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The analysis of dynamic gene expression patterns in peripheral blood of multiple sclerosis patients indicates possible diagnostic and prognostic biomarkers

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ABSTRACT

Introduction: Among numerous invasive procedures for the research of biomarkers, blood-based indicators are regarded as marginally non-invasive procedures in the diagnosis and prognosis of demyelinating disorders, including multiple sclerosis (MS). In this study, we looked into the blood-derived gene expression profiles of patients with multiple sclerosis to investigate their clinical traits and linked them with dysregulated gene expressions to establish diagnostic and prognostic indicators.

Methods: We included 51 patients with relapsing-remitting MS (RRMS, n = 31), clinically isolated syndrome (CIS, n = 12), primary progressive MS (PPMS, n = 8) and a control group (n = 51). Using correlational analysis, the transcriptional patterns of chosen gene panels were examined and subsequently related with disease duration and the expanded disease disability score (EDSS). In addition, principal component analysis, univariate regression, and logistic regression analysis were employed to highlight distinct profiles of genes and prognosticate the excellent biomarkers of this illness.

Results: Our findings demonstrated that neurofilament light (*NEFL*), tumor necrosis factor α (*TNF-α*), *Tau*, and *clusterin (CLU)* were revealed to be increased in recruited patients, whereas the presenilin-1 (*PSEN1*) and cell-surface glycoprotein-44 (*CD44*) were downregulated. Principal Component Analysis revealed distinct patterns between the MS and control groups. Correlation analysis indicated co-dependent dysregulated genes and their differential expression with clinical findings. Furthermore, logistic regression demonstrated that *Clusterin* (AUC=0.940), *NEFL* (AUC=0.775), *TNF-α* (AUC=0.817), *Tau* (AUC=0.749), *PSEN1* (AUC=0.6913), and *CD44* (AUC=0.832) had diagnostic relevance. Following the univariate linear regression, a significant regression equation was found between EDSS and *IGF-1* (R^2 *adj* = 0.10844; p= 0.0060), *APP* (R^2 *adj* = 0.1107; p= 0.0098), and *PSEN1* (R^2 *adj* = 0.1266; p=0.0102).

Conclusion: This study exhibits dynamic gene expression patterns that represent the significance of specified genes that are prospective diagnostic and prognostic biomarkers for multiple sclerosis.

Abbreviations: **CIS**, Clinically isolated syndrome; **CNS**, Central nervous system; **CSF**, Cerebrospinal Fluid; **MRI**, Magnetic resonance imaging; **MS**, Multiple sclerosis; **PPMS**, Primary Progressive multiple sclerosis; **qRT-PCR**, Quantitativereal time polymerase chain reaction; **RRMS**, Relapse remitting Multiple sclerosis; **SPMS**, Secondary progressive multiple sclerosis; **CLU**, Clusterin; **APP**, Amyloid precursors proteins; **NEFL**, Neurofilament light; **TNF-α**, Tumor necrosis factor-α; **AVP**, Arginine vasopressin; **PSEN1**, Presenilin1; **IGF-1**, Insulin-like growth factor-1; **CD44**, Cell-surface glycoprotein-44; **PCA**, Principal Component Analysis.

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1. Introduction

Multiple sclerosis (MS) is a central nervous system neurological condition (CNS). It is also defined as an inflammatory response-mediated neurodegenerative condition characterized by the loss of myelin protein from nerve cell axons (Willis et al., 2015). MS is known as the common cause of disability among the young population. It has twice the prevalence rate in women than in men (Confavreux and Vukusic, 2006). MS is a heterogeneous phenotypical disease because patients of this disease have varying degrees of severity, as well as varying degrees of remission with contrasting extents of recovery (Compston and Coles, 2008; Trapp and Nave, 2008).

Based on clinical variability, MS is classified into four phenotypic: relapse remitting ms (RRMS), progressive relapse remitting ms (PRMS), primary progressive ms (PPMS), and secondary progressive ms (SPMS). Approximately 87% of people are originally diagnosed with RRMS, whereas 10–15% are initially diagnosed with SPMS. Around 65% of RRMS patients eventually develop SPMS. This is referred to as the second stage of MS (Confavreux and Vukusic, 2006). PPMS is the least common type of MS, with only 5% of patients diagnosed with it (Fisniku et al., 2008). When a patient has their first episode of MS, they are classified as having a clinically isolated condition (Polman et al., 2011). There are multiple risk factors for MS, which include Epstein-Barr virus (EBV) infection (Ascherio, 2013), vitamin D insufficiency (Ascherio and Munger, 2007), smoking (Wingerchuk, 2012), and a high salt diet (Farez et al., 2015).

MS pathogenesis is distinguished by an autoimmune response against antigens of the central nervous system (CNS), which results in the continuous activation of inflammatory processes (Selter and Hemmer, 2013). Following the activation of the inflammatory response, myelin-reactive T cells cross the blood-brain barrier (BBB) into the central nervous system. After they cross the blood-brain barrier, the activation of microglia and astrocytes results in the recruitment of inflammatory mediators such as IL-1, IL-6, TNF-α (Ichiyama et al., 1998; Han et al., 2016; Dette and Boettcher, 2017; Jelcic et al., 2018). That's why long-term activation of microglia and macrophages and increased production of cytokines, degrade myelin protein and causes aggravated neuroinflammation, thus causing disintegration of essential components of the neuronal architecture (Inglese, 2006; Rübsamen et al., 2021).

MS is a chronic, progressive immune-mediated central nervous system (CNS) disorder defined by neurodegeneration produced by inflammation, which leads to demyelination and axonal loss (Hemmer et al., 2015). In terms of early MS diagnosis and management, we still have a long way to go. As a result, better biomarkers for MS detection are critically and urgently needed (Chung et al., 2012). Multiple sclerosis biomarkers can help in diagnosis, prognosis of disease progression, and determining the outcome of medication response. Despite the need for biomarkers and extensive effort to develop them, biomarker validation and clinical application in multiple sclerosis remain unresolved. There is still a massive distinction among exploratory biomarkers proposed, verified biomarkers, and biomarkers employed in clinical settings. Therefore, this work employed gene expression profiles from patients diagnosed with multiple sclerosis, to assess the distinguishing factors between the disease group and the healthy group. To accomplish this goal, a selected gene panel which includes Clusterin (*CLU*), amyloid precursor protein (*APP*), neurofilament light (*NEFL*), tumor necrosis factor-α (*TNF-α*), *Tau*, arginine vasopressin (*AVP*), Insulin-like growth factor-1 (IGF-1), Presenilin-1 (*PSEN1*), and Cell-surface glycoprotein-44 *(CD44)* was used to identify new indicators to develop diagnostic and prognostic models that will aid in diagnosis and would assess in predicting disease progression. To understand the role of these genes involved in neurodegeneration (*NEFL, Tau, APP, PSEN1*, and *CLU*), neuroinflammation (*TNF-α* and *CD44*), and neuromodulation (*IGF-1* and *AVP*), , the elaborated pathway was designed on these genes and other genes that have a significant role in the pathogenesis, cell signalling cascade involved in the process of axonal damage and neuronal disintegration (Fig. 1). We hypothesized that several of these genes dysregulation could be influencing each other and could be related to disease activity and disease duration. We hoped to use peripheral blood as an easily accessible source to develop markers of disease identification and disease activity in patients with multiple sclerosis.

2. Materials and methods

2.1. Inclusion and exclusion criteria

Sequential blood samples were taken from patients with RRMS, PPMS, and CIS who matched the inclusion criteria, as well as healthy controls. Patients experiencing complaints of fatigue, coordination problems, vision problems, and bowel and bladder dysfunction, were consecutively recruited from the outpatients' door of the Punjab Institute of Neurosciences from 2019 to 2021. Fifty one patients were selected diagnosed following MS diagnosis according to the revised criteria of McDonald (Thompson et al., 2018). All recruited patients met the following inclusion criteria; patients who were at baseline or had not taken disease-modifying therapies; and patients with no records of neurological documentation, includingmedical history; brain magnetic resonance imaging (MRI); the treatment registered to them and EDSS (Kurland et al., 1963). Those patients were excluded who had a treatment with glucocorticoids within one month of enrolment, DMTs within six months, a history of immunosuppressive treatment (mitoxantrone, cyclophosphamide), chronic infectious and autoimmune illnesses, neoplasms, and acute infections within one month of enrollment were all in the exclusion criteria. Initially, 70 individuals were screened for this study, but later on 51 patients were selected because they met inclusion criteria. Nine patients were excluded from this study because they had taken disease-modifying therapies from the last six months. Three individuals were not chosen because they were under the age of 18, had COVID-19, and lacked neurological data.

For the recruitment of healthy participants, 51 individuals were selected with age $\geq$ 18 years old and with no other neurological disorders or inflammatory diseases.

2.2. Sample collection and RNA extraction

Whole blood (200μL) was obtained through peripheral venepuncture and PBMCs were isolated using density gradient centrifugation (Sigma, Poole, UK) and stored in Trizol reagent (MRC, Catalog No. RT111, USA). Following that, samples collected in the Trizol reagent were processed for RNA extraction. Later on, 500 μL of Chloroform and 0.5mAcetic acid were added to the samples using Trizol reagent for RNA extraction, and the samples were centrifuged at 15,000 rpm for 15 min at 4 °C. Following centrifugation, the top phase was transferred to a clean microfuge tube and an equivalent amount of 100% isopropanol was added. For 20 min, the mixtures were exposed to precipitation at − 20 °C. Again, the content of the tube was centrifuged at 13,000 rpm for 10 min at 4 °C until the clear pellets were recovered. The obtained RNA was extracted in triplicate and measured using a NanoDrop spectrophotometer and gel documentation gear from (Thermo Fisher, USA). In a final volume of 20 μL, single-stranded cDNA was synthesized from 1 μg of total DNAase treated RNA using the Thermo Fisher Scientific, RevertAid First Strand cDNA Synthesis Kit (Catalog No. K1622, USA). Using agarose gel electrophoresis, the cDNA products were screened for genomic DNA contamination.

2.3. Real-time polymerase chain reaction

Quantitative real-time PCR was carried out using the Real-Time PCR System (CFX96, Bio-Rad, USA). The cycling protocol was set as follows: For 40 cycles, 1 min at 95 °C and 30 s at 60 °C. The following cycling procedure was established: For 40 cycles, 1 min at 95 °C and 30 s at 60 °C were used. The procedure for preparing the reaction mixture was

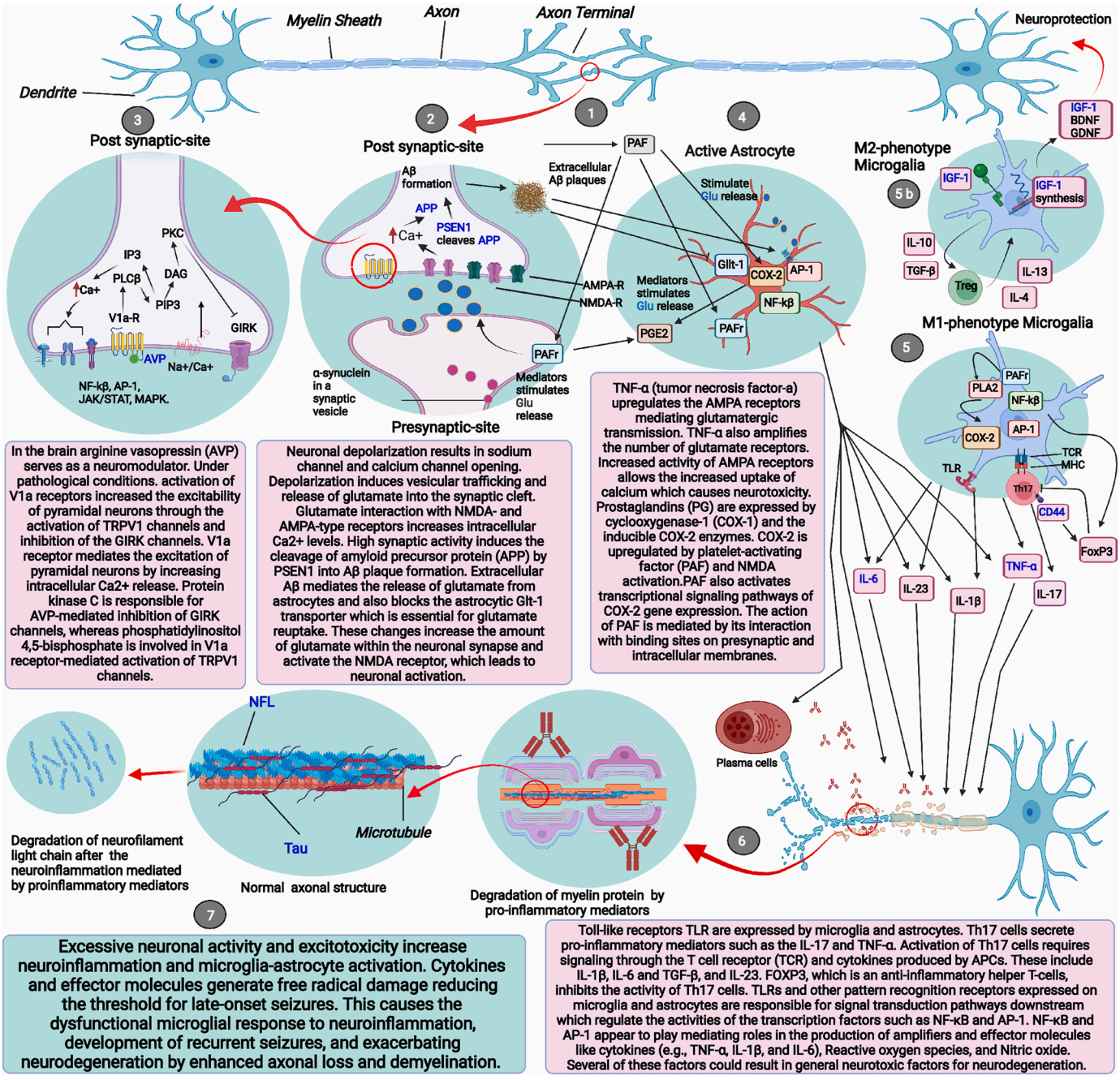

Fig. 1. Overview of targeted genes involved in multiple sclerosis. Details of these pathways are discussed in this figure. Genes selected for this study are indicated in blue color (Created with BioRender.com).

supplied by the SYBR Green I qPCR kit (Catalog No. K0221, Thermo Fisher, USA). Experiments were carried out in duplicates to assure the transparency and quality of the results. Data obtained from qRT-PCR was analyzed and calibrated with housekeeping genes *HPRT1* and *GAPDH* in patient samples. Before including them as a final endogenous control, four endogenous controls (named *HPRT1, GAPDH, TBP,* and *β-actin*expression levels) were measured in given conditions. Finally, those genes were selected that were stably expressed in all experiments. Undermentioned formulas were used to measure the change between the calibrator and the sample of interest:

Fold change $= 2^{-\Delta\Delta Ct}$. (Livak and Schmittgen, 2001).

Log_2fold change $=$ Log(Fold change, 2). (Schmittgen and Livak, 2008).

2.4. Statistical analysis

Statistical analyses were performed with the help of Graph prism V9.0 (GraphPad Software Inc., San Diego, CA, USA). To determine the distribution of the data, the Kolmogorov–Smirnov test of normality was utilized as the sample size was n $\geq$ 50. Upon finding the distribution of the data, non-parametric tests opted for the dataset. The Mann-Whitney U test was used to compare the differences between the delta CT of the two groups. Therefore, no adjustment was built, as no multiple comparisons were made. Continuous data are presented with mean and standard deviation (mean $\pm$ S.D). Before principal component analysis (PCA), the dataset was transformed (Z-score normalization and mean centering). Correlations matrices were calculated using the Spearman rank correlation to analyze the relationship between elevated gene

expression, and clinical outcomes. Univariate linear regression analysis including gene expressions levels, gender, disease duration, and tobacco exposure as independent variables and EDSS as dependent variables were run to identify the best predictors of disease progression. Moreover, logistic regression was exploited to measure the extent of the predictive power of differentially expressed genes. Receiver operating characteristic (ROC) curves were derived from logistic regression to investigate the discriminatory power of selected genes in the disease group compared to control group. All statistical tests were two-tailed, and the significance level was set at p-value = 0.05.

3. Results

3.1. Patients attributes

The detailed attributes of recruited individuals are summarized in Table 1. We included 51 patients with relapsing-remitting multiple sclerosis (RRMS, n = 31), clinically isolated syndrome (CIS, n = 12), and primary progressive MS (PPMS, n = 8). Fifty one control people were selected for this investigation. Furthermore, the gender ratio shows that this condition affects 1.5:1 females compared to males. At the time of onset, the average age was 29.53 ± 13.21 years (mean ± S.D). The average illness duration, on the other hand, was shown to be 1.92 ± 3.247 years (mean ± S.D). The mean EDSS obtained was 2.47 ± 1.274 (mean ± S.D). Clinical data also indicated that the majority of the patients were registered for the disease-modifying medications, such as Interferon beta-1a administration, and Dexamethasone, but they did not take the medication because of the financial situation (Table 2). Only 59% of patients with MS in 51 instances were using Pregabalin, a medicine used to treat neuropathic pain. Lactulose, a laxative, was used by 47% of the patients. Also, antidepressants and other anxiety-management drugs were given to 41% of the recruited individuals. When the tobacco exposure was investigated, it was determined that eight patients were actively exposed to tobacco, with seven of them having RRMS and one having PPMS. Tobacco was introduced passively to 29 patients. Of these, 17 belonged to the RRMS group, 6 were diagnosed with CIS, and the other 6 were PPMS individuals. The never-exposed group consisted of 13 people with MS from all subtypes. There was only one patient in the preceding exposure group.

3.2. MS and control groups are successfully separated by distinct expression profiles of selected genes

The expression levels of each of the chosen genes were examined, and dysregulated profiles were generated. *CLU, NEFL, TNF-α,* and *Tau* were found to be considerably elevated when compared to control participants. Furthermore, *IGF-1, PSEN1,* and *CD44* expression levels were lower in recruited patients compared to controls. Each gene's dysregulated expression patterns are depicted in Fig. 2a and b. However, the expressions of *APP* and *AVP* were not significantly different in patients with multiple sclerosis compared to controls.

Table 1
Characteristics of recruited patients.

Demographic characteristics	Recruited patients (51)	Control group (51)
Gender ratio	20 M/31 F	14 M/36 F
Age at onset (mean ± S.D)	29.53 ± 13.21	–
Age (mean ± S.D)	31.08 ± 12.41	29.89 ± 8.60
Disease duration years (mean ± S.D)	1.92 ± 3.247	–
EDSS	2.47 ± 1.274	–
Active smokers %(n)	16% (8)	8%(4)
Passive smokers %(n)	57% (29)	20%(10)
Ex Smokers %(n)	2%(1)	–
Non-Smokers %(n)	26%(13)	72%(36)

Table 2
Clinical feature of patients with MS subtypes.

Clinical Features	RRMS (31)	CIS (12)	PPMS (8)
Gender ratio	12 M/19 F	4 M/8 F	4 M/4 F
Age at onset (mean ± S.D)	30.99 ± 15.00	30.08 ± 10.8	21.62 ± 5.4
Age (mean ± S.D)	31.9 ± 14.0	29 ± 13.795	25.79 ± 6.54
Disease duration (mean ± S.D)	1.23 ± 2.05	0.58 ± 0.426	4.5 ± 2.10
EDSS	2.36 ± 0.65	1.25 ± 0.58	4.5 ± 1.164
Tobacco Exposure			
Active exposure %(n)	14% (7)	–	2% (1)
Passive exposure %(n	33% (17)	11.5% (6)	11.5% (6)
Previously exposed %(n)	–	2% (1)	–
Never exposed %(n)	14% (7)	10% (5)	2% (1)
Given Treatment			
Pregabalin	8%(4)	41%(21)	%(5)
Lactulose	4%(2)	29%(15)	14%(7)
Baclofen	–	6%(3)	4%(2)
Clonazepam	8%(4)	39%(20)	8%(4)
Escitalopram	4%(2)	23%(12)	6%(3)
Diazpam	12%(6)	17%(9)	6%(3)

We accentuated this research by creating a discriminating expression profile of differentially expressed genes between the MS and healthy groups. A principal component analysis was used to attain this purpose. The combination resulted in two non-overlapping clusters (Fig. 4f). The greatest contributions to this clustering were genes coding for axonal structural integrity, such as *Clusterin, NEFL, Tau,* and *PSEN1*, as well as genes responsible for the immune response (*TNF-α*, and *CD44*) (Fig. 4g).

3.3. Correlation analysis of differentially expressed gene profiles

Having established the unique profiles of the diseased and healthy groups next, we looked at how selected gene patterns are interdependent on one another. We discovered that just a small proportion of the investigated genes were substantially linked with each other (Fig. 3e). Among several genes, IGF-1 has shown noteworthy correlation with *APP* (r= 0.420, p= 0.0024) and *PSEN1* (r= 0.4431, p=0.0011). Gene encoding *APP* showed significant positive correlation with *PSEN1* (r= 0.600, p< 0.0001), and *CD44* (r= 0.390, p= 0.0049). Significant correlation was found between genes coding for axonal structure such as *NEFL* and *Tau* (r= 0.350, p=0.0118). However, non significant correlation was found between *PSEN1* and *NEFL* (r= 0.2550, p=0.0710), *NEFL* and *APP* (r= 0.240, p= 0.0873), and *PSEN1*, and *CD44* (r= 0.2711, p= 0.054).

3.4. Disparate profiles of selected genes have shown a significant correlation with EDSS, indicating potential prognostic markers

Half of the selected genes have shown an association with clinical parameters. While investigating the correlation of dysregulated expression profiles of genes with clinical attributes, a significant correlation was found between *Clusterin* and disease duration (r= −0.3053, p= 0.0294) (Fig. 3a). Moreover, respective genes have shown their association with EDSS, such as significant correlation was found between *IGF-1* and EDSS (r= 0.5193, p< 0.0001) (Fig. 3b), *PSEN*1 and EDSS (r= 0.4445, p= 0.0011) (Fig. 3c), and *APP* and EDSS (r= 0.4559, p= 0.008) (Fig. 3d).

To prominently emphasize the significance of *IGF-1, PSEN1* and *APP* in predicting disease progression, we further investigated their relationship using univariate linear regression. Univariate analysis results are summarized in Table 3. The results of this analysis indicated that significant regression equation was found between EDSS and *IGF-1* (R^2 *adj* = 0.1266; β:0.08371; 95% CI= 0.02513–0.1423; p=0.0060), *APP* (R^2 *adj* = 0.1107; β:0.1171; 95% CI= 0.02956–0.2047; p=0.0098), and *PSEN1* (R^2 *adj* = 0.1095; β:0.1222; 95% CI= 0.03034–0.2141;

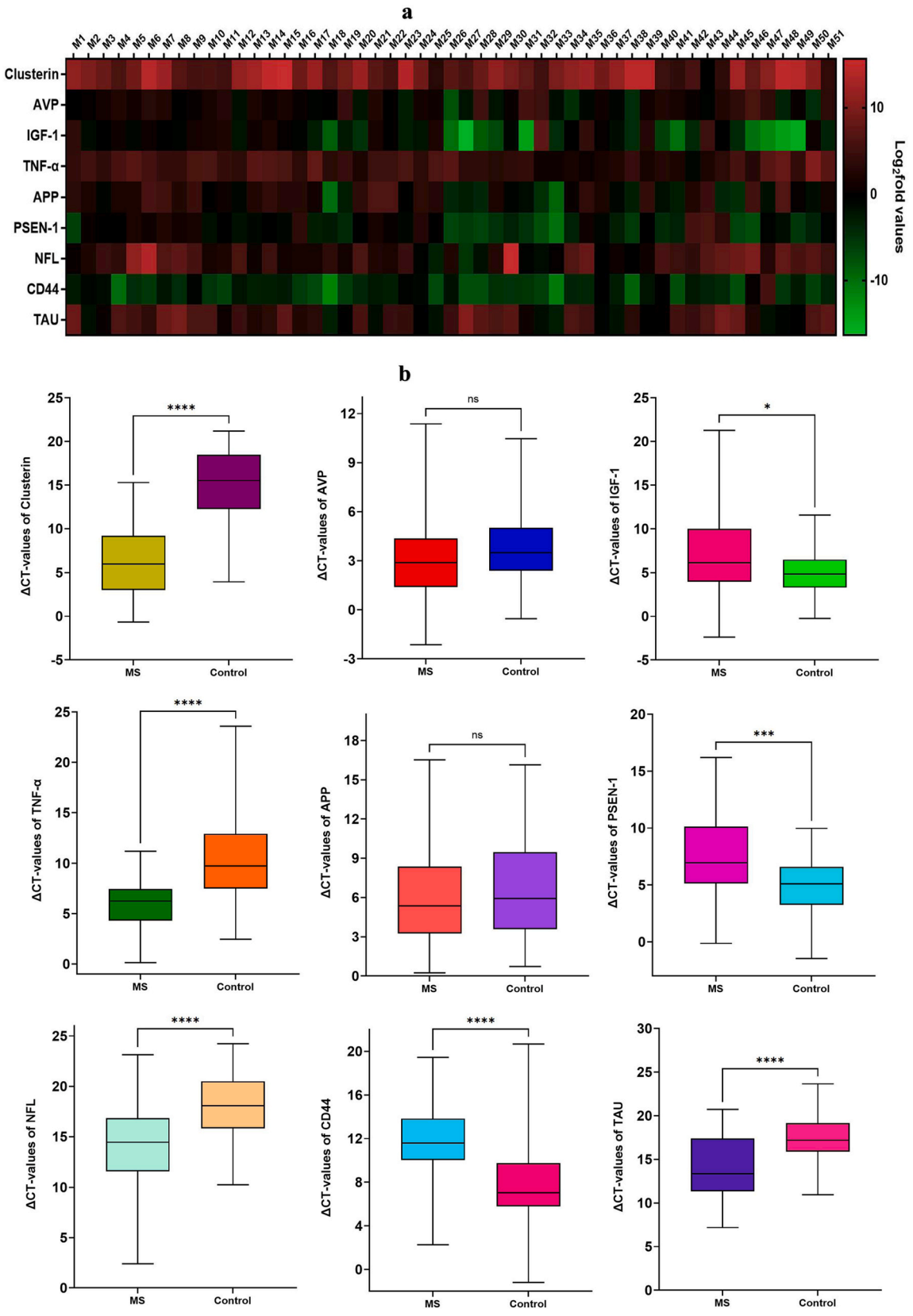

Fig. 2. Differentially expressed profiles of selected genes. **(a)** illustrates a heatmap of 9 differentially expressed genes in 51 individuals diagnosed with MS. Disparate profiles of dysregulated genes are elucidated in **(b)**. Interquartile range box are representing ΔCt-values of recruited patients in comparison to the control group. Smaller ΔCt-values indicate higher expression, while larger ΔCt-values indicate lower expression of the respective group. * : $p < 0.05$; * *: $p < 0.01$;* ** : $p < 0.001$; * ** *: $p < 0.0001$.

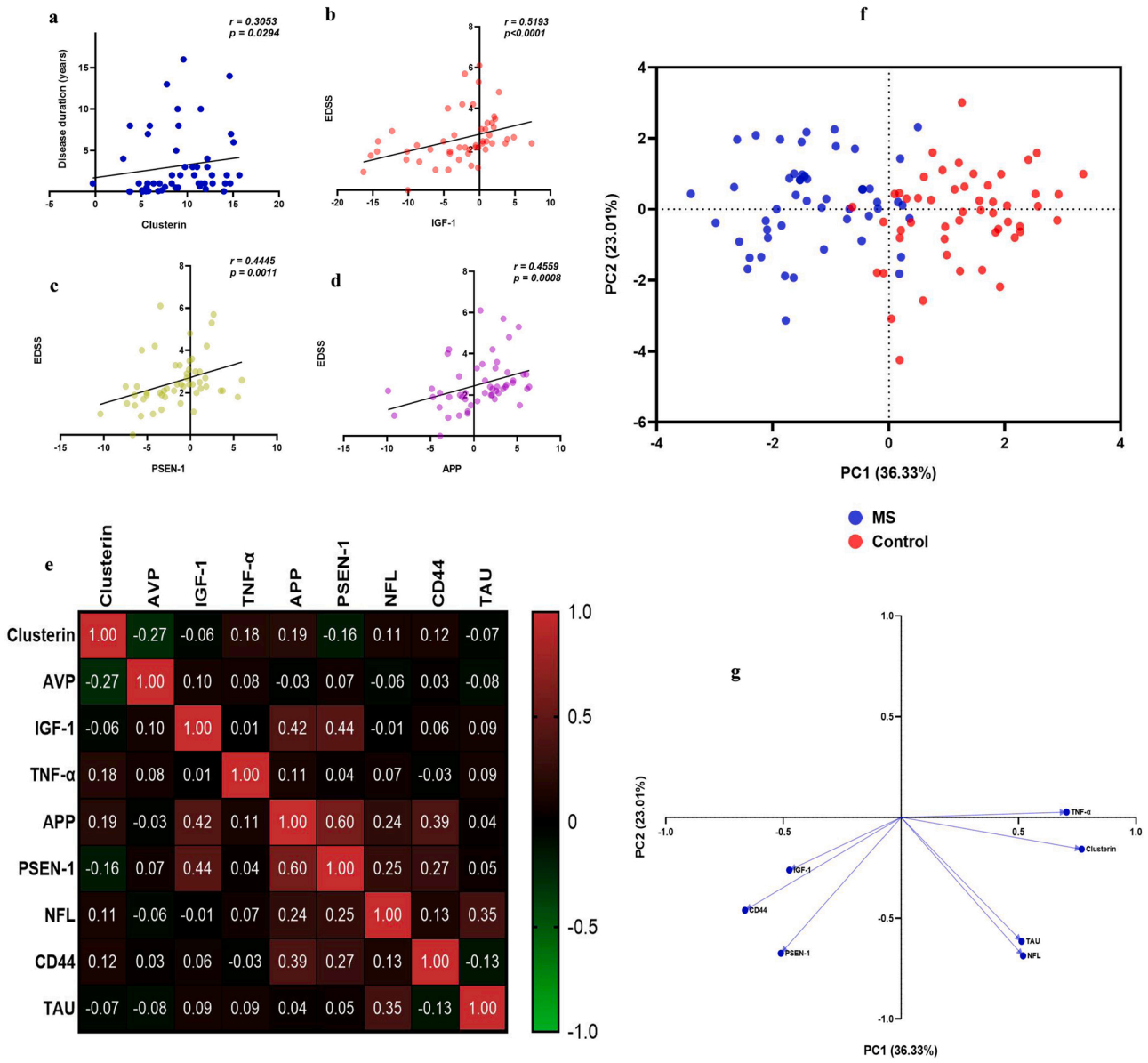

Fig. 3. Correlational analysis between differentially expressed genes, clinical variables, and principal component analysis was performed to associate the distinguished profiles of selected groups and to establish the correlation of genes dysregulation with each other and with clinical variables. A significant correlation was established between **(a)** clusterin and disease duration, **(b)** *IGF-1* and EDSS, **(c)** *PSEN1* and EDSS, and **(d)** *APP* and EDSS using Spearman's rank correlation coefficient. Correlational matrix **(e)** was constructed between dysregulated gene expression to reveal the co-dependency of genes. The individual plot is illustrated in **(f)**. Distinct clusters were produced which successfully segregated disease samples from control samples. Samples are represented by dots and each color represents a group of individuals who were recruited. Panel **(g)** is representing a variable plot. Arrows graphically represent the direction of each contributing gene. The longer the arrow is, the more it influences the variance.

p=0.0102).

Thus, a noteworthy association of these genes with EDSS indicated their prospective prognostic pattern to measure the coarse of disease development in the individuals diagnosed with multiple sclerosis.

3.5. Using logistic regression to develop diagnostic factors from distinct gene expression profiles

The area under the curve (AUC) of ROC analysis was used to assess the discriminatory performance of data between MS and control groups. The findings of a logistic regression analysis of given genes with confidence interval and standard error are presented in Fig. 4. The given figure is explains the performance of each gene selected and the performance of each model generated by a single gene. The most discriminatory and outstanding predictors with positive predictive power (PPP) and negative predictive power (NPP) were *PSEN1* (AUC= 0.6913; PPP= 65.12%; NPP= 61.02%) (Fig. 4a), *Tau* (AUC= 0.749; PPP= 73.33%; NPP= 68.42%) (Fig. 4b), *Clusterin* (AUC= 0.940; PPP= 90.20%; NPP= 90.20%) (Fig. 4c), *CD44* (AUC= 0.832; PPP= 76.92%; NPP= 78.00%) (Fig. 4d), *TNF-α* (AUC= 0.817; PPP= 75.47%; NPP= 77.55%) (Fig. 4e), and *NEFL* (AUC= 0.775; PPP= 70.21%; NPP= 67.27%) (Fig. 4f). However, *IGF-1* (AUC= 0.644; PPP= 61.90%; NPP= 58.33%) (Fig. 4g) has shown weak discriminatory levels.

4. Discussion

MS pathogenesis commences when the immune system targets central nervous system antigens, triggering an auto-reactive T-cell response (Hafler et al., 2005; Selter and Hemmer, 2013). T lymphocytes representing myelin become activated and breach the blood-brain barrier

Table 3
Linear regression analysis.

Variable	Univariate Analysis				
	Estimate	95% CI	p-value	R^2	R^2 Adj.
Clusterin	0.05662	-0.0350–0.1483	0.2206	0.0304	0.01068
AVP	0.03562	-0.0899–0.1612	0.5713	0.0065	-0.01369
IGF-1*	**0.08371**	**0.02513–0.1423**	**0.0060**	**0.1441**	**0.1266**
TNF-α	0.01805	-0.1421–0.1782	0.8217	0.0010	-0.01934
APP*	**0.1171**	**0.02956–0.2047**	**0.0098**	**0.1285**	**0.1107**
PSEN1*	**0.1222**	**0.03034–0.2141**	**0.0102**	**0.1273**	**0.1095**
NEFL	-0.005921	-0.0924–0.0800	0.8911	0.0003	-0.02001
CD44	0.02457	-0.0874–0.131	0.6627	0.0039	-0.01641
TAU	-0.01229	-0.107–0.0828	0.7963	0.0013	-0.01901
Tobacco Exposure	0.5299	-0.1720–1.232	0.1357	0.04486	0.02537
Disease duration*	**0.2430**	**0.1618–0.3242**	**< 0.0001**	**0.4246**	**0.4129**
Gender	-0.002419	-0.7087–0.7038	0.9945	0.000001	-0.02041

* Significant results of independent variables are in bold.

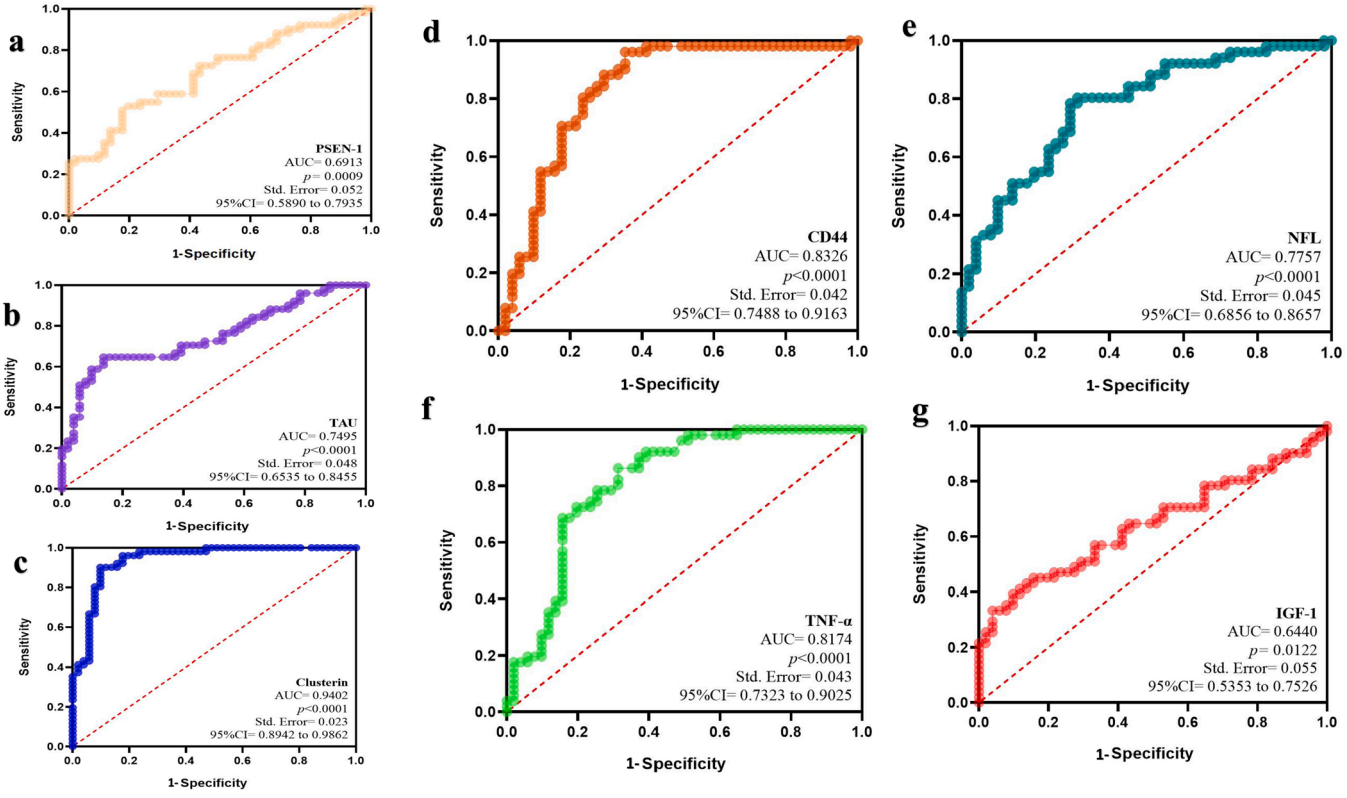

Fig. 4. Logistic regression analysis of potential predictive markers to establish a discriminatory power between disease group and control group. The given figure is explaining the performance of each gene selected i.e. (**a**) *PSEN1*, (**b**) *Tau*, (**c**) *Clusterin*, (**d**) *CD44*, , (**e**) *NEFL*, (**f**) TNF-alpha, and (**g**) IGF-1.

(BBB) and enter the central nervous system (Dette and Boettcher, 2017). They allow additional inflammatory mediators to recruit, resulting in the activation of microglia and macrophages, which eventually leads to demyelination (Jelcic et al., 2018; Inglese, 2006). Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) are two examples of particular proinflammatory mediators. Numerous studies have found that inflammatory mediators impair neuronal function by increasing neurodegeneration (Cheng and Mattson, 1995; Lobo-Silva et al., 2016). Our results showed that *TNF-α* levels were increased in the MS group compared to the control group. Another study also observed a similar trend in the monocytes of individuals diagnosed with MS. On the contrary note, they investigated RRMS patients receiving disease-modifying therapies (DMTs), concluding that DMTs didn't decrease the production of inflammatory cytokines in patients with MS (Fiedler et al., 2017). The release of TNF-α is also a key, positive regulator of CD44 hyaluronan binding in peripheral blood monocytes (Levesque and Haynes, 1997). We also investigated the *CD44* levels and, they were found to be greater in the MS group than in the control group. Several additional investigations have suggested the similar dysregulation of *CD44* gene in multiple sclerosis (Teder et al., 2002; Liang et al., 2007; van der Windt et al., 2010). CD44 is a type I transmembrane glycoprotein that has been linked to a variety of immune-related biological functions. It is involved in alternative splicing, post-translational modifications, and a variety of physiological functions, including cellular adhesion, myelopoiesis, lymphopoiesis, angiogenesis, and cytokine release (Ponta et al., 2003).

Axonal damage is thought to be the primary cause of disability in individuals with MS. Neurofilaments and Tau proteins, which are exclusively produced in nerve cells, are key structural elements of axons and dendrites (Bjartmar and Trapp, 2001). In other neurodegenerative illnesses, Tau and NEFL are disintegrated when an axonal injury occurs

(Román et al., 2012a, 2012b). Multiple studies have found that elevated levels of plasma *NEFL* and *Tau* are associated with the worst cognition and neuroimaging measures of cortical thickness, cortical atrophy, white matter hyperintensity (WMH), or white matter integrity across neurodegenerative diseases, including multiple sclerosis (Jaworski et al., 2012; Kuhle et al., 2017; Khalil et al., 2018; Lewczuk et al., 2018; Mielke et al., 2019). Tau is a well-known diagnostic biomarker, and NEFL is recognized as both a diagnostic and prognostic indicator for multiple sclerosis (Jaworski et al., 2012; Kuhle et al., 2017; Yuan et al., 2017; Gaetani et al., 2019). In this study, *Tau* levels were disclosed to be increased in the disease group when compared to the control. Previously, one study indicated the CSF levels of Tau protein in MS patients. However, they found a significant correlation between EDSS and tau in reported patients. Their results indicated that tau proteins are thought to represent neuronal damage, and tau leaks into the CSF compartment following axonal injury. They concluded that brain atrophy appeared early in the course of MS and was found in CIS patients who progressed to MS (Jaworski et al., 2012). In accordance with several studies, *NEFL* levels in this study were uncovered to be elevated in the disease group. The release of *NEFL* bolstered the two concurrent neurodegenerative processes in MS: the first as a result of chronic brain-diffuse neuroinflammation and the second as a result of acute localized inflammatory activity during plaque development (Kuhle et al., 2017; Yuan et al., 2017; Gaetani et al., 2019). One piece of evidence revealed the correlated *NEFL* levels of CIS, RRMS, and PPMS patients with blood-CSF barrier damage and relapses. Furthermore, they associated the *NEFL* levels with disability in earlier CIS and RRMS (Kuhle et al., 2017). However, the findings of our study revealed a poor association between *NEFL* and disease advancement, indicating that the majority of the patients were at baseline or were avoiding disease-modifying medications.

IGF-1 is implicated in immune system modulation, oligodendrocyte proliferation, and survival (Ahmed et al., 2016). In this study, IGF-1 levels were shown to be downregulated in the Multiple Sclerosis group, which may or may not explain the neurotoxicity and implications of long-term remyelination. One report, linked an increase in serum *IGF-1* to the early stages of MS (Hosback et al., 2007). However, another study conducted by Nageeb et al., couldn't observe the significant difference between individuals with MS and the control group (Nageeb et al., 2018). One piece of evidence corroborated our findings and concluded that there was a considerable rise in blood *IGF-1* levels in MS patients when compared to controls (Ghassan et al., 2017). In our investigation, *IGF-1* levels were shown to be substantially associated with EDSS. Interestingly, despite non-significant levels of IGF-1 in the MS group, Nageeb and his associates identified a substantial negative connection with EDSS, disease duration, and frequency of attacks, indicating the presence of a link between serum *IGF-1* levels and disability accumulating in people with MS (Nageeb et al., 2018).

A study conducted and investigated the CSF levels of *PSEN1* and they found that these levels were significantly increased in Alzheimer's patients (García-Ayllón et al., 2013). Recently, a study conducted measured the PBMCs levels of *PSEN1* in RRMS patients with IFN-β therapy and patients without IFN-β therapy. They found that there was a significant increase in the expression of *PSEN1* indicating the ability of IFN-β ability to induce the expression of proteases such as *PSEN1* that have been shown to cleave the transmembrane of cell surface receptor IFNAR (Aliaga-Gaspar et al., 2021). According to our findings, *PSEN1* expression levels in all types of MS were downregulated. Furthermore, they were significantly correlated with EDSS. There is a deficiency of evidence of *PSEN1* related investigation in multiple sclerosis, therefore, further research is needed to explore this area.

Previously, a study conducted while examining the CSF of patients diagnosed with MS suggested that the abundance of clusterin was abundant in the CSF of patients with MS. They explained it by implicating its role as a carrier of several proteins across the blood-brain and CSF barrier, such as amyloid beta. The amyloid precursor protein is upregulated during an axonal injury in MS, and clusterin may clear it and prevent aggregation of β-amyloid, eventually ending up in the CSF to control ongoing inflammation in the MS brain by functioning as a complement inhibitor (van Luijn et al., 2015). Until now, there was no indication of clusterin expression in the peripheral blood of patients diagnosed with MS. In this study, our results showed that *CLU* levels were upregulated in the peripheral blood of the MS group when compared to the control group.

Myelin loss and disintegration of myelin basic protein were associated with an increase in amyloid beta peptides (Roher et al., 2002). Several other studies (Mitew et al., 2010; Ou-Yang and Van Nostrand, 2013) have associated the reduced manifestation of myelin basic protein gene (*MBP*) in the region presenting amyloid beta accumulation and decreased amyloid beta deposition in white matter areas exhibiting greater expression of *MBP27*. A study conducted by Pietroboni and his co-workers investigated the levels of amyloid beta precursors in CSF of patients with MS. They found that the levels of amyloid beta precursors were significantly higher in patients with MS. High levels have been linked to ongoing neurodegeneration (Pietroboni et al., 2017). On the contrary, *APP* expression patterns haven't shown significant dysregulation in our dataset. A major reason for this is we recruited most of the patients which were recently diagnosed. Despite non-significant dysregulation, correlation analysis had disclosed the association of *APP* expression patterns with EDSS, indicating that the dysregulated expression of this gene is related with a measure of disease progression. This behavior signifies its important role to become a prognostic marker. Thus, further studies are needed to confirm this predictive activity.

Another neuromodulator that has an impact on the vulnerability of inflammatory and immunological diseases is Arginine Vasopressin (AVP). AVP is a stress hormone released from the neurohypophysis due to an imbalance in osmotic and homeostatic regulation. During this feedback mechanism, corticotropin-releasing hormone and ADH are discharged from the hypothalamus and pituitary corticotrophs (Kern et al., 2013). Several clinical data produced had supported the hyperactivity of the HPA axis and increased release of AVP in most of the patients with multiple sclerosis (Erkut, 1998; Melief et al., 2013; Baranowska-Bik et al., 2015). Contrarily, results produced by our data do not significantly discriminate the MS group from the control group. One piece of evidence indicated that enhanced expression of *AVP* is strongly associated with disease duration (Melief et al., 2013). However, this was not the case in our study as most of the study participants were recently diagnosed. These investigator reached this conclusion by investigating human brain tissues and CSF. Their findings disclosed that HPA axis activation and pronounced release of AVP, as seen in MS patients, may have a direct influence on lesion activity, molecular pathways, and enhanced disease severity. Furthermore, higher secretion of *AVP* in MS may be associated with high levels of neurodegeneration, whereas low expression was linked with reduced neurodegeneration (Melief et al., 2013).

The goal of this study was to look at blood-derived differentially expressed gene patterns to construct viable biomarkers. Therefore, those genes were selected (*CLU, TNF-α, PSEN1, NFL, CD44, Tau, IGF-1, APP,* and *AVP*) that have been reported earlier in white matter lesions (Wilczak and De Keyser, 1997;Bitsch et al., 2000; van Luijn et al., 2015; Saraste et al., 2021), hypothalamus (Huitinga et al., 2004), CSF of patients with multiple sclerosis (Jaworski et al., 2012) or had a significant reported role in pathological processes of neurodegenerative disease (Hosback et al., 2007). Various statistical methods were used for the targeted genes to broaden the scope of previously recognized neurodegenerative indicators. *PSEN1* and *CLU* were quantified, and for the first time, they have been shown to be a diagnostic biomarker of multiple sclerosis Several genes in this study have shown to be the new blood-based prognostic biomarkers of Multiple sclerosis such a such as *APP, PSEN1*, and *IGF-1*. Certain gene expressions patterns were shown to be associated with one another using correlational analysis such as *IGF-1* association with *APP, PSEN1, APP* association with *PSEN1, CD44,* and *NFL* with *Tau* .

Nonetheless, this study possesses potential limitations. The number of variables investigated may be expanded to further assess the potential usefulness of a single diagnostic marker. In addition, the severity of the disease must be assessed with the time between follow-ups. Furthermore, biofluids such as CSF and neuroimaging tests are required to validate the use of identified biomarkers. Finally, a larger number of patients may have boosted the statistical power of this study. Our top objective was to include patients who met tight selection criteria throughout their recruitment.

In conclusion, our study suggests the use of blood-derived *clusterin*, *NEFL, TNF-α, Tau, CD44, Tau,* and *IGF-1* for the diagnosis of multiple sclerosis. Furthermore, *APP, PSEN1,* and *IGF-1* will help in identifying those patients who will have disease severity. This study utilized multiple statistical methodologies to broaden the perspective of clinical data with dysregulated gene expressions. This provided the most comprehensive understanding of dysregulated gene expression profiles and how their expression patterns impact each other's expressions as well as the clinical manifestations of individuals with multiple sclerosis.

Finally, these findings could stimulate more research to dissect further the involvement of these genes in the mechanisms of disease parthenogenesis and aggressiveness, causing fatigue, and cognitive impairment in people diagnosed with multiple sclerosis. Our findings hold the future prospects to explore them in the animals models that how these genes are co-regulating blood based expression in the patients of multiple sclerosis.

Ethical statement

The study was carried out following the Ethical Review Committee's (ERC-70–2021) instructions and was authorized by the Institutional Review Board (IRB-307/07–2021-B) of Forman Christian College (A Chartered University), Lahore, Pakistan. Before taking part in the study, each participant signed a written permission form. The research was done following the Helsinki Declaration.

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CRediT authorship contribution statement

RR was majorly involved in the conduct of the study, data analysis, and writing manuscript. FM interpreted the clinical reports and helped in data analysis. RA helped in the sample collection and experimental conduct. SM diagnosed the patients and helped in sample collection. KM provided clinical support in the Punjab Institute of Neurosciences and edited the draft of the manuscript. DNB designed, analyzed, and reviewed the manuscript.

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Declaration of Competing Interest

The authors involved in the study have represented no conflict of interest. Declarations of interest are none.

Authors agreement

All authors have provided consent for the publication of the study and its results.

Data availability

The datasets used during the current investigation are accessible upon reasonable request from the corresponding author.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.molimm.2022.05.002.

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