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BDNF-AS as a promising downregulated biomarker for multiple sclerosis pathogenesis and diagnosis

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Abstract

Multiple sclerosis (MS) is a prevalent neurological disorder affecting the central nervous system, with a global incidence exceeding 2.5 million cases. Brain-derived neurotrophic factor-antisense (*BDNF-AS*), a long non-coding RNA, has been identified as a factor that negatively influences the expression of brain-derived neurotrophic factor, a neurotrophin protein that exhibits heightened expression within actively demyelinating lesions in MS. This study aims to assess the relative expression of *BDNF-AS* across all MS subtypes, evaluate its diagnostic accuracy, and explore correlations between *BDNF-AS* expression and disease parameters. Quantitative real-time polymerase chain reaction was employed to quantify the expression levels of *BDNF-AS* in the serum samples of 54 individuals diagnosed with various types of MS and 20 healthy controls. Statistical analyses were performed to assess the correlation and diagnostic efficacy of *BDNF-AS* expression levels. The expression of *BDNF-AS* was markedly reduced in individuals with MS compared to the control group ($P < 0.01$). Notably, the highest expression levels were observed in patients diagnosed with secondary progressive MS. Using a defined cutoff value of 0.31, and the findings suggest that *BDNF-AS* expression in serum has notable potential as a specific and sensitive diagnostic marker for MS. In conclusion, this study provides a comprehensive evaluation of *BDNF-AS* across all MS subtypes, highlighting its diagnostic accuracy and the association between elevated *BDNF-AS* expression and disease progression in secondary progressive MS. Further research is needed to validate these results.

Keywords: Multiple sclerosis; Brain-derived neurotrophic factor; Brain-derived neurotrophic factor-antisense; Relapsing-remitting multiple sclerosis; Secondary progressive multiple sclerosis; Primary progressive multiple sclerosis; Gene expression

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1. Introduction

Neurodegeneration refers to the progressive loss of neuronal function within the central nervous system (CNS), affecting millions of people and posing a major medical challenge worldwide.[1] Multiple sclerosis (MS) is a neurodegenerative condition that results in the degeneration of neuronal axons and gliosis within the CNS.[2] According to the 2013 MS Atlas, the Middle Eastern, and North African nations are classified as low- to moderate-risk regions for MS.[3] It is characterized by ongoing inflammation within the CNS, which results in tissue damage, leading to brain and spinal cord atrophy and irreversible neurological impairment.[4] MS is considered the most prevalent neurological disease among young adults, affecting more than 2.5 million people globally.[5] In Africa and the Middle East, Egypt has the highest incidence of MS, with 59,671 documented patients.[6]

The majority (~85 – 90%) of MS cases initially present a relapsing-remitting disease course (i.e., RRMS), whereas 10% of cases develop primary progressive MS (PPMS). About 90% of RRMS patients eventually progress to secondary progressive disease (i.e., SPMS).[7] The causes of progressive MS are not as well understood as those of relapsing forms, which involve disruptions in the immune system.[8] While inflammation is the primary driver of MS activity, neurodegeneration is also thought to play a major role in the disease progression.[9]

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides that do not have the functional ability to code for proteins.[10,11] The functions of lncRNAs, such as those of protein-coding genes, are diverse and can vary greatly.[12] LncRNAs have been shown to play a role in various significant biological processes through interactions with DNA or chromatin, modulation of regulatory or signaling pathways, and the regulation of other types of ribonucleic acid (RNAs).[13] Some lncRNAs may function as decoys, binding to effector proteins and preventing them from carrying out their intended functions by diverting them from their target sites.[10] Certain lncRNAs contain so-called "microRNA (miRNA) response elements," which are complementary binding sites for miRNAs. These lncRNAs are known as competitive endogenous RNAs (ceRNAs), which act as molecular sponges for miRNAs, inhibiting their ability to reach target genes.[14] Despite typically having lower expression levels than messenger RNAs, lncRNAs often display more distinctive expression patterns specific to certain tissues and cells, suggesting that they play crucial roles in cell-specific pathways.[15]

The CNS is composed of a variety of cell types with distinct morphologies and functions. Neurotrophic lncRNAs are believed to influence the genetic and epigenetic regulation of the expression of genes involved in the CNS.[16,17] Numerous lncRNAs expressed in the CNS have been found to play critical roles in the functions of neural cells, neurodevelopment, and the regeneration and differentiation of neural stem cells.[18] Brain-derived neurotrophic factor-antisense (*BDNF-AS*), a type of lncRNA, is known for its role in regulating the expression and function of the neurotrophin protein brain-derived neurotrophic factor (BDNF) within the CNS.[19,20]

The downregulation of *BDNF-AS* has been shown to inversely upregulate BDNF protein levels, resulting in a pro-neuronal effect by stimulating neuronal cell growth and differentiation.[21,22] This effect is believed to be mediated by BDNF, which is recognized as a critical regulator of neuronal plasticity, making it one of the most significant proteins in this context.[23] Importantly, BDNF is overexpressed by immune cells in MS lesions,[24] and other studies have demonstrated that BDNF protein levels are reduced simultaneously in the serum or plasma of MS patients compared to controls.[25-30] Therefore, the level of *BDNF-AS* has become an important focus of research in MS in investigating its role in regulating BDNF in MS and its potential pathophysiological function.

To our knowledge, the expression of *BDNF-AS* has not been thoroughly investigated in neurological diseases within patient blood samples; it has only been analyzed in cell models and postmortem patient tissues. Moreover, the only study on *BDNF-AS* in MS focused on whole blood from RRMS patients, where no significant differences were found compared to controls.[31] Therefore, our study aims to be the first to comprehensively investigate the expression of *BDNF-AS* across different MS subtypes and explore its relationship with disease prognostic parameters. This research will contribute to understanding the role of BDNF in MS and examine the diagnostic and prognostic potential of *BDNF-AS* in MS.

2. Methods

2.1. Subject recruitment

This study involved 54 individuals diagnosed with MS at multiple stages of the disease alongside 20 healthy volunteers, carefully matched for gender and age. Recruitment occurred between January and May 2021, with participants sourced from the MS Research Unit at Cairo University Hospital in Kasr Al-Ainy, Cairo, Egypt. Written informed consent was obtained from all participants, both patients and controls, before their involvement. Ethical approval (approval number: 19922) was granted by the Board of Ethical Committee, Suez University, Egypt, ensuring compliance with the ethical principles outlined in the Declarations of Helsinki (1964) and its subsequent revisions.[32]

2.2. Characteristics of subjects

All participants were diagnosed with MS based on the guidelines set forth by the International Panel on the Diagnosis of MS, employing the McDonald's criteria (2010).[33] A total of 74 participants were included in the study, comprising 31 cases from the RRMS group, 13 from the SPMS group, 10 from the PPMS group, and 20 healthy volunteers as controls. The RRMS and SPMS groups were subdivided as follows: 16 with relapsing RRMS, 15 with remitting RRMS, eight with relapsing SPMS, and five with remitting SPMS. The degree of clinical disability was evaluated using the Kurtzke expanded disability status scale (EDSS).[34]

2.3. Exclusion criteria

Individuals were deemed ineligible for the research if they had any of the following conditions: cancer, liver disease, diabetes mellitus, autoimmune disorders, hypertension, cerebrovascular disease, pregnancy, infectious or inflammatory diseases, a history of alcohol abuse, or smoking, based on the specified eligibility criteria.

2.4. Demographics and disease characteristics in MS subjects

Clinical data collected from all participants in this study were as follows:

(i) Frequency of relapses in the previous 3 years
(ii) Age and sex
(iii) EDSS score
(iv) History of family MS or other autoimmune diseases
(v) Current disease status (relapse/remitting) at the time of sampling
(vi) History and duration of MS
(vii) Patient's clinical history.

2.5. Specimen collection

Five milliliters of blood were drawn from both MS patients and the 20 healthy volunteers. The blood samples were incubated for 30 min at 37°C and then centrifuged for 10 min at 3000 rpm. The resulting serum was divided into two fractions and stored at −80°C until required for analysis.

2.6. RNAs extraction

RNAs were extracted using the QIAGEN serum and plasma RNA extraction kit (catalog number 217184, Hilden, Germany). A 200 µL serum aliquot was processed according to the manufacturer's instructions. Approximately 14 µL of RNA was eluted, and the purity and concentration were assessed using the One/One^{C} Microvolume ultraviolet-Vis NanoDrop™ Spectrophotometer (ThermoFisher, Singapore). The eluted RNA was stored at −80°C until needed.

2.7. Complementary deoxyribonucleic acid (cDNA) synthesis

cDNA was synthesized from 2.5 µg of total RNA using the QIAGEN RT² First Strand Kit (catalog number 330404, Hilden, Germany), following the manufacturer's guidelines. The process involved three stages: (i) genomic DNA removal at 42°C for 5 min, (ii) reverse transcription at 37°C for 60 min to optimize reverse transcriptase activity, and (iii) enzyme denaturation at 95°C for 5 min. All thermal cycles were performed using the SimpliAmp™ Thermal Cycler (Applied Biosystems, Singapore).

2.8. Real-time polymerase chain reaction (PCR)

Real-time PCR was used to evaluate the relative gene expression of the target gene (*BDNF-AS*) and the housekeeping gene (*GAPDH*). Pre-designed primers from QIAGEN (catalog number 330701, Hilden, Germany) were utilized, with the following identifications for each gene: *GAPDH* (LPH15126A-200) and *BDNF-AS* (LPH15814A-200). The amplification was carried out using QIAGEN's RT² SYBR Green ROX quantitative PCR Mastermix (catalog number 330520, Hilden, Germany). Forty amplification cycles were performed, each consisting of 15 seconds at 95°C and 1 min at 60°C. After amplification, a melting curve analysis was performed to assess the PCR products. All processes were carried out using the Real-Time PCR QuantStudio™ 1 (Applied Biosystems, Singapore).

2.9. Relative gene expression

The relative expression of *BDNF-AS* was calculated for each sample using the double-delta cycle threshold (Ct) method. The Ct values of all samples were normalized against *GAPDH*. The fold change for each sample was calculated and compared between patients and normal controls to investigate the relative expression of *BDNF-AS*.

2.10. Statistical analysis

Statistical analyses were conducted using Statistical Package for the Social Sciences Statistics (IBM, version 26, USA). A normality test was performed on all data to differentiate between non-parametric and parametric data. The non-parametric Kruskal–Wallis test was utilized to evaluate variations among groups. Multiple comparison tests were applied to identify the source of any significant differences between groups. The non-parametric Spearman's correlation test was used for correlation analysis. Receiver operating characteristic (ROC) curve analysis was also performed.[35] A $P < 0.05$ was considered statistically significant. Microsoft software was used to create all graphs and tables.

3. Results

3.1. Demographics and disease characteristics in MS subjects

When comparing age and sex across the disease groups and the control group, no differences were observed, indicating that all groups were age- and sex-matched. Regarding family history of MS, only 2 cases (6.5%) within the RRMS group and 1 case (7.7%) within the SPMS group reported a family history of the disease. The relapse/remitting classification within the RRMS and SPMS groups revealed 16 relapse RRMS cases, eight relapse SPMS cases, 15 remitting RRMS cases, and five remitting SPMS cases. The number of relapses within the past 3 years (median ± interquartile range) was 2 ± 2 for RRMS and 4 ± 2 for SPMS. The mean ± standard deviation of the EDSS scores were 3.05 ± 2.74 for RRMS, 6.00 ± 1.02 for SPMS, and 5.55 ± 1.14 for PPMS. The demographic and clinical characteristics of all included subjects and controls are summarized in Table 1.

3.2. Evaluation of glyceraldehyde-3-phosphate dehydrogenase as a suitable reference gene

The suitability of *GAPDH* as a reference control gene was assessed by examining the average Ct across all MS patients and healthy control volunteers. The mean Ct values (± standard error [S.E.]) for *GAPDH* were as follows: 25.97 ± 0.058 for control samples, 23.69 ± 0.78 for PPMS, 24.59 ± 0.34 for RRMS-relapse, 24.58 ± 0.37 for RRMS-remitting, 25.03 ± 0.74 for SPMS-relapse, and 27.36 ± 0.49 for SPMS-remitting. The normality of the data was confirmed using the non-parametric Shapiro–Wilk and Kolmogorov–Smirnov tests, with $P = 0.153$ and 0.200, respectively. A parametric one-way analysis of variance was conducted to examine differences in the Ct values of *GAPDH* across all groups, revealing no statistically significant differences ($P = 0.068$). These findings are illustrated in Figure 1, which shows the expression of *GAPDH* in all MS patient groups and healthy controls.

3.3. Relative expression of *BDNF-AS*

The lncRNA *BDNF-AS* was found to be significantly downregulated ($P < 0.01$) in MS patients compared to control subjects. The fold change relative to the control (mean ± S.E) was 0.19 ± 0.06 for PPMS, 0.33 ± 0.07 for RRMS-relapse, 0.30 ± 0.08 for RRMS-remitting, 0.33 ± 0.11 for SPMS-relapse, and 0.83 ± 0.20 for SPMS-remitting. Multiple comparison tests revealed that all groups were significantly different from the control, except for the SPMS-remitting group, which showed no significant difference from the controls and exhibited the highest expression among all groups. However, there were no

Table 1. Disease characteristics and demographics of multiple sclerosis subjects and controls

Group	Age[a]	Age at onset[a]	Gender[c]		Family history[c]		Status[c]		MS duration[a]	Frequency of relapses[b,d]	EDSS[a]
			Male (%)	Female (%)	Yes (%)	No (%)	Relapse (%)	Remitting (%)			
RRMS (n=31)	31.10±9.04	25.68±7.54	13 (42)	18 (58)	2 (6.5)	29 (93.6)	16 (51.6)	15 (48.4)	5.11±4.65	2±2	3.05±2.74
SPMS (n=13)	46.38±9.49	31.23±7.67	6 (46)	7 (54)	1 (7.7)	12 (92.3)	8 (61.5)	5 (38.5)	15.12±8.66	4±2	6.00±1.02
PPMS (n=10)	45.10±10.49	34.30±4.16	4 (40)	6 (60)	0 (0)	10 (100)	-	-	10.8±9.9	-	5.55±1.14
Control (n=20)	37.70±16.84	-	9 (45)	11 (55)	0 (0)	20 (100)	-	-	-	-	-
P-value	0.382	-	0.883		-	-	-	-	-	-	-

Notes: [a]Mean±standard deviation; [b]Median±interquartile range; [c]Frequency; [d]Within the last 3 years. Abbreviations: EDSS: Expanded disability status scale; MS: Multiple sclerosis; PPMS: Primary progressive multiple sclerosis; RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis.

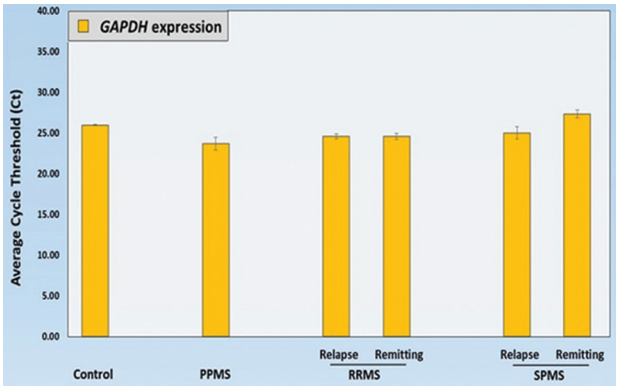


Figure 1. Average cycle threshold (Ct) values of *GAPDH* in multiple sclerosis patients and healthy controls. The normality of the data was assessed using the non-parametric Shapiro–Wilk and Kolmogorov–Smirnov tests, yielding non-significant $P = 0.153$ and 0.200, respectively. A parametric one-way analysis of the variance test showed no statistically significant differences in the Ct values of *GAPDH* across all groups ($P = 0.068$). Error bars represent standard errors.
Abbreviations: PPMS: Primary progressive multiple sclerosis; RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis.

significant differences between the MS groups themselves. These results are depicted in Figure 2.

3.4. ROC curve analysis

Based on the above findings, a ROC curve was generated, and the area under the curve (AUC) was calculated to evaluate the diagnostic value of *BDNF-AS*. The calculated AUC (mean ± S.E.) was 0.869 ± 0.041, with a 95% confidence interval of 0.789 – 0.948. The optimal cutoff point, determined by the highest Youden index value across all possible cutoffs, was 0.31, which corresponds to 75.93% sensitivity and 100% specificity. These findings are summarized in Figure 3.

3.5. Correlation analysis

Spearman's non-parametric correlation test was used to examine the relationship between the relative expression of the *BDNF-AS* gene and various factors, including EDSS, age, MS duration, age at onset, and frequency of relapses in the previous 3 years. The analysis revealed a significant, moderately positive correlation with age at onset of MS ($r = 0.384$, $P < 0.01$), but no correlation was found with any of the other factors. These findings, along with the correlation parameters, are presented using the four-parametric logistic regression model, as shown in Figure 4.

4. Discussion

The investigation into the expression of *BDNF-AS* was prompted by its established role in the negative regulation of the neurotrophic protein BDNF, which exhibits complex and intriguing behavior in the context of MS and other neurodegenerative diseases.[36] First, it was found that immune cells secrete BDNF in MS lesions but only in those with active myelin degeneration.[24] Second, despite this finding, studies have shown that BDNF levels in the serum/plasma, cerebrospinal fluid (CSF), and peripheral blood mononuclear cells are lower in MS patients.[25-30] Interestingly, BDNF levels in the SPMS group were found to be lower than in the RRMS and the PPMS groups.[27] A similar pattern of low BDNF levels in the serum has been observed in other neurological diseases, such as schizophrenia,[37,38] depression,[39,40] Alzheimer's disease,[41,42] and Huntington's disease.[43,44] In contrast, autism has been associated with higher serum levels of BDNF.[45]

A study by Modarresi *et al.*[46] demonstrated that inhibiting *BDNF-AS* can increase endogenous BDNF protein expression, stimulate neurite formation, and promote neuronal differentiation. Therefore, in our study, we aim to comprehensively examine the levels of *BDNF-AS* in the serum of MS patients across all subtypes of the disease. Interestingly, we found that *BDNF-AS* levels were significantly lower in the serum of MS patients compared to healthy controls. This observation may reflect a downregulation mechanism that inhibits *BDNF-AS* to upregulate BDNF protein expression in MS lesions, potentially stimulating synaptic plasticity, and myelin regeneration in MS plaques as part of an attempt to recover from the demyelinating event characteristic in MS.

One interesting finding that might contradict this explanation is the downregulation of BDNF itself in the serum of MS patients.[25-29] This observation could challenge our hypothesis that the downregulation of *BDNF-AS* facilitates overexpression of BDNF in MS. However, the reduced levels of BDNF in the serum or CSF of MS patients.[28,29] may suggest that BDNF is predominantly localized within MS lesions rather than being secreted into body fluids. Therefore, the overexpression of BDNF resulting from *BDNF-AS* downregulation may occur only in MS lesions, where this mechanism is activated. Alternatively, this observation may indicate the presence of another compensatory mechanism that mimics the effects of *BDNF-AS* in inhibiting BDNF. Nevertheless, our initial hypothesis remains the most plausible explanation, as any compensatory mechanism would likely influence BDNF levels in MS lesions, which is not observed given the higher expression of BDNF in these lesions.

Our finding of lower expression of the *BDNF-AS* in MS, whereas BDNF remains downregulated, is in line with a study by Gharzi *et al.*[31] on RRMS patients, which found that the correlation between BDNF and *BDNF-AS* may not always be negative. This finding may extend to

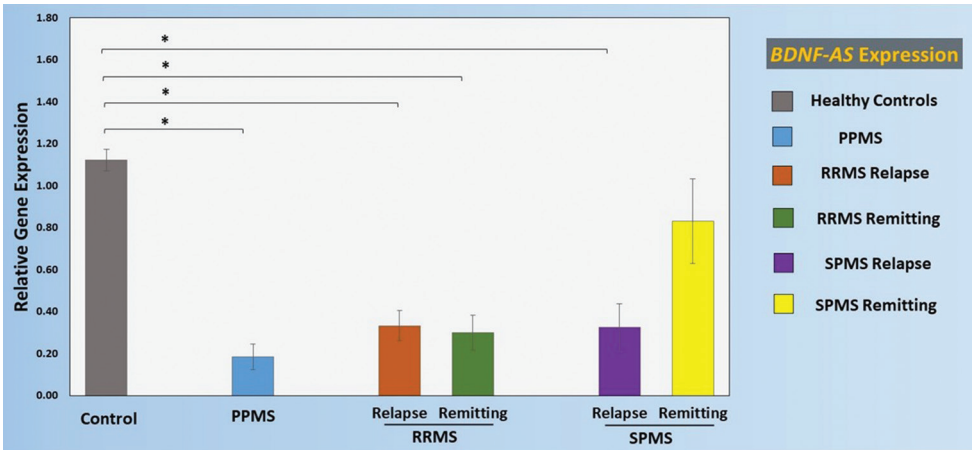


Figure 2. Relative expression of brain-derived neurotrophic factor-antisense multiple sclerosis subjects compared to healthy controls. Data are presented as mean and standard error. The figure shows that PPMS, RRMS-relapse, RRMS-remitting, and SPMS-relapse are statistically downregulated compared to the control group. However, SPMS-remitting is not statistically different from the controls. Additionally, no significant differences were observed among the multiple sclerosis types themselves.
Abbreviations: PPMS: Primary progressive multiple sclerosis; RRMS: Relapsing-remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis.

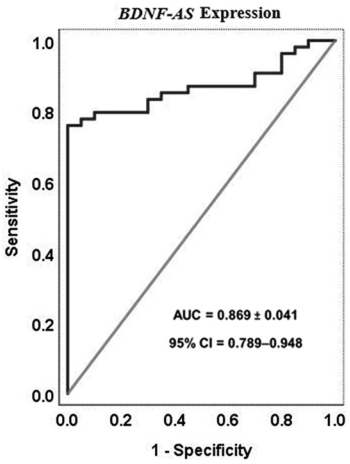


Figure 3. Diagnostic performance of brain-derived neurotrophic factor-antisense using receiver operating characteristic curve analysis. The optimal cutoff was calculated to be 0.31, with a sensitivity of 75.93%, specificity of 100%, and an overall AUC of 0.869 ± 0.041.
Abbreviations: AUC: Area under the curve; CI: Confidence interval.

other diseases, such as bipolar disorder, gastric cancer, and breast cancer, where studies have suggested that a positive correlation between BDNF and *BDNF-AS* may also exist despite the known negative regulation of BDNF by *BDNF-AS*.[19,31]

Previous studies have demonstrated that BDNF is highly produced in RRMS, whereas its expression is lower in progressive MS.[24,26] This finding could be explained by hypothesizing that in progressive MS, BDNF is more negatively regulated by *BDNF-AS*. One potential consequence of this regulation is the progression of neuronal degeneration in progressive types of MS. Our study supports this hypothesis, as the SPMS group exhibited the highest *BDNF-AS* expression among MS subtypes and was the only group not showing significant downregulation compared to controls. On the other hand, the PPMS group demonstrated low *BDNF-AS* expression, suggesting that the progression of the disease in this group may involve a different mechanism than the one hypothesized for the SPMS group.

Moreover, we found that *BDNF-AS* expression displayed higher specificity and sensitivity for MS diagnosis, which may prove useful in diagnosing MS. We also determined a relative expression cutoff of 0.31, which demonstrated moderate sensitivity and high specificity. However, our study failed to find significant differences between the RRMS, PPMS, and SPMS groups, which may be necessary for distinguishing between different MS types.

5. Limitations and future research

It is important to acknowledge that every study has its limitations, which often present valuable opportunities for future research and deeper exploration. First, the sample size for each group was constrained by rigorous exclusion criteria (Section 2.3), designed to ensure high specificity by excluding cases with confounding medical conditions that could influence lncRNA expression. Moreover, although the samples were drawn from the MS Unit at Kasr Al-Ainy in Cairo – a center that attracts patients from across Egypt due to the scarcity of specialized MS facilities – the geographic scope of the study was inherently limited to a single country. While this regional focus provides a representative view of MS patients within Egypt, it highlights the need for broader, multicenter studies

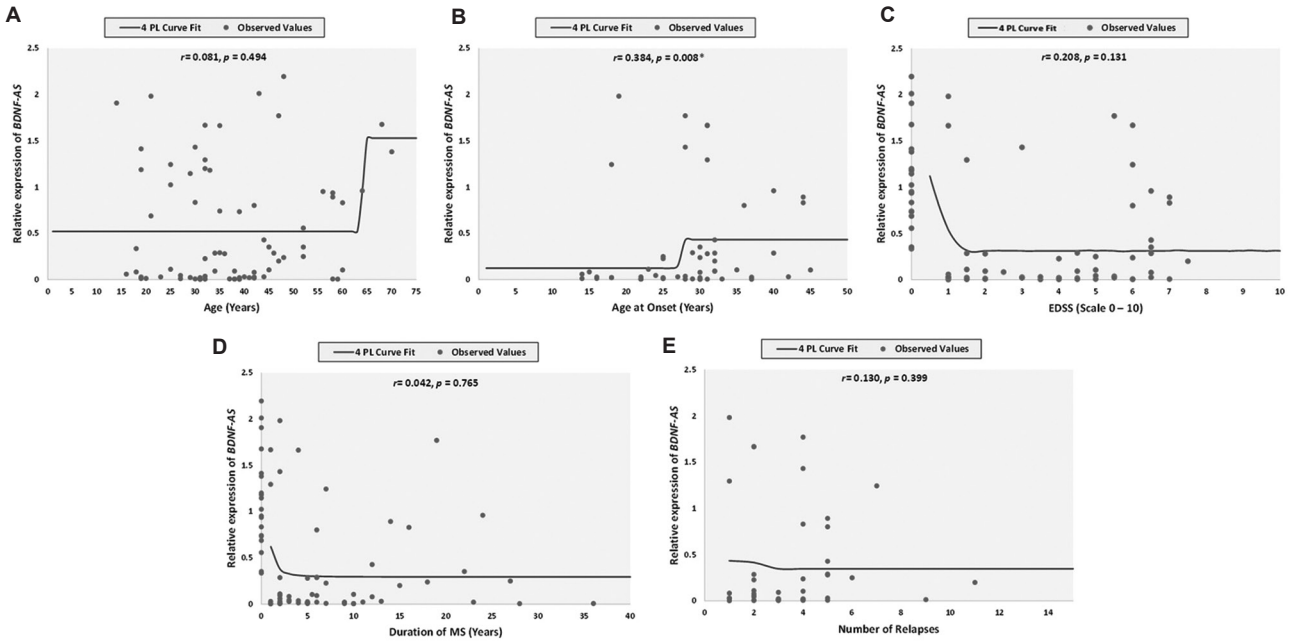


Figure 4. Correlation of brain-derived neurotrophic factor-antisense (*BDNF-AS*) expression with (A) age, (B) age at onset, (C) Expanded Disability Status Scale (EDSS), (D) multiple sclerosis (MS) duration, and (E) incidence of relapses in the preceding 3 years. The four-parametric logistic (4PL) model was used to fit the data, and Spearman's test was applied to investigate the non-parametric correlation parameters. *BDNF-AS* expression had a moderately positive and significant correlation with age at onset of MS ($r = 0.384$, $P < 0.01$).
Notes: *P*: *P*-value; r: Correlation coefficient.

to validate the generalizability of these findings across diverse populations. In addition, the variation in treatment regimens among patients with different MS subtypes raises intriguing questions about the potential impact of therapeutic approaches on *BDNF-AS* expression. This question highlights the importance of future investigations to examine the interplay between treatment modalities and lncRNA expression.

While this study highlights the downregulation of *BDNF-AS* across various MS subtypes, the precise mechanisms underlying this phenomenon remain incompletely understood. Of particular interest is the marked elevation of *BDNF-AS* expression in SPMS compared to other groups, particularly PPMS, which raises important questions regarding its role in disease progression. Although limited funding constrained our ability to explore this aspect further, future studies investigating these differences could yield critical insights into the molecular pathways and key regulatory mechanisms driving MS pathophysiology and progression. Finally, measuring BDNF protein levels alongside *BDNF-AS* expression would offer a more comprehensive understanding of the regulatory network. Although financial constraints precluded this aspect of the study, we have addressed this limitation by integrating data from existing literature to contextualize our findings (Section 4). Expanding such investigations in future studies could significantly enhance our understanding of the interplay between BDNF and *BDNF-AS* in MS pathophysiology and progression.

While these considerations represent opportunities for further exploration rather than limitations, they underscore the need for continued research to build upon the foundation established by our study.

6. Conclusion

The expression of *BDNF-AS* has been identified as a potential biomarker for the diagnosis of MS, demonstrating high specificity and sensitivity through its downregulation in the serum of MS patients. Furthermore, our findings suggest that elevated levels of *BDNF-AS* are associated with disease progression in secondary progressive MS, indicating that targeting the downregulation of this lncRNA could serve as a potential therapeutic strategy for MS. While these results are promising, further studies with larger sample sizes and across various MS subtypes are necessary. Future research should also investigate the expression of *BDNF-AS* in cell cultures, as well as brain and spinal cord samples from MS patients, to better understand its relationship with BDNF protein in CNS lesions and to gain deeper insights into disease mechanisms and potential treatment options.

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None.

Conflict of interest

The authors declare that they have no competing interests.

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Methodology: Ahmed Kamal, Menha Swellam, Eslam M. El-Nahrery
Writing-original draft: Ahmed Kamal
Writing-review & editing: Menha Swellam, Eslam M. El-Nahrery

Ethical approval and consent to participate

The authors declare that this research adhered to the ethical principles outlined in the Declarations of Helsinki (1964) and its subsequent amendments [21]. Ethical approval (approval number: 19922) was granted by the Board of Ethical Committee, Suez University, Egypt. In addition, written informed consent was obtained from all patients and healthy controls after the nature of the study was fully explained.

Consent for publication

All patients were assured of data protection and privacy, except for their analytical data and clinical status, which were included in this study with their informed consent for use and publication.

Availability of data

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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