosis, hammer toes, neuropathic pain, fatigue, sensory disturbance (slow, not progressive)	Supportive therapy (exercise, muscle training, balancing)

of AIDP (major variants of GBS) with 83% sensitivity and 93% specificity. Whereas, PLR only showed 74% sensitivity and 70% specificity (61). NLR also studied for its correlation with the degree of weakness in several muscles assessed through the Medical Research Council (MRC) score (62). High levels of NLR were seen in the lower MRC score upon admission and increased baseline disability among GBS patients (62).

More recent findings indicate monocyte-lymphocyte ratio (MLR) along NLR as a better prognostic marker for GBS (63). NLR and MLR are significantly higher in GBS patients compared to healthy controls. Moreover, NLR and MLR are found to be tremendously increased in severe group (63). On the other hand, Piccolo, a multidomain zinc finger protein that is involved in synaptic active zones and synaptic vesicle trafficking was shown to present in sera of GBS patients. High serological levels of Piccolo were associated with better outcomes in GBS patients (65).

Ganglionic nicotinic acetylcholine receptors (gAChR) are nicotinic receptors that assist synaptic transmission in peripheral autonomic ganglia. Over the last decades, the autoantibodies toward gAChR have been associated with autoimmune dysautonomia (72). Several GBS patients experience autonomic dysfunctions such as cardiac arrhythmia and urinary retention (31). Detection of autoantibodies against gAChR could measure the risk of developing debilitating autonomic dysfunction. High level of α3 or β4 subunits of gAChR was detected in 13.6% of GBS patients with autonomic symptoms through luciferase immunoprecipitation (66).

TABLE 2 | Recent biomarkers of Peripheral Demyelinating diseases.

Disease	Biomarkers	References
Guillain-Barre Syndrome	Neutrophil-lymphocyte ratio	(61) (62) (63)
	Platelet-lymphocyte ratio	(61)
	Monocyte-lymphocyte ratio	(63)
	Serum IgG	(64)
	Piccolo protein	(65)
	Anti-ganglionic nicotinic acetylcholine receptor	(66)
Chronic inflammatory demyelinating polyradiculoneuropathy	Neurofascin-155	(67)
	Contactin-1	
	Contactin-1/Contactin-associated protein 1/2	
	P_0, PMP22	
	Neuronal cell adhesion molecule	
	Gliomedin	
	Subunit of sodium channel at node of Ranvier (NavB1, NavB2)	
	Serum IgG-Fc sialylation	(68)
Anti-MAG neuropathy	anti - SGPG	(69)
POEMS syndrome	Vascular endothelial growth factor (VEGF)	(50)
	Serum free light chain	(70)
	Serum heavy/light chain	(70)
Charcot-Marie Tooth disease	PMP22, P_0, MFN2, GJB1 mutations	(55)

Articles were selected from 2015-2018 from SCOPUS database.

Biomarkers of CIDP

Proteomic analysis of the CSF was used to isolate potential biomarker target specific to CIDP (73). Unfortunately, the disease specificity of the identified protein was low. The captured proteins include transferrin, proapolipoprotein, retinal binding protein and transthyretin (73). Several autoantibodies, including NF155, CNTN1, and contactin-1/contactin-associated protein 1 (CNTN1/CASPR1) complex also have been associated with the pathogenesis of CIDP (67).

Autoantibodies against ganglioside antibodies, myelin protein (P0, PMP22), nodal proteins (NF155, CNTN1, CNTN1/CASPR1, CNTN2/CASPR2 complex, neuronal cell adhesion molecule (NCAM)), gliomedin and two subunits of sodium channel at nodes of Ranvier (NavB1, NavB2) have been investigated in CIDP patients. Among these patients, 11 expressed anti-ganglioside antibody reactivity (anti-GM1, anti-GD1b), 4 reacted to CNTN1 (6.2%), 3 reacted to NF155 (6.2%), 1 against CNTN1/CASPR1 complex (1.5%) and 1 against PMP22 (67).

Current literature also associates reduced sialylation of IgG-Fc with increased clinical severity of CIDP. Sialylation and galactosylation of IgG-Fc were significantly lower in CIDP patients. Treatment with IVIG, on the other hand, increased the levels of sialylated IgG-Fc and concurrently attenuated the disease severity (68).

Biomarkers of Anti-mag Neuropathy

The diagnosis of Anti-MAG Neuropathy is through the detection of autoantibodies against MAG. Early findings from Latov's laboratory detected anti-MAG IgM in the sera of almost more than half of the anti-MAG patients (74). Some anti-MAG IgM also co-reacted with acidic glycolipid in the ganglioside fraction of the peripheral nerves, GM1, GD1a and were identified as sulfoglucuronyl glycosphingolipid (SGPG) (69). In clinically indistinguishable anti-MAG neuropathy without seropositivity of MAG and SGPG, the IgM may react to gangliosides such as GD1b, GT1b, and GQ1b (75, 76). Anti-MAG titers could also be correlated with prognosis of the neuropathy. High baseline and increasing anti-MAG titer correlated with higher chances of recurrence (77).

Biomarkers of POEMS Syndrome

VEGF, an angiogenic factor, is markedly elevated in POEMS syndrome patients and also referred as one of the major criteria for diagnosis of POEMS syndrome (50). Clinical improvement and prolonged relapse-free survival were reported among patients with normalized serum level of VEGF (78).

Biomarkers of Charcot-Marie Tooth Disease

Diagnostic markers for CMT disease are usually determined through genetic testing. Once the suspicion of CMT disease is made through clinical judgement and electrophysiological studies.

Diagnosis is confirmed through testing for mutations on PMP22 (CMT1A, most common), GJB1 (CMTX1), P0 (CMT1B), and MFN2 (CMT2A).

MYELIN SHEATH-ASSOCIATED MARKERS

During demyelination, various components of myelin and axon are being released as a result of the damage toward the Schwann cells. Myelin sheath consists of approximately 70 % of lipids such as gangliosides, phospholipids, sphingomyelin and 30% of proteins such as myelin [P0, PMP22, myelin protein 2 (P2)] and nodal proteins (neurofascin, gliomedin, contactin) (**Figure 2**) (**Table 3**) (13). These components can be detected in CSF, serum and even peripheral nerve biopsies (71), which can potentially indicate the degree of demyelination during the disease progress and also the efficacy of the treatment as reflected by the degree of remyelination.

Glycolipids such as gangliosides are one of the major lipid components of the myelin sheath. Situated in the

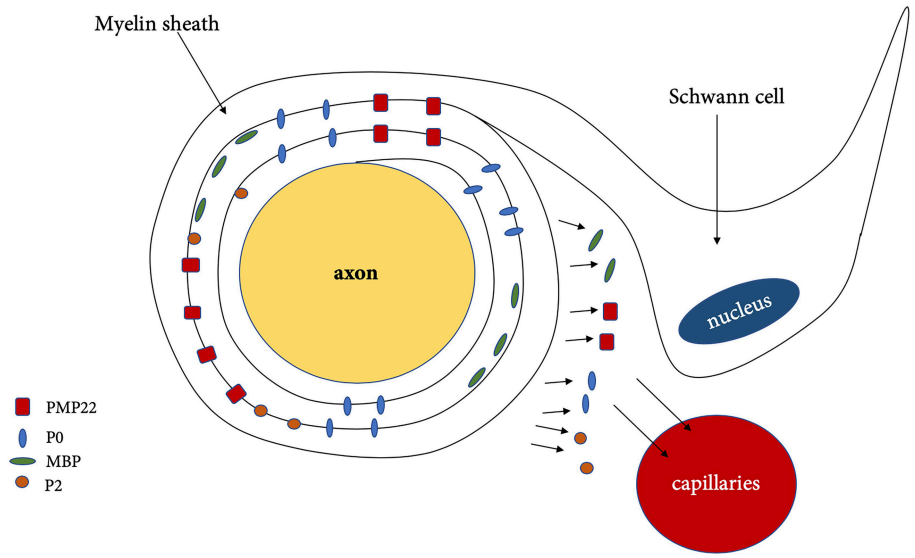

FIGURE 2 | Demyelination and released of myelin associated protein. PMP22, peripheral myelin protein 22; P0, myelin protein zero; MBP, myelin basic protein; P2, myelin protein 2.

plasma membrane with the hydrophilic carbohydrate moiety exposed extracellularly, gangliosides have a greater propensity for autoimmune reaction, especially in GBS, CIDP, and anti-MAG neuropathy (26). Thus, detection of antibody towards gangliosides such as anti-LM1, anti-Hex-LM1, anti-GT1b, anti-SGPG, anti-galactocerebroside in some GBS patients could serve as a diagnostic tool. In parallel to this, the presence of antibodies towards ganglioside complex (GD1a/GD1b, GD1b/GT1b) in serum has been associated with better prognosis of GBS in terms of disease severity (81).

Association of autoimmune diseases such as systematic lupus erythematosus, scleroderma with GBS (96) led researchers (Nakos et al) to ascertain the role of few antiphospholipid antibodies which include phosphatidic acid (PA), cardiolipin, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglyecerol (PG), and cardiolipin. The antibody levels were shown to decrease following 1-day treatment with γ-globulin (IVIG) and increased 2 days following cessation of treatment (82). This indicates that serum levels of anti-PI and anti-cardiolipin antibodies may be useful to monitor the response of the patient toward treatment with IVIG in GBS patients (82).

Sphingomyelin, another myelin-enriched lipid was reported to be significantly higher among PDD patients (GBS and CIDP) (83). The ability to distinguish between demyelinating variant from axonal variant supports the connotation that sphingomyelin is specific biomarker for peripheral myelin breakdown. In addition, the techniques used to detect and quantify sphingomyelin were also reliable, cost-effective with good sensitivity and specificity (83).

Although the lipid/protein ratio and lipid constituents of myelin sheath in both CNS and PNS are similar, the distribution and type of myelin proteins in PNS are different. Myelin sheath consists of two compartments, compact myelin (dense area around axon) and non-compact myelin (**Figure 3**). The compact myelin consists of intraperiod line and the major dense line (MDL). The myelin- specific protein in PNS includes P0, PMP22, and P2.

P0 is a transmembrane glycoprotein that stabilizes the intraperiod line through homophilic binding to another P0 protein (97). Knockout P0 mice were shown to undergo severe hypomyelination and also demonstrated thin, non-compacted myelin sheath with axonal degeneration. In addition, the mice also exhibited tremors, convulsion and deficits in motor coordination (98). P2 protein, also participate in fusion of the MDL in compact myelin (99). PMP22 is a transmembrane protein (100, 101) that is synthesized by Schwann cells and makes up 2–5% myelin protein (102).

Myriad studies have reported the development of antibodies toward P0, PMP22, and P2, especially among GBS and CIDP patients (84, 86–88, 92). The antibodies toward P0 were only present in small proportion of patients with GBS and CIDP and therefore were not useful as a diagnostic test (84). Antibodies toward P0 were only detected in 22% of CIDP and 19% of GBS patient (88). Other studies also reported lower levels of P0 antibodies using various techniques including ELISA, Immunoblot, and Western blot (85, 86, 89). In contrary, some studies reported absence of immune response or no significant response toward P0 (87, 90).

Compared to P0 antibodies, detection of P2 antibodies was reported to be more common in some cases of GBS and CIDP (84). Subsequent studies revealed trace level of IgG to anti-P2 in GBS patients during the peak stage of the disease. The detected trace level of P2 in most patients with GBS and CIDP across

TABLE 3 | List of myelin associated biomarkers.

	Markers	Disease	Sample	References
Myelin sheath lipid markers	LM-1, Hex-LM1, GT1b, SGPG	GBS	Serum	(79) (26, 80)
	GD1a, GD1b	GBS	Serum	(81)
	PC, PI, PG, PS, PE, PA, cardiolipin	GBS	Serum	(82)
	Sphingomyelin	GBS, CIDP	CSF	(83)
Myelin sheath protein markers	P_0	GBS, CIDP	Serum	(84)
		CIDP	Serum	(85)
		CIDP	Serum	(86)
		GBS, CIDP	Serum	(87)
		GBS, CIDP	Serum	(88)
		GBS, CIDP	Serum	(89)
		GBS	Serum	(90)
	P2	GBS, CIDP	Serum	(84)
		GBS, CIDP	Serum	(89)
	PMP22	CMT1A, CMT2, CIDP, anti-MAG, Miller Fisher Syndrome	Serum	(91)
		GBS, CIDP	Serum	(92)
		GBS, CIDP	Serum	(87)
Myelin sheath nodal protein markers	Paranodin	CIDP	Biopsy	(93)
	NF186, gliomedin, NCAM	GBS, CIDP	Serum	(94)
	NCAM	PDN, axonal PN, non-inflammatory diabetic PN	Serum	(95)

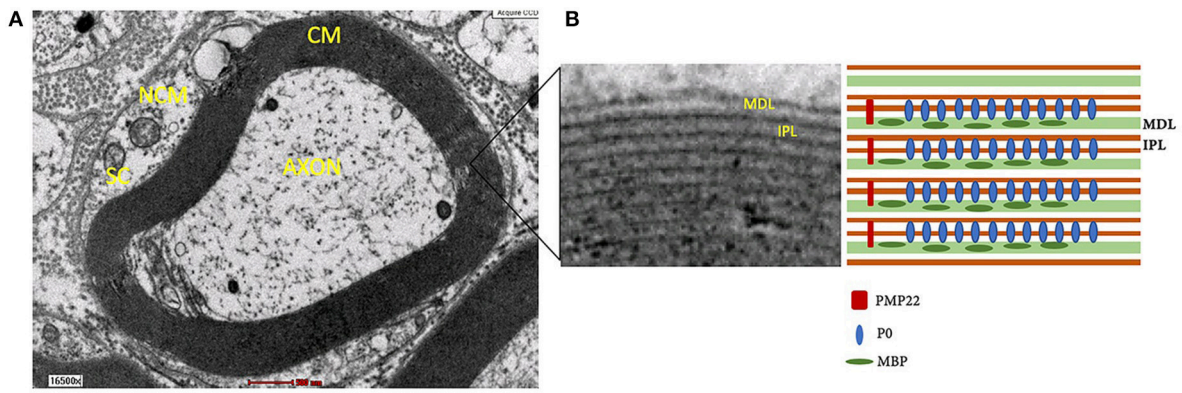

FIGURE 3 | (A) Structure of myelin sheath of Schwann cell around axon under electron microscope. **(B)** Compact myelin that consist of major dense line and intraperiod line. NCM, non-compact myelin; CM, compact myelin; SC, Schwann cell; MDL, major dense line; IPL, intraperiod line; P0, myelin protein zero; MBP, myelin basic protein; PMP22, peripheral myelin protein 22.

all stages of the diseases were within the same range as in the control group (84, 89). Therefore, P2 is not sensitive and specific biomarkers for demyelinating diseases.

PMP22 was detected in 70% of CMT1 and 60% of CMT2 cases. Surprisingly, no significant difference in the immune response of PMP22 from healthy donors and patients with acquired neuropathies (91). Similarly, another study also reported antibodies against PMP22 in 52% of GBS and 35% of CIDP patients (92). Contrary to these findings, absence of an immune response to PMP22 P0 and Cx32 proteins were reported in GBS and CIDP patients (87). These discrepancies were probably due to different usage of PMP22 antigen and also different patient groups that might reflect distinct immunoreactivity toward PMP22.

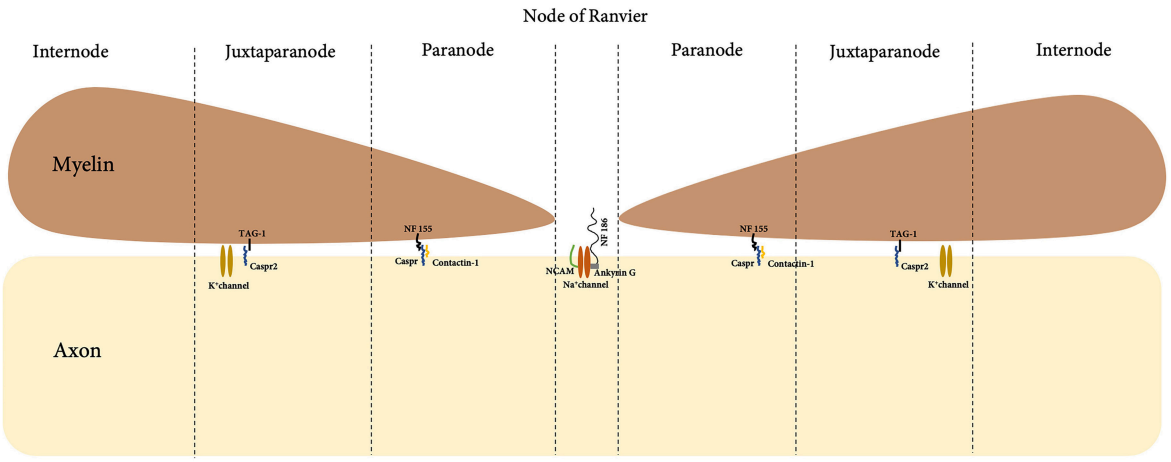

FIGURE 4 | Anatomical compartment of the node of Ranvier and myelin.

MYELIN NODAL PROTEIN

In a myelinated fiber, there are multiple internodes of Schwann cells, which are separated by the node of Ranvier. A detailed and magnified look into the boundaries of the node of Ranvier with Schwann cell internode further shows detailed compartment that viewed the node, paranode and juxtraparanode (**Figure 4**). Nodal proteins such as contactin, neurofascin and NCAM were identified at these regions and they took part in the formation of the septate-like junction that closely in contact with axons (67).

Abnormalities of the nodal proteins were reported in the pathogenesis of CIDP (103). Paranodin, a nodal protein involved in axoglial contacts was tested through immunostaining of biopsies from patients with CIDP. Evaluation of positively-stained paranodin biopsies led to the correct diagnosis of CIDP in 70% of the reported cases (93). Further laboratory investigations revealed IgG autoimmunity towards myelin nodal proteins, neurofascin-186 (NF186), gliomedin, contactin and NCAM in 43% of GBS ($n = 100$) and 30% of CIDP ($n = 50$) patients (94). In addition, passive transfer of anti-gliomedin IgG to Lewis rat induced progressive neuropathy characterized by conduction defect and demyelination of spinal nerves (104). This has led the authors to suggest that myelin nodal proteins may play a role in induction of demyelination. Furthermore, clinical remission seen in these animals was parallel with the gradual decrease of IgG titers (94), suggesting that levels of antibody titers toward gliomedin could serve as a biomarker for disease remission.

Another study by Niezgoda et al. compared the level of serum NCAM in peripheral demyelinating neuropathy (PDN), axonal polyneuropathy (PN), non-inflammatory diabetic PN and healthy controls. In the study, Overall Neuropathy Limitation Scale (ONLS) and electrophysiological analysis comprises of motor and sensory studies were employed for clinical assessment. Significant increase in NCAM was seen among PDN group ($n = 40$ GBS, 29 CIDP, 11 Multifocal Motor Neuropathy) compared to other groups. In patients with PDN but not PN and non-inflammatory diabetic PN, serum NCAM levels had a high positive correlation to ONLS and negative correlation to motor conduction velocity. Thus, it has been concluded that NCAM detection could serve as a specific marker for peripheral nerve immune-mediated demyelination and sensitive marker for peripheral nerve involvement (95).

SUMMARY

Poor prognosis remained a dilemma in the management of the PDDs. Many people remain underdiagnosed or diagnosed when disease is already advanced. Moreover, the biomarkers that currently being used develop late in the disease process. Therefore, understanding the physiology of myelination and SC biology is vital to help further delineate the mechanisms involved in the pathogenesis of PDD. Autoantibodies against several types of gangliosides, phospholipids, glycoproteins, and nodal proteins have been shown to be present in numerous PDDs. However, their serum levels are yet to be correlated with a clinical course, and prognosis. A reliable biomarker should be sensitive and specific. Can peripheral autoantibodies that develop against the myelination-associated proteins or lipids in the early stage of PDDs be appropriate biomarker candidates? Future studies should explore this premise to discover time-sensitive biomarkers for early detection of PDDs.

AUTHOR CONTRIBUTIONS

KK and JK performed the literature search and drafted the manuscript. MY, RI, and SD reviewed and finalized the manuscript. The figures were designed by KK.

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Association Between *Aldehyde dehydrogenase-2* Polymorphisms and Risk of Alzheimer's Disease and Parkinson's Disease: A Meta-Analysis Based on 5,315 Individuals

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Objective: A number of studies have reported that aldehyde dehydrogenase-2 (*ALDH2*) polymorphisms maybe associated with the risk of Alzheimer's disease (AD) and Parkinson's disease (PD). However, the results of such studies are inconsistent. We therefore conducted a meta-analysis to clarify the association between *ALDH2* polymorphisms and the risk of AD and PD.

Methods: Five online databases were searched and the relevant studies were reviewed from inception through May 10, 2018. Odds ratios (ORs) and corresponding 95% confidence intervals (CIs) were calculated in each genetic model of the general population and various subgroups. Furthermore, we simultaneously performed heterogeneity, cumulative, sensitivity, and publication bias analyses.

Results: Overall, nine case-control studies involving 5,315 subjects were included in this meta-analysis. Potential associations were found between the *ALDH2* rs671 G>A polymorphism and the risk of AD (A vs. G: OR = 1.46, 95%CI = 1.01–2.11, $P = 0.05$, $I^2 = 84.2\%$; AA vs. GG: OR = 2.22, 95%CI = 1.03–4.77, $P = 0.04$, $I^2 = 79.2\%$; AA vs. GG+GA: OR = 1.94, 95%CI = 1.03–3.64, $P = 0.04$, $I^2 = 71.1\%$). In addition, some similar results were observed in other subgroups. Moreover, no significant association between *ALDH2* polymorphisms and PD risk.

Conclusions: In conclusion, our meta-analysis indicated that the *ALDH2* rs671 G>A polymorphism plays an important role in AD development.

Keywords: *Aldehyde dehydrogenase 2*, neurodegenerative disorders, Alzheimer's disease, Parkinson's disease, polymorphism

INTRODUCTION

Neurodegenerative disorders are a family of heterogeneous disorders, in which progressive degeneration occurs in the structure and function of the central or peripheral nervous system (1). Neurodegenerative disorders would lead to progressive cognitive and motor disabilities, such as senile dementia, balance disorder, movement dyskinesia, and muscular tension abnormalities (2).

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common neurodegenerative disorders (3).Currently, there are more than 5 million patients with AD and this number is projected to increase to 7.1 million by 2025 (4). Indeed, the World Health Organization has predicted that AD and other causes of dementia, as well as such as PD and amyotrophic lateral sclerosis will overtake cancer to become the second leading cause of death following cardiovascular disease (5). Further, as the population ages, AD and PD will increasingly place substantial burdens on not only the families of individuals with these conditions, but also on society as a whole.

AD and PD are closely related to aging, can have multiple causative factors, such as oxidative damage, abnormal protein deposition, and neuroinflammation (6). Other factors, including atherosclerosis (7), diabetes (8), neuroinflammation (9), and various environmental effects (10), have also been proposed as interactive factors for AD and PD (11). Moreover, increasing evidence suggests that genetic abnormities, including genetic mutations, play important roles in the development of AD and PD (12, 13).

One such gene that is thought to be involved in AD and PD is the aldehyde dehydrogenase-2 (*ALDH2*) gene. This gene is located on chromosome 12q24.12, comprises 13 exons and 12 introns, and encodes an important biologically active enzyme, ALDH2. This enzyme participates in the metabolism and detoxification of aldehyde, and it can metabolize short-chain aliphatic aldehydes and converted acetaldehyde into acetate. Moreover, ALDH2 is involved in the metabolism of other biogenic aldehydes, such as 4-hydroxynonenal, 3,4-dihydroxyphenylacetaldehyde, and 3,4-dihydroxyphenylglycoaldehyde (14). Recent studies have indicated that ALDH2 exerts protective effects on the cardio-cerebral vascular system and central nervous system. Single nucleotide polymorphisms (SNPs) of the *ALDH2* gene have been reported to be associated with the risks for several diseases, such as coronary artery disease, ischemic stroke, digestive system cancer and allergic asthma. The A allele (*ALDH2*2*) of rs671, inherent in mainly the East Asian population, deregulates the ALDH2 activity intrinsically, and induces the accumulation of acetaldehyde. In 2000, Kamino et al. conducted the first case-control study in the Japanese population and found that the A allele and GA/AA genotypes are associated with an increased risk of AD (15). Subsequently, studies focusing on the association between *ALDH2* polymorphisms and the risk of AD and PD have been continually published, but the results are inconsistent. Therefore, we conducted a meta-analysis of all available studies to investigate the precise association between the *ALDH2* polymorphisms and the risk of AD and PD.

MATERIALS AND METHODS

This meta-analysis of observational studies was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines (16). All included data were collected from published studies, and no ethical issues were involved.

Search Strategy

Five online databases (PubMed, Embase, Web of Science, Chinese national knowledge infrastructure and Wanfang) were used to search for related studies on the association between *ALDH2* polymorphisms and the risk of AD and PD from inception through May 10, 2018. Only the studies published in English and Chinese were included. The bibliographies of the collected studies and relevant reviews were also checked to identify potential additional articles. The following search terms and strategy was adopted (e.g., in PubMed):

#1 *Aldehyde dehydrogenase 2*
#2 *Aldehyde Dehydrogenase-2*
#3 *ALDH2*
#4 rs671
#5 rs4767944
#6 rs441
#7 #1 OR #2 OR #3 OR #4 OR #5 OR #6
#8 polymorphism
#9 variant
#10 mutation
#11 #8 OR #9 OR #10
#12 neurodegenerative disorders
#13 Alzheimer's disease
#14 Parkinson's disease
#15#12 OR #13 OR #14 OR #15
#16 #7 AND #11 AND #15.

Eligibility Criteria

The following were our inclusion criteria: (1) observation studies focusing on the association between *ALDH2* polymorphisms and the risk of AD and PD; (2) studies containing sufficient data on the genotype in the control groups to evaluate crude odds ratios (ORs) and 95% confidence intervals (CIs); (3) studies published in English or Chinese, and (4) if overlapping or duplicate data were found on the same theme, only the largest or most recent sample data were included. The exclusion criteria included: (1) case report or review articles; (2) molecular biology research; (3) studies without efficient data; and (4) studies with duplicated or overlapping data.

Data Extraction and Quality Evaluation

Two authors (Chen and Huang) independently reviewed the included studies, and the following information was extracted and recorded for analysis: the first authors' name, publication date, study country, control design, genotyping method, sample sizes of the cases and controls, frequency data for the genotype distribution, assessment of Hardy-Weinberg equilibrium (HWE) in control, minor allele frequency, and disease type. The modified Newcastle-Ottawa scale (NOS) was used to evaluate the quality of all included studies (17). The scores ranged from 0 points (worst) to 11 points (best) (**Supplementary Table 1**). Studies with a score of 8 points or higher were classified as high quality.

Statistical Analysis

We calculated the crude ORs and 95% CIs to assess the statistical power of the association between *ALDH2* polymorphisms and the risk of AD and PD. For example, the following five genetic

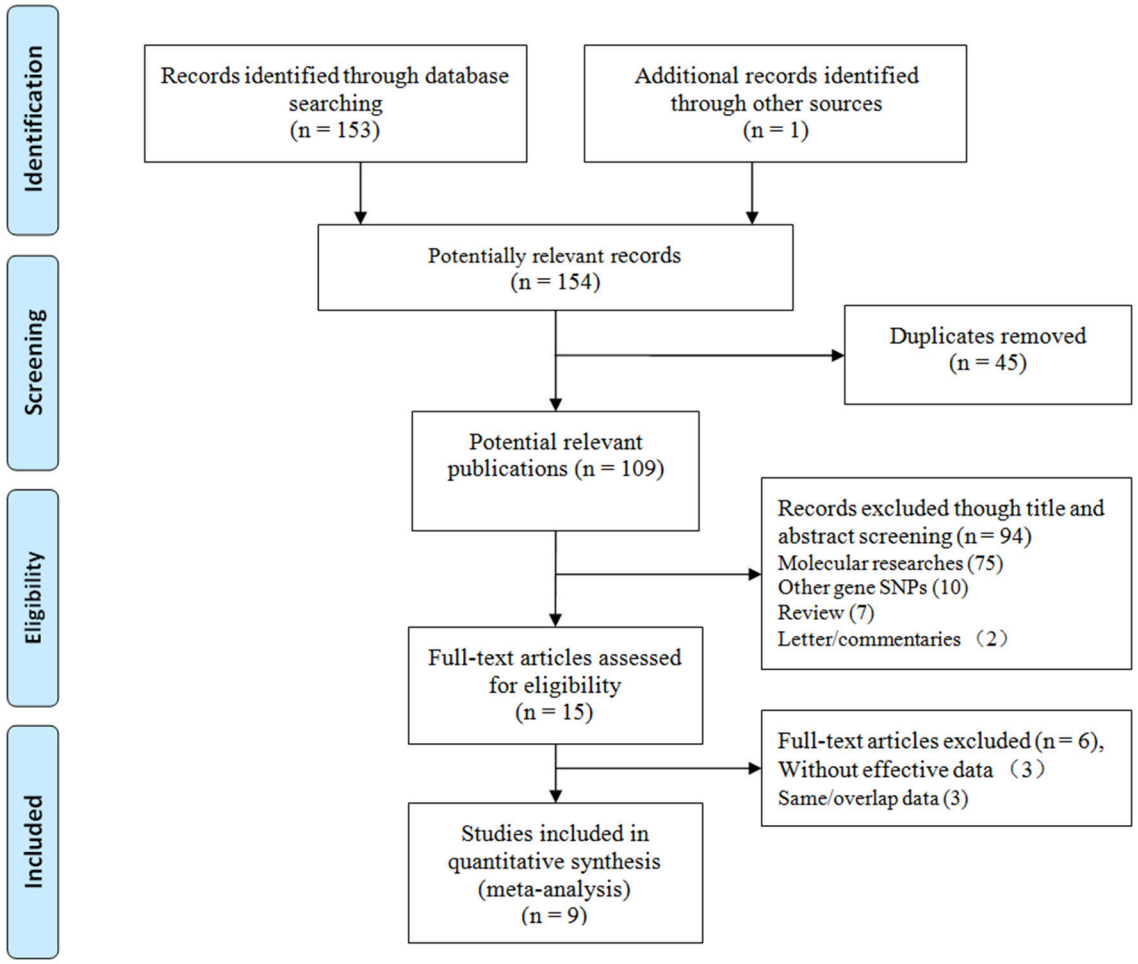

FIGURE 1 | Flow diagram of the study selection process.

models of the rs671 G>A locus were used: allele contrast (A vs. G), co-dominant models (GA vs. GG and AA vs. GG), dominant model (GA+AA vs. GG), and recessive model (AA vs. GG+GA). Heterogeneity among the included studies was examined using Cochran's Q tests and I^2-tests (18). A fixed-effects model was adopted when I^2 was ≤40%, but a random-effects model was adopted when I^2 was > 40%(19, 20). Subgroup analyses were performed according to the HWE status, study country, disease type, control design (population-based and hospital-based), subject number, NOS evaluation and gender diversity. Meta-regression was conducted to identify which factors contributed to the existing heterogeneity. A cumulative meta-analysis was performed to assess the statistical tendency of the results. Sensitivity analysis was used to examine the stability of the results by sequentially removing each study individually. Potential publication biases were assessed with Egger's linear regression test and Begg's funnel plots (21, 22). All statistical analyses were performed using STATA version 14.0 (Stata Corporation, College Station, TX, USA). Statistical significance was set at <0.05 (two-sided).

RESULTS

Study Characteristics

We initially identified 154 relevant articles through our systematic literature search. The selection process is shown in **Figure 1**. According to the eligibility criteria, 45 studies were excluded following duplicate screening, 94 studies were removed after the subsequent title and abstract reviews, and 9 studies were eliminated because of deficient data, and/or similar/overlapping data. Finally, nine publications (11 independent studies) involving 2,283 patients and 3,032 controls were included (15, 23–30). Three common SNPs were reported; eight studies focused on the rs671 G>A polymorphism (15, 23–29), two studies focused on the rs4767944 C>T polymorphism (27, 30), and one study focused on the rs441 T>C polymorphism (27). In all included studies, most subjects were from East Asians countries including China, Japan, and Korea, except for one study that was performed in the Iranian population (30). The HWE assessment of control revealed that there were two studies that deviated from or lacked the HWE index in the rs671

TABLE 1 | Characteristics of case-control studies on ALDH2 polymorphisms and AD and PD risk.

References	Country	Control design	Genotype method	Case	Control	Genotype distribution						P for HWE	MAF	NOS evaluation	Disease type
						Case			Control						
Rs671						GG	GA	AA	GG	GA	AA				
Kamino et al. (15)	Japan	HB	PCR-RFLP	447	447	232	183	32	280	138	29	0.04	0.22	7	AD
Kim et al. (23)	Korea	PB	PCR-RFLP	80	610	60[a]	20[b]		435[a]	175[b]		NA	NA	7	AD
Wang et al. (24)	China	HB	PCR-RFLP	188	223	54	92	42	124	84	15	0.88	0.26	7	AD
Zhou et al., (25)	China	HB	PCR-RFLP	106	100	65	32	9	54	38	8	0.72	0.27	7	AD
Komatsu et al. (26)	Japan	PB	TaqMan	158	130	81	62	15	67	54	9	0.67	0.28	8	AD
Ma et al., (29)	China	HB	PCR-RFLP	115	236	72	30	13	177	52	7	0.20	0.14	7	AD
Zhang et al. (27)	China	HB	PCR-RFLP	584	582	321	236	27	339	208	35	0.68	0.24	9	PD
Zhao et al. (28)	China	HB	PCR-RFLP	115	214	71	32	12	157	52	5	0.78	0.14	7	PD
Rs4767944						CC	CT	TT	CC	CT	TT				
Zhang et al. (27)	China	HB	PCR-RFLP	584	582	92	316	176	62	301	219	0.01	0.63	8	PD
Madadi et al. (30)	Iran	HB	PCR-RFLP	490	490	339	143	9	344	133	13	0.97	0.16	8	PD
Rs441						TT	TC	CC	TT	TC	TT				
Zhang et al. (27)	China	HB	PCR-RFLP	584	582	281	278	25	293	256	33	0.02	0.28	8	PD

HWE in control.
NA, Not available.
[a]Data of the GG genotype; [b]Data of the GA/AA genotypes.
HB, Hospital or healthy based; PB, Population based.
AD, Alzheimer's disease; PD, Parkinson's disease.

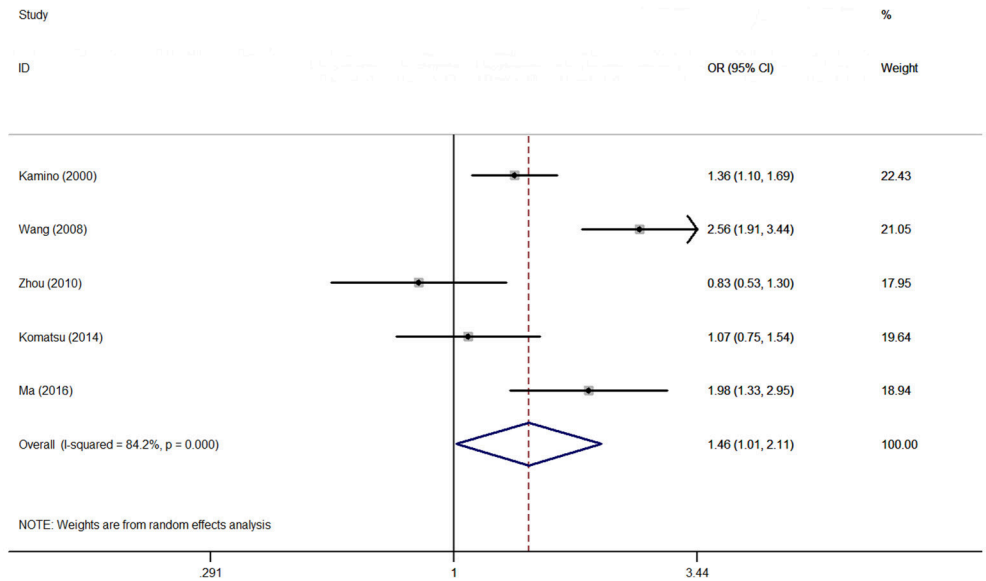

FIGURE 2 | OR and 95% CIs of the associations between ALDH2 rs671 G>A polymorphism and AD risk in A vs. G model.

G>A polymorphism (15, 23), and one study each that deviated from the HWE in the s4767944 C>T polymorphism (27) and rs441T>C polymorphism (27). The characteristics of all included studies are presented in **Table 1**.

Quantitative and Subgroup Analyses

Association between the rs671 polymorphism and the risk of AD and PD. There were six case-control studies involving 2,840 subjects focused on the association between the rs671 polymorphism and AD risk. The aggregated results indicated an increased risk of the rs671 G>A polymorphism in patients with AD (A vs. G: OR = 1.46, 95%CI = 1.01–2.11, P = 0.05, I^2 = 84.2% (**Figure 2**); AA vs. GG: OR = 2.22, 95%CI = 1.03–4.77, P =0.04, I^2 = 79.2%; AA vs. GG+GA: OR = 1.94, 95%CI = 1.03–3.64, P =0.04, I^2 = 71.1%). Subgroup analyses also revealed an increased AD risks in Chinese (AA vs. GG: OR = 3.15, 95%CI = 1.03–9.65, P =0.04, I^2 = 79.6%; AA vs. GG+GA: OR = 2.75, 95%CI = 1.23–6.14, P =0.02, I^2 = 63.0%) and Japanese (A vs. G: OR = 1.54, 95%CI = 1.00–2.36, P =0.05, I^2 = 17.1%) on the basis of country difference. The subsequent analysis based on the HWE status and other subgroup revealed the similar increased associations (**Table 2**, **Supplementary Figure S1** for other models) Heterogeneity was observed in all five genetic models; the meta-regression analysis was conducted with the above-mentioned stratified factors and did not identify any factor that contributed to the existing heterogeneity (e.g., A vs. G model: P =0.89 for HWE status, P =0.53 for study country, P =0.53 for control design, P =0.89 for subject number, and P =0.53 for NOS evaluation).

The accumulative analysis presented fluctuating findings and the results tending to show a potential association by Ma et al. (29) (**Figure 3** for A vs. G model, **Supplementary Figure S2** for other models). A sensitivity analysis was conducted by removing each included study, and fluctuating results were observed after several studies were removed (**Figure 4** for A vs. G model, **Supplementary Figure S3** for other models).

Publication biases were investigated, and the results did not show any obvious asymmetry in the five funnel plots (**Figure 5** for A vs. G model, **Supplementary Figure S4** for other models). All results were confirmed with Egger's linear regression test (A vs. G, P =0.76; GA vs. GG: P =0.40; AA vs. GG, P =0.94; GA+AA vs. GG, P =0. 42; AA vs. GG+GA, P =0.77).

Two case-control studies involving 1,495 subjects focused on the association between the rs671 polymorphism and PD risk and presented a negative relationship (**Table 2**).

Association between the rs4767944 and rs441 polymorphisms and the risk of PD. Two studies with 2,146 subjects focused on the association between the rs4767944 polymorphism and the risk of PD. The results of our aggregated analysis showed that the rs4767944 C>T polymorphism increased the PD risk in the homozygous model, but not in other genetic models. Only one study focused on the association between the rs441 polymorphisms and the risk of PD. The related information about the rs441 polymorphism is presented in **Table 1** (Quantitative calculation was not conducted).

DISCUSSION

AD and PD are the most important neurodegenerative disorders. The most intensively studied neurodegenerative disease is AD, which is known to be an established cause of aging-associated dementia, accounting for 60–70% of all neurodegenerative cases (31). Autopsy reports have demonstrated that the brain tissues of individuals with AD exhibit atrophied neurons, intraneuronal neurofibrillary tangles, and amyloid plaques. The

TABLE 2 | Summary ORs and 95% CI of ALDH2 polymorphisms and AD and PD risk.

	N*	OR	95% CI	*P*	I^2(%)	OR	95% CI	*P*	I^2(%)	OR	95% CI	*P*	I^2(%)	OR	95% CI	*P*	I^2(%)	OR	95% CI	*P*	I^2(%)
rs671		**A vs. G**				**GA vs. GG**				**AA vs. GG**				**GA+AA vs. GG**				**AA vs. GG+GA**			
AD Total	6	1.46	1.01–2.11	0.05	84.2	1.35	0.91–1.99	0.13	73.9	2.22	1.03–4.77	0.04	79.2	1.36	0.90–2.03	0.14	81.0	1.94	1.03–3.64	0.04	71.1
HWE–yes	4	1.48	0.88–2.49	0.14	87.4	1.26	0.72–2.21	0.41	79.4	2.57	1.01–6.53	0.05	78.1	1.45	0.77–2.73	0.25	86.0	2.23	1.18–4.60	0.01	61.4
HWE–no	2	1.36	1.10–1.69	0.01	NA	1.60	1.21–2.12	0.001	NA	1.33	0.78–2.27	0.29	NA	1.19	0.64–2.18	0.58	76.4	1.11	0.66–1.87	0.69	NA
COUNTRY																					
China	3	1.64	0.87–3.10	0.12	88.3	1.39	0.67–2.85	0.38	83.0	3.15	1.03–9.65	0.04	79.6	1.63	0.74–3.62	0.23	87.9	2.75	1.23–6.14	0.02	63.0
Japan	2	1.54	1.00–2.36	0.05	17.1	1.28	0.77–2.13	0.33	69.7	1.34	0.85–2.12	0.20	0	1.31	0.87–1.98	0.20	59.6	1.18	0.76–1.85	0.46	0
CONTROL DESIGN																					
HB	4	1.57	1.02–2.42	0.04	86.0	1.47	0.95–2.27	0.09	74.6	2.48	0.98–6.30	0.06	83.4	1.63	0.99–2.67	0.05	82.7	2.10	0.96–357	0.06	77.6
PB	2	1.07	0.75–1.54	0.71	NA	0.95	0.58–1.55	0.84	NA	1.38	0.57–3.35	0.48	NA	0.93	0.65–1.32	0.68	0	1.41	0.60–3.34	0.43	NA
SUBJECTS																					
>500	2	1.36	1.10–1.69	0.01	NA	1.60	1.21–2.12	0.001	NA	1.33	0.78–2.27	0.29	NA	1.19	0.64–2.18	0.58	76.4	1.11	0.66–1.87	0.69	NA
<500	4	1.48	0.88–2.49	0.14	87.4	1.26	0.72–2.21	0.41	79.4	2.57	1.01–6.53	0.05	78.1	1.45	0.77–2.73	0.25	86.0	2.23	1.18–4.60	0.01	61.4
NOS EVALUATION																					
NOS < 8	5	1.57	1.02–2.42	0.04	86.0	1.47	0.95–2.27	0.09	74.6	2.48	0.98–6.30	0.06	83.4	1.43	0.90–2.28 .92–2.18	0.13	82.9	2.10	0.96–357	0.06	77.6
NOS ≥8	1	1.07	0.75–1.54	0.71	NA	0.95	0.58–1.55	0.84	NA	1.38	0.57–3.35	0.748	NA	1.01	0.64–1.61	0.96	NA	1.41	0.60–3.34	0.43	NA
GENDER																					
Male	2	1.52	1.06–2.16	0.02	0	1.70	1.07–2.70	0.03	14.3	1.81	0.73–4.46	0.20	0	1.72	1.10–2.67	0.02	0	1.47	0.60–3.58	0.40	0
Female	2	0.90	0.40–2.10	0.79	83.4	0.94	0.35–2.57	0.891	81.5	0.93	0.37–2.32	0.88	43.8	0.91	0.33–1.2.49	0.86	84.3	0.98	0.57–1.69	0.95	0
PD Total	2	1.38	0.77–2.45	0.28	85.2	1.23	0.98–1.52	0.07	0	1.95	0.31–12.23	0.47	89.3	1.30	0.90–1.92	0.15	53.6	1.18	0.29–11.16	0.52	89.4
Rs4767944		T vs. C				CT vs. CC				TT vs. CC				CT+TT vs. CC				TT vs. CC+CT			
PD Total	2	0.87	0.67–1.14	0.31	70.9	0.89	0.58–1.36	0.60	71.3	0.57	0.40–0.80	<0.01	0	0.83	0.51–1.36	0.46	80.3	0.71	0.56–0.90	0.01	0

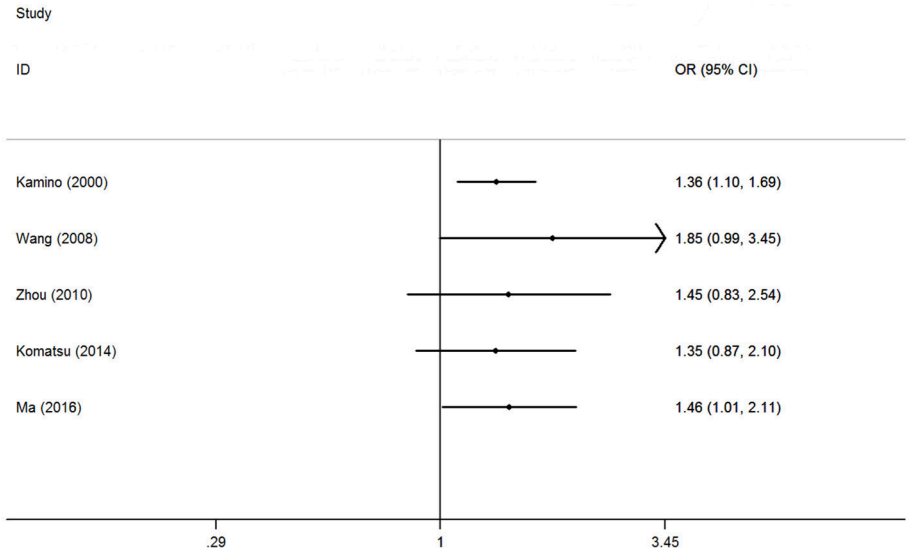

FIGURE 3 | Cumulative meta-analyses according to publication year in A vs. G model of ALDH2 rs671 G>A polymorphism and AD risk.

amyloid beta accumulation promotes oxidative stress and leads to mitochondrial dysfunction. As for PD, it is a chronic neurodegenerative disease without a clear etiology and is characterized by the classic triad of tremors, bradykinesia, and rigidity (32). Research has shown that >50% of patients with PD will develop dementia within 10 years after the initial diagnosis (33). The prominent pathological changes that occur in patients with PD are the degeneration of dopamine neurons in the mesencephalon, which causes a decrease of the dopamine content in the striatum.

Alcohol consumption is one of the most common habits of human beings. Alcohol is characterized by a high affinity for water and can be quickly distributed throughout the body after rapid absorption into the blood from the gastrointestinal tract. The majority of ethanol metabolism occurs in the liver and ALDH2 has a strong effect on acetaldehyde metabolism and accumulation (34). Current evidence suggests that alcohol intake can have two opposing effects on health. Specifically, consuming small doses of alcohol is believed to confer protection and decrease the risk of cardiovascular system dysfunction (35), while consuming large doses of ethanol can increase the alcohol content in the serum and seriously threaten human health, leading to cardiovascular disease and various oncological diseases. Most studies suggest that long-term and excessive alcohol consumption may damage the central nervous system cells and impair cognitive function (36).

Several important SNP loci have been identified and studied in the *ALDH2* gene. These SNPs change the nucleotide bases of the human genome, thus altering the protein expression levels and biological activity. The rs671 polymorphism is caused by a single-nucleotide mutation from G to A, which encodes an amino acid change from glutamate to lysine. Rs671is the most well-known dysfunctional SNP, and both the GA and AA genotypes severely reduce the activity of the ALDH2 enzyme and impair ethanol metabolism (37). The mutant genotypes and allele frequencies of rs671 are markedly different among different populations and are particularly more prevalent in East Asian populations (Chinese, Japanese and Korean individuals) than in other populations (38, 39). Many studies have demonstrated that the *ALDH2* rs671 G>A polymorphism maybe associated with the development of various diseases, such as digestive system cancer (40), diabetes (41), coronary heart disease (42), and ischemic stroke (43), especially in East Asians.

Since 2000, an increasing number of studies have focused on the relationship between *ALDH2* polymorphisms and the risk of AD. Kamino et al. conducted the first case-control study and found that the rs671 A allele would increase the risk for AD in the Japanese population (OR = 1.6, 95% CI = 1.19–2.03), and this

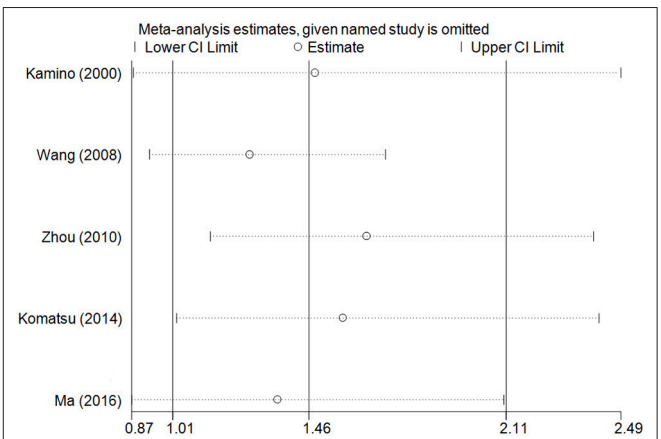

FIGURE 4 | Sensitivity analysis through deleting each study to reflect the influence of the individual dataset to the pooled ORs in A vs. G model of ALDH2 rs671 G>A polymorphism and AD risk.

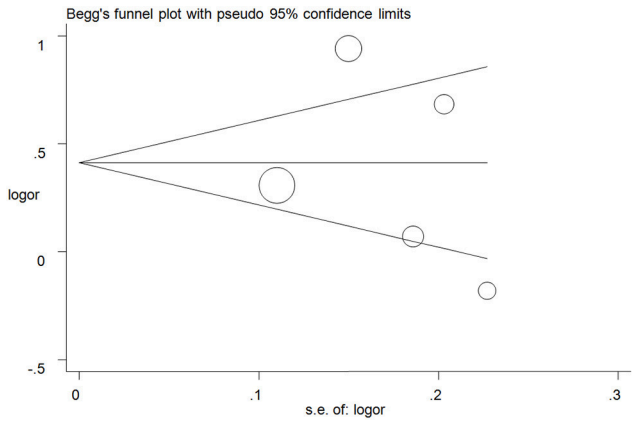

FIGURE 5 | Funnel plot analysis to detect publication bias for A vs. G model of ALDH2 rs671 G>A polymorphism and AD risk. Circles represent the weight of the studies.

trend was observed in both males and females. Wang et al. found an apparently increased AD risk in Chinese individuals with the rs671 A-allele (OR = 3.11, 95%CI = 2.06–4.69). Similarly, Zhao et al. and Ma et al. reported the similar elevated risk for PD (28) and AD with the rs671G>A mutation (29). However, Zhou et al., Komatsu et al., and Zhang et al. did not find any potential relationship between the rs671 G>A polymorphism and the risk of AD. Therefore, the discrepancies in these result promoted us to conduct this meta-analysis to investigate the precise association based on published studies.

To our knowledge, this is the first meta-analysis on the association between the *ALDH2* rs671 G>A polymorphism and the risk of AD and PD. All results suggest that the polymorphism locus of *ALDH2* rs671 G>A may be a potential risk factor for AD but not for PD in the East Asians. The current evidence indicated that the carriers with AA genotype were more dangerous compared with the GG genotype, which was also coincides with the decrease of ALDH2 protein activity that caused by allele A mutation. Moreover, the elevated risks we identified were also observed in some subgroups, such as in male groups. Among East Asians, males account for the majority of people who consume alcohol. The interactive effects of alcohol consumption and the deficient ALDH2 enzymatic activity caused by the rs671 mutation may contribute to AD development in males. Two of the studies we evaluated focused on the rs4767944 polymorphism, and the results revealed a slightly protective effect of this polymorphism against PD. In addition, no positive association was identified between rs441 polymorphism and the risk of PD with only one study. However, given the limited number of studies and included participants for this polymorphism, the results might not reflect the real relationship. Therefore, additional studies examining the association between the rs4767944 and rs441 polymorphisms and the risk of PD are necessary.

Given the inherent deficiencies in meta-analyses, this study has some limitations that should be considered when interpreting our findings. First, all subjects in the included studies were from East Asian countries. As such, the pooled results of this meta-analysis only reflect the East Asian population and thus are not generalizable to other ethnicities. Second, a slightly large sample size was only available for the *ALDH2* rs671 G>A polymorphism, but not for the rs4767944 and rs441 polymorphisms. The investigations of the associations of each of the three polymorphisms with the risk of AD and PD were conducted independently without adjusting for gene-gene interactions, such as through a haplotype analysis. Third, the interactions with some factors, such as body mass index, blood pressure, and unhealthy living habits, were not examined because of the lack of accurate individual information in the included studies. Finally, heterogeneity was observed in some of the genetic models in all included studies, and the meta-regression failed to identify any factors that contributed to the heterogeneity. Future studies that address these limitations will be required before any concrete conclusions about the relationships between these polymorphisms and AD and PD can be made.

In conclusion, the present results indicate that the *ALDH2* rs671 G>A polymorphism may be a potential risk factor for AD. Additional case-control studies are needed to investigate the underlying mechanism of the potential risk.

AUTHOR CONTRIBUTIONS

JC, WH, and LZ conceived the study and wrote the draft of the paper. JC, WH, and Y-YH searched the databases and extracted the data. C-HC and G-BJ analyzed the data and reviewed the manuscript. All the authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00290/full#supplementary-material

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BRIEF RESEARCH REPORT
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Potential Utility of ^{123}I-MIBG Scintigraphy as a Predictor of Falls in Parkinson's Disease

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Background: Falls are associated with poor prognosis in patients with Parkinson's disease (PD). Although several factors related to falls were reported in patients with PD, objective predictors of falls are not identified. We aimed to determine whether ^{123}I-meta-iodobenzylguanidine (MIBG) cardiac scintigraphy could be a useful biomarker to predict falls.

Methods: Forty-five patients with PD were enrolled in this study. These subjects were followed up more than 5 years after MIBG scintigraphy and were divided into two groups: one with decreased uptake of MIBG and the other without decreased uptake of MIBG. The cut-off value for the delayed heart-to-mediastinum ratio was 1.8. Kaplan-Meier analysis and a log-rank test were performed to test the predictive power of MIBG cardiac scintigraphy for falls. Univariate analysis was selected because we did not have appropriate data for adjustment, such as motor and cognitive assessment.

Results: The group with decreased uptake of MIBG had a significantly higher incidence of falls than that without decreased uptake of MIBG ($P = 0.022$, log-rank test).

Conclusions: Although the limitations of this study were lack of several key factors including motor and cognitive assessment, MIBG cardiac scintigraphy may be used to predict falls in patients with PD.

Keywords: Parkinson's disease, falls, MIBG, prognosis, motor symptom

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INTRODUCTION

Falls predict an unfavorable prognosis due to poor motor function in patients with Parkinson's disease (PD), which are attributed to considerable factors including motor and non-motor impairments (1). Although it is not easy to overcome falls, several interventions have been developed. These include administration of acetylcholinesterase inhibitors (2) or the prodrug of epinephrine (3), and Tai chi (4) or other types of exercise (5). Early identification of patients at high risk for falls is essential to ensure that the above interventions are undertaken before the patient is bed-ridden due to fall-related injury. There is thus a need for the identification of risk factors that allow prediction of falls.

In terms of a risk factor for a poor prognosis, ^{123}I-meta-iodobenzylguanidine (MIBG) cardiac scintigraphy has been reported to be a predictor of dementia in PD (6). Patients with PD tend to have reduced uptake of MIBG, although this measure varies widely (7). We hypothesized that the wide-range of MIBG uptake might help define subgroups of patients with PD with difference in prognosis. To test our hypothesis, we investigated the relationship between MIBG uptake and falls using data from 45 patients with PD at the Tokushima University Hospital.

MATERIALS AND METHODS

Participants

A total of 438 patients with PD were identified from the medical records of inpatients and outpatients at the Tokushima University Hospital between April 1st, 2007 and September 30th, 2017. Ultimately, 45 patients who were diagnosed with clinically established or probable PD according to the internationally established PD criteria (8), and were followed up for more than 5 years after MIBG evaluation were included in this study. These patients were divided into two groups: one with decreased uptake of MIBG [delayed heart-to-mediastinum ratio [H/M ratio] $<$ 1.8], and the other without decreased uptake of MIBG (delayed H/M ratio $\geq$ 1.8). We considered the following variables: sex, age at onset, age at MIBG evaluation, disease duration, Hoehn-Yahr stage, daily levodopa dose at MIBG evaluation, use of dopamine agonist at MIBG evaluation, severity and frequency of falls, and follow-up period after MIBG evaluation. Disease duration was defined as the period from the onset of motor symptoms to the time of MIBG evaluation. Severe falls were defined by the need for medical care after injuries. Injuries due to falls and the number of subjects with injury were summarized in the **Supplementary Table 1**. The present study protocol (number 3118) was approved by the local ethical committee at Tokushima University Hospital in March 2018.

MIBG Imaging

MIBG imaging was performed 15 min (early) and 3–4 h (delayed) after intravenous injection of ^{123}I-MIBG (111 mBq). The H/M ratio was calculated according to the standard protocol, as previously described (9).

Statistical Analysis

All comparisons between the two groups were performed using Mann-Whitney *U*-tests and Fisher's exact probability tests for continuous and categorical variables, respectively. Jonckheere-Terpstra Test was performed to clarify the relationship between MIBG uptake and frequency of falls. For trend analysis, the subjects were divided into three groups according to MIBG uptake (group 1, delayed H/M ratio $<$ 1.8; group 2, 1.8 $\leq$ delayed H/M ratio $\leq$ 2.7; group 3, 2.7 $<$ delayed H/M ratio). Analyses were performed using SPSS statistics software (IBM; Armonk, NY). The predictive power of MIBG cardiac scintigraphy data for falls was evaluated using Kaplan-Meier analysis. Survival curves were compared using the log-rank test. This analysis was computed using R software (http://www.r-project.org/). $P < 0.05$ were considered statistically significant.

TABLE 1 | Characteristics of patients with PD in the study.

Characteristics	Decreased uptake of MIBG ($n = 29$)	Without decreased uptake of MIBG ($n = 16$)	*P*
Male (*n*)	17	9	0.562
Age at onset	62.7 (9.9)	58.8 (15.5)	0.307
Age at evaluation	67.7 (9.0)	61.3 (15.1)	0.132
Disease duration (*m*)	49.5 (52.6)	31.7 (32.7)	0.203
Hoehn-Yahr stage	1.95 (0.81)	1.77 (0.64)	0.357
Follow-up period (*m*)	89.6 (16.4)	87.8 (19.2)	0.669
Daily levodopa dose (mg)	141.4 (180.3)	82.3 (106.5)	0.188
Use of dopamine agonist (*n*)	13	5	0.286
Early H/M ratio	1.63 (0.25)	2.52 (0.47)	* <0.001
Delayed H/M ratio	1.35 (0.22)	2.60 (0.79)	* <0.001

The results are shown as mean ± standard deviation.
**Indicates a significant difference between the group with decreased uptake of MIBG and that without decreased uptake of MIBG.*
PD, Parkinson's disease; MIBG, ^{123}I-meta-iodobenzylguanidine; n, number; m, months; H/M, heart-to-mediastinum.

RESULTS

We enrolled 45 patients with PD in this study based on the inclusion criteria. Each step of the recruitment process is presented in **Supplementary Figure 1**. The patient characteristics are summarized in **Table 1**. There were no significant differences in sex, age at onset, age at MIBG evaluation, Hoehn-Yahr stage, follow-up period, daily levodopa dose at MIBG evaluation, or use of dopamine agonist at MIBG evaluation between the groups. The group with decreased uptake of MIBG had a higher incidence of falls than the group without decreased uptake of MIBG ($P = 0.022$, log-rank test; **Figure 1**). Next, we investigated the effect of MIBG uptake on severity and frequency of falls. There was no significant difference in the incidence of severe falls between groups divided by MIBG uptake ($P = 0.081$, log-rank test; **Supplementary Figure 2A**). Frequency of falls was significantly associated with MIBG uptake in the delayed phase ($P = 0.017$, Jonckheere-Terpstra test; **Supplementary Figure 2B**).

DISCUSSION

An MIBG-defined imaging phenotype was demonstrated to predict falls in patients with PD in the present study. This supports the potential utility of MIBG as an objective biomarker for prognosis of motor function.

The ideal biomarker is based on a risk factor that is a continuous variable and easy to measure across institutes. MIBG scintigraphy may be an easy and objective biomarker to predict falls, as uptake is standardized using a phantom in this technique. Another candidate biomarker is the β-amyloid 42 level in cerebrospinal fluid, which is associated with gait progression (10). A multifactorial model including MIBG uptake and β-amyloid 42 concentration could lead to development of a biomarker for prediction of falls.

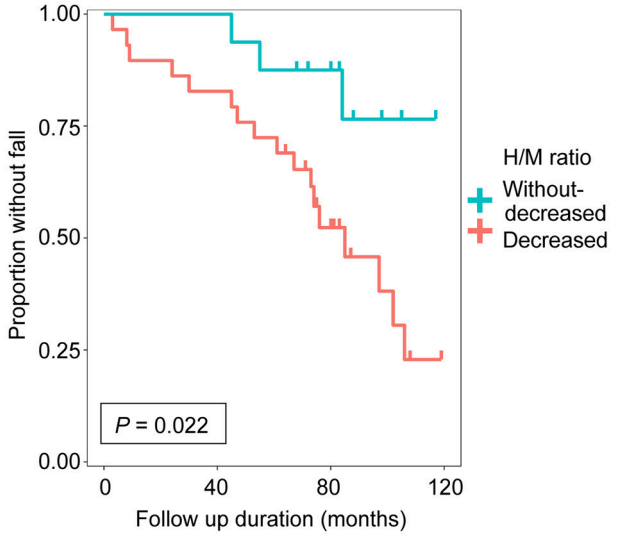

FIGURE 1 | Cumulative fall-free survival based on delayed heart-to-mediastinum ratio (H/M ratio) was shown. The group with decreased uptake of ^{123}I-meta-iodobenzylguanidine (MIBG) ($n = 29$, delayed H/M ratio < 1.8) and the group without decreased uptake of MIBG ($n = 16$, delayed H/M ratio ≥ 1.8).

Reduced MIBG uptake has been reported to be related to dementia and hallucination (6, 11). Motor dual-tasking deficits may predict falls (12), and cholinesterase inhibitors, which are used to treat cognitive impairment, have been reported to improve gait stability (2). Taken together, multiple lines of evidence suggest the presence of a significant association between cognitive function and falls. Considering that both regular falls and cognitive impairment may determine time to death (13), reduced MIBG uptake might predict an unfavorable prognosis in PD. Decreased cardiac uptake of MIBG reflects degeneration of postganglionic presynaptic nerve terminals in the adrenergic nervous system, where the presence of alpha-synuclein aggregates has been pathologically confirmed (14). It is reasonable that orthostatic hypotension is implicated in falls, and that falls are alleviated by droxidopa, which is a prodrug of norepinephrine (3). The relationship between MIBG scintigraphy and falls is understandable, as reduced uptake of MIBG reflects autonomic dysfunction, including orthostatic hypotension. Although MIBG uptake has been reported to be heterogeneous in PD (7), a significant reduction in MIBG uptake might indicate that alpha-synuclein aggregates are distributed widely in the whole body, including the brain. That said, in terms of pathology, decreased uptake of MIBG might be considered as a poor prognostic factor, which was further supported by clinical evidence that decreased uptake of MIBG was associated with cognitive impairment, hallucination, autonomic dysfunction and REM sleep behavior disorder (15). This study adds falls to the above-mentioned list.

The most important subtype of falls from the viewpoint of prognosis is thought to be recurrent/regular falls. Falls have been classified into two subtypes: falling forward and falling backward or sideways. The subtypes are thus based on the direction of the fall, which is reflective of the mechanism underlying each type of fall (16). However, it remains unknown whether there are differences in MIBG uptake and other factors between these subtypes.

The limitations of our study are moderate number of subjects and the use of univariate analysis due to lack of several key factors including cognitive assessment, autonomic function test, gait freezing, motor score, direction of the fall and the relation between falls and drugs, which may have biased the results. Future investigation will be carried out in a prospective cohort using multivariate analysis. This will allow us to adjust for confounders, such as age, sex, disease duration, disease severity, levodopa equivalent dose, mood, hallucination, dementia, and types of falls.

In summary, MIBG scintigraphy may predict motor prognosis as well as cognitive prognosis in patients with PD. Further prospective studies are needed to validate the present findings using multivariate analysis.

ETHICS STATEMENT

This study was retrospective cohort study where the data were produced as a part of standard patient care. In accordance with the Ethics Committee of the Tokushima University Hospital, written informed consent was not required.

AUTHOR CONTRIBUTIONS

WS conceived the idea for this research. NM and WS designed the experiments. WS, SH, TF, YI, and RK recruited the patients. NM, WS, and SH analyzed the data. WS and NM wrote the first draft of the manuscript, with important contributions from YO, HO, YI, MH, and RK. All authors provided input for the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2019.00376/full#supplementary-material

Supplementary Figure 1 | A flow diagram for the present study was shown. The diagram illustrates the inclusion and exclusion criteria used. Data from forty-five subjects with Parkinson's disease were analyzed. PD, Parkinson's disease; MIBG, ^{123}I-meta-iodobenzylguanidine.

Supplementary Figure 2 | Cumulative severe fall-free survival based on delayed heart-to-mediastinum ratio (H/M ratio) was shown **(A)**. The group with decreased uptake of ^{123}I-meta-iodobenzylguanidine (MIBG) ($n = 29$, delayed H/M ratio < 1.8) and the group without decreased uptake of MIBG ($n = 16$, delayed H/M ratio ≥ 1.8). The relationship between delayed H/M ratio and frequency of falls were shown **(B)**. The subjects were divided into three groups according to MIBG uptake (group 1, delayed H/M ratio < 1.8; group 2, $1.8 \leq$ delayed H/M ratio ≤ 2.7; group 3, $2.7 <$ delayed H/M ratio).

Supplementary Table 1 | Injuries due to falls and the number of subjects.

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