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RESEARCH

Association of serum eosinophil-derived neurotoxin with amyotrophic lateral sclerosis

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Introduction: The exact pathogenesis of amyotrophic lateral sclerosis (ALS) is vague, with neuroinflammation having a part in the process. Eosinophil-Derived Neurotoxin (EDN) is a secretory protein that has ribonuclease activity. Higher levels of EDN in ALS patients suggest it may take part in the pathogenesis of ALS as an inflammatory biomarker and serve as an ALS indicator. Measuring serum EDN level in ALS patients may be helpful for early diagnosis as well as early therapeutic trials. In this study, we aimed to find the association of EDN with ALS.

Methodology: This case-control study was executed in the Department of Neurology, Bangladesh Medical University, Dhaka, from December 2019 to March 2021. A total of 54 subjects were enrolled; among them, 27 were patients with ALS and 27 were controls who were age- and sex-matched. According to El Escorial diagnostic criteria, ALS patients were categorized into three groups: definite, probable (lab-supported), and possible ALS. The severity of patients was evaluated by the ALS functional rating scale-revised (ALSFRS-R). Then quantitative estimation of serum EDN level of both cases and controls was done by Enzyme Linked Immunoassay (ELISA) Kit in the Department of Microbiology and Immunology Laboratory, BSMMU, Dhaka.

Results: The mean EDN in ALS cases (13.5 $\pm$ SD 4.05) was higher than the control group (5.72 $\pm$ SD 3.25) (p-value $<$ 0.001). The EDN level was significantly increased by 2.36-fold in the sera of ALS patients as compared to the control group. EDN level also differentiates definite, probable (lab supported), and possible ALS from the control group ($EDN_{Definite}$ – 13.60 $\pm$ 3.84, EDN_{CG} – 5.72 $\pm$ 3.25; $p < 0.001$). $EDN_{Probable}$ – 14.43 $\pm$ 4.27, EDN_{CG} – 5.72 $\pm$ 3.25; $p < 0.001$, and $EDN_{Possible}$ – 9.97 $\pm$ 3.84, EDN_{CG} – 5.72 $\pm$ 3.25; $p < 0.05$). Receiver operating characteristic curve analysis revealed that with a cut-off value of 10.3 ng/ml, EDN reliably differentiated ALS patients from the control group, exhibiting 77.8% sensitivity and 88.9% specificity.

Conclusion: EDN was significantly increased in patients with ALS than the control. Thus, EDN level measurement might help in early diagnosis of ALS as well as a possible therapeutic trial.

Keywords: eosinophil-derived neurotoxin, EDN, amyotrophic lateral sclerosis, ALS functional rating scale-revised, motor neuron disease (MND)

Introduction

Motor neuron disease (MND) is a relentlessly progressive neurodegenerative disorder affecting the motor neurons in varying combinations, first described in the 1870s by Jean Martin Charcot as Charcot's sclerosis (1). The age-standardized prevalence rate of MND in 2019 was 3.37 (95% UI, 2.9–3.87) per 100,000 people (2). The global prevalence of MND increased by 1.91% [95% UI, 0.61–3.42] as compared to 1990 and is expected to increase further (2).

MND is categorized into various subtypes according to their symptoms and signs. The most prevalent and severe type of adult-onset MND is ALS, characterized by the simultaneous presence of upper and lower motor neuron signs (3). ALS is a continuously advancing disorder that is currently without a cure, with an incidence rate of 1.7 per 100,000 person-years (2).

Neuroinflammation is a key trait of various neurodegenerative conditions, including ALS, characterized by the accumulation of numerous activated microglia, astrocytes, and T cells adhering to post-capillary venules (4). In ALS patients from Northern India, there were significant increases in inflammatory markers like tumor necrosis factor alpha, interferon-γ, and nitric oxide (5). These inflammatory response-inducing factors in ALS could serve as potential biomarkers. Damage-associated molecular patterns (DAMPs) are known to significantly activate macrophages and T lymphocytes (6). Additionally, eosinophil-derived neurotoxin (EDN), another DAMP, shows elevated levels in the sera of ALS patients (7).

ALS is a clinical diagnosis. There is a pronounced delay between the presentation and diagnosis. But no study regarding ALS has been done so far in Bangladesh to find out the biomarker which has an important role in the pathophysiology of ALS, and that may be helpful for early diagnosis of ALS as well as early initiation of therapeutic trials. This study attempts to evaluate whether there is any association of serum EDN level with ALS as well as to correlate different clinical parameters of ALS, including types of ALS, with EDN among the ALS patients of Bangladesh.

Methodology

This case-control study was carried out in patients purposively selected from the Inpatient, Outpatient, and Neuromuscular Disorder Clinic of Bangladesh Medical University (BMU). The patients with a clinical diagnosis of ALS (definite, probable, probable lab-supported, and possible cases) who had no family history, according to Revised El Escorial diagnostic criteria, were included as cases in the study (8). Patients with allergic asthma and acute infection were excluded. Age- and sex-matched healthy persons or patients not fulfilling revised El Escorial diagnostic criteria without any allergic asthma or acute infection were enrolled as controls.

Before commencing the study, ethical clearance was obtained from the departmental Technical Committee and the Institutional Review Board of BMU [No. BSMMU/2020/1717]. The importance and procedure of the study were elaborated to all participants. Informed written consent was also taken from all the respondents. Data were collected through face-to-face interviews. Demographic profile, clinical history, physical and electrophysiological findings, and laboratory reports of each subject were recorded in a pre-formed data collection sheet. Subjects' right to withdraw from the study at any point in time was ensured, and information obtained from study subjects was kept confidential, except for research purposes only.

Functional status was evaluated by the ALS Functional Rating Scale-Revised (ALSFRS-R) (9). Serum EDN was measured quantitatively by an Enzyme Linked Immunoassay (ELISA) Kit in the Department of Microbiology and Immunology, BMU, Dhaka.

Freshly drawn blood by standard venipuncture procedure was collected into a plastic red screw-capped vial labeled with the patient's identification number and allowed to clot for 2 hours at room temperature before centrifugation for 10 minutes at 1000 × g at 2–8°C. Then the appropriate amount of serum was dispensed into a plastic screw-capped vial (Eppendorf tube) labeled with the patient's identification number (**Figure 1**). Identification was marked as capital letter C for the control group and S for ALS patients (cases). Samples were stored at −20°C for final analysis at the Department of Microbiology and Immunology Lab, BSMMU, Dhaka.

The EDN ELISA Kit quantifies human EDN using the sandwich ELISA method. Prior to the main experiment, a pilot study was conducted beforehand to assess the validity and suitability of the sample dilution ratio, utilizing standards and a limited number of samples. All reagents were prepared according to the instructions in the manual. Reagents and samples were allowed to reach room temperature before analysis. **Figure 2** illustrates the assay procedure. The concentration of human EDN was ascertained by using a dose-response curve derived from the reference standards (**Figure 3**).

Serum samples were measured with the EDN ELISA Kit of My Biosource.com (Catalog No.: MBS765798). The maximum detection range is 40 ng/ml, the minimum detection range is 0.625 ng/ml, and the sensitivity is 0.375 ng/ml. The kit identifies human EDN in samples. There was no significant cross-reactivity between human EDN and analogues, and the coefficient of variation was less than 10%.

The comparison of continuous variables and categorical variables between two groups was executed using the unpaired t-tests and chi-square tests, respectively. The comparison of continuous variables among three or more groups was conducted using analysis of the variance

FIGURE 1 | Stored serum in a labeled Eppendorf tube.

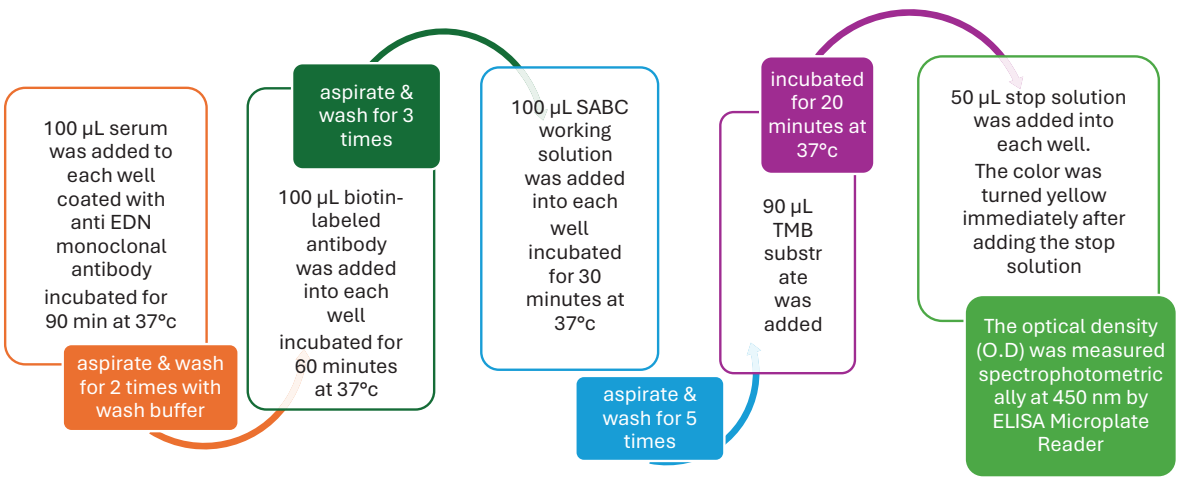

FIGURE 2 | Flowchart showing assay procedure.

(ANOVA). Correlation analyses were performed using the Pearson's correlation test. Receiver Operating Characteristic (ROC) curve analysis was conducted to assess the diagnostic utility of EDN in ALS. The p-value of less than 0.05 was considered significant.

Results and observations

In this study, 27 ALS patients (cases) were enrolled along with 27 age- and sex-matched healthy controls. The demographic profiles (age, sex, and residence) were well matched between ALS patients and the control group, as the differences were insignificant (**Table 1**). That suggests they were taken from the same population.

Out of 27 patients, 14 (51.9%) were definite, 10 (37%) were probable (lab supported), and 3 (11.1%) were possible ALS in this study. In respect to severity, among 27 ALS patients, 12 (44.4%) were in the mild stage, 11 (40.7%) were in the moderate stage, 2 (7.4%) were in the severe stage, and 2 (7.4%) were in an advanced stage of severity (**Figure 4**).

The mean EDN in ALS cases (13.5 ± SD 4.05) was higher than in the control group (5.72 ± SD 3.25), with a p-value of <0.001. The EDN level was increased by 2.36-fold in the sera of ALS patients compared to the control group, which was statistically significant (**Figure 5**). **Table 2** indicates that the EDN level was significantly higher in definite, probable (lab-supported), and possible ALS than in the control group ($EDN_{Definite}$– 13.60 ± 3.84, EDN_{CG} – 5.72 ± 3.25; $p < 0.001$; $EDN_{Probable}$ – 14.43 ± 4.27, EDN_{CG} – 5.72

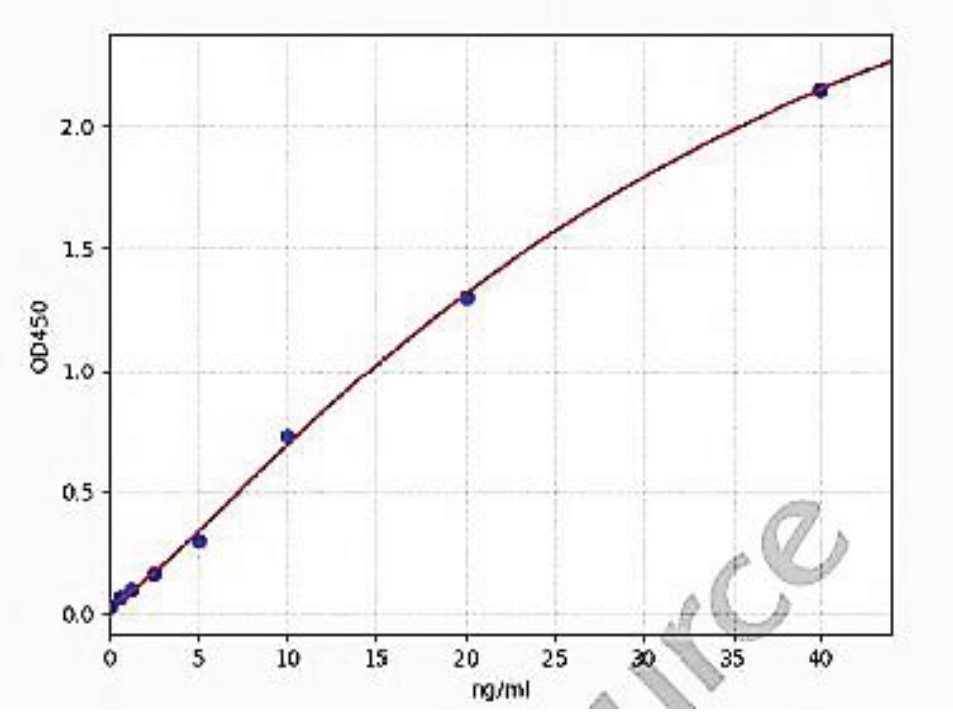

FIGURE 3 | Calibration curve for identifying the concentration of human EDN.

TABLE 1 | Distribution of study subjects by age, sex and residence (n = 54).

Variables	Sub-category	ALS patients (n = 27) No. (%)	Comparison group (n = 27) No. (%)	p-value
Age group (years)	<30	7 (25.9%)	5 (18.5%)	0.119^{ns}
	31–50	10 (37.0%)	19 (70.4%)	
	>50	10 (37.0%)	3 (11.1%)	
	Mean ± SD	43.3 ± 14.9	38.0 ± 8.9	
	Range	18–67	18–55	
Sex	Male	18 (66.7%)	20 (74.1%)	0.551^{ns}
	Female	9 (33.3%)	7 (25.9%)	
	Male:Female ratio	2:1	2.9:1	
Residence	Urban	13 (48.1%)	15 (55.6%)	0.586^{ns}
	Rural	14 (51.9%)	12 (44.4%)	

Data were expressed as frequency, percentage within parenthesis, mean ± SD, range and ratio.
Unpaired t-test and Chi-squared Test (χ^2) was done to analyze the data.
ns: not significant; ALS: amyotrophic lateral sclerosis.

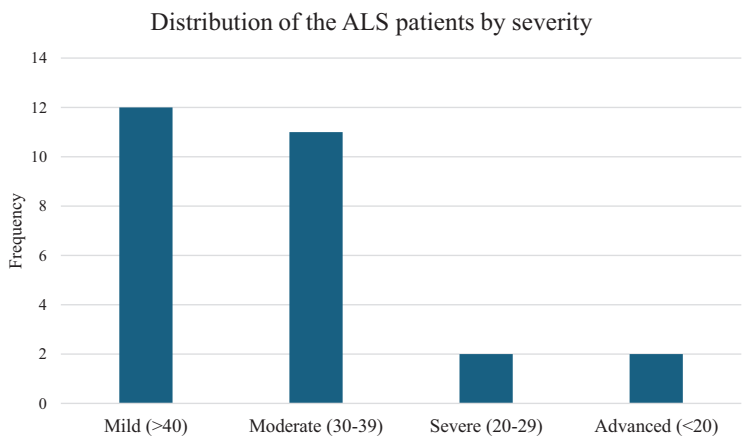

FIGURE 4 | Bar diagram showing the distribution of the ALS patients by severity.

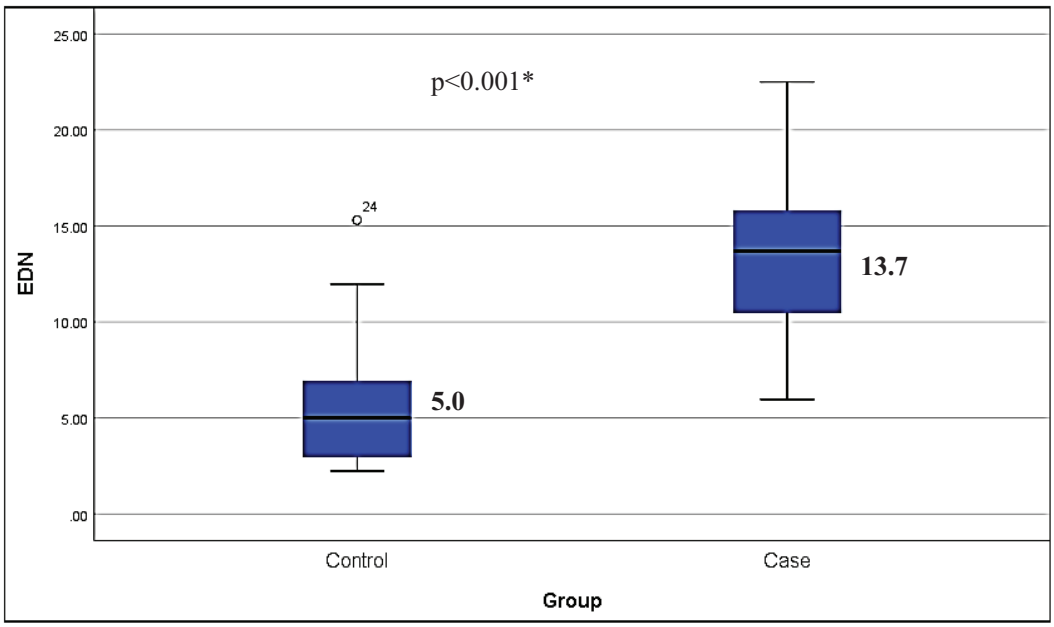

FIGURE 5 | Box-whisker plot showing EDN of cases (ALS patients) and controls. *significant.

TABLE 2 | Comparison of EDN among the types of ALS and the control group (n = 54).

Variable	Type of ALS			Control (n = 27)	p-value
	Definite (n = 14)	Probable (lab supported) (n = 10)	Possible (n = 3)		
EDN (ng/ml)	13.60 ± 3.84	14.43 ± 4.27	9.97 ± 3.84	5.72 ± 3.25	<0.001*
Median	14.30	14.0	9.1	5.0	
Definite vs. control					<0.001*
Probable vs. control					<0.001*
Possible vs. control					<0.041*

Data were expressed as mean ± SD and median, ANOVA test was done to analyze the data among groups and Unpaired t-test was done between groups.
*significant, ALS: amyotrophic lateral sclerosis.

± 3.25; $p < 0.001$; and $EDN_{Possible}$ – 9.97 ± 3.84, EDN_{CG} – 5.72 ± 3.25; $p < 0.05$).

EDN had a positive correlation ($r = +0.225$, $p = 0.258$) with the severity of the disease, but the strength was weak and not statistically significant (**Figure 6**). A serum EDN level at the cutoff value of 10.3 ng/ml aids in the diagnosis of ALS, with a sensitivity of 77.78%, specificity of 88.89%, positive predictive value of 87.50%, negative predictive value of 80.00%, and accuracy of 83.33% (**Table 3**). The ROC revealed that with a cutoff value of 10.3 ng/ml, EDN reliably differentiated ALS from the control group [AUC = 0.929, $p < 0.001$, sensitivity = 77.8%, specificity = 88.9%] (**Figure 7**).

TABLE 3 | Performance of EDN as a diagnostic test for ALS.

		Group		Total
		Case	Control	
EDN (ng/ml)	>10.3	21 (true positive)	3 (false positive)	24
	<10.3	6 (false negative)	24 (true negative)	30
Total		27	27	54

Discussion

This case-control study evaluated the association of EDN with amyotrophic lateral sclerosis (ALS) and predicted its role in the pathogenesis of ALS as an inflammatory biomarker. For the research, a total of 27 patients (ALS cases) and 27 controls were selected after fulfilling the inclusion and exclusion criteria. They were interviewed by specific questionnaire to find out the association.

EDN is an 18.6 kDa single-chain polypeptide, expressed predominantly in eosinophils but also detected in neutrophils (10). EDN is a secretory protein which has both ribonuclease and antiviral activity (11, 12). The in vivo effect of EDN is to induce ataxia and paralysis (13). It also damages the myelinated neurons when injected intrathecally (13). In ALS, excessive EDN may be an auto-aggressive factor

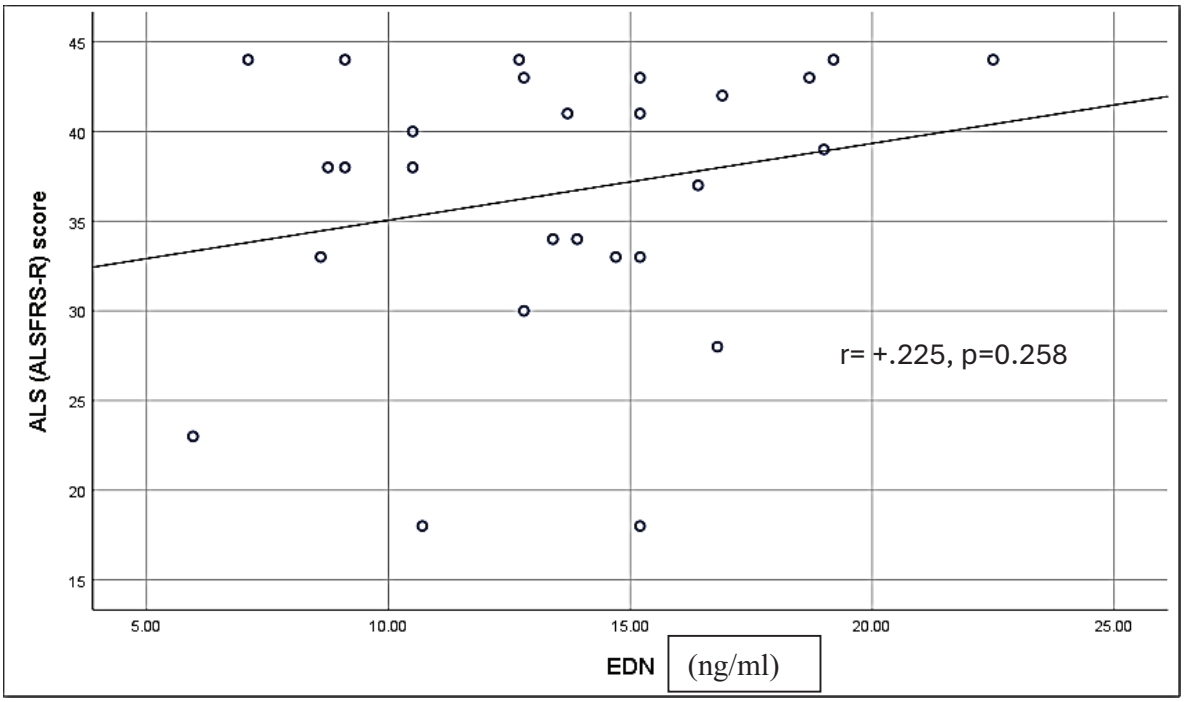

FIGURE 6 | Scatter diagram showing correlation of EDN with severity of ALS (ALSFRS-R).

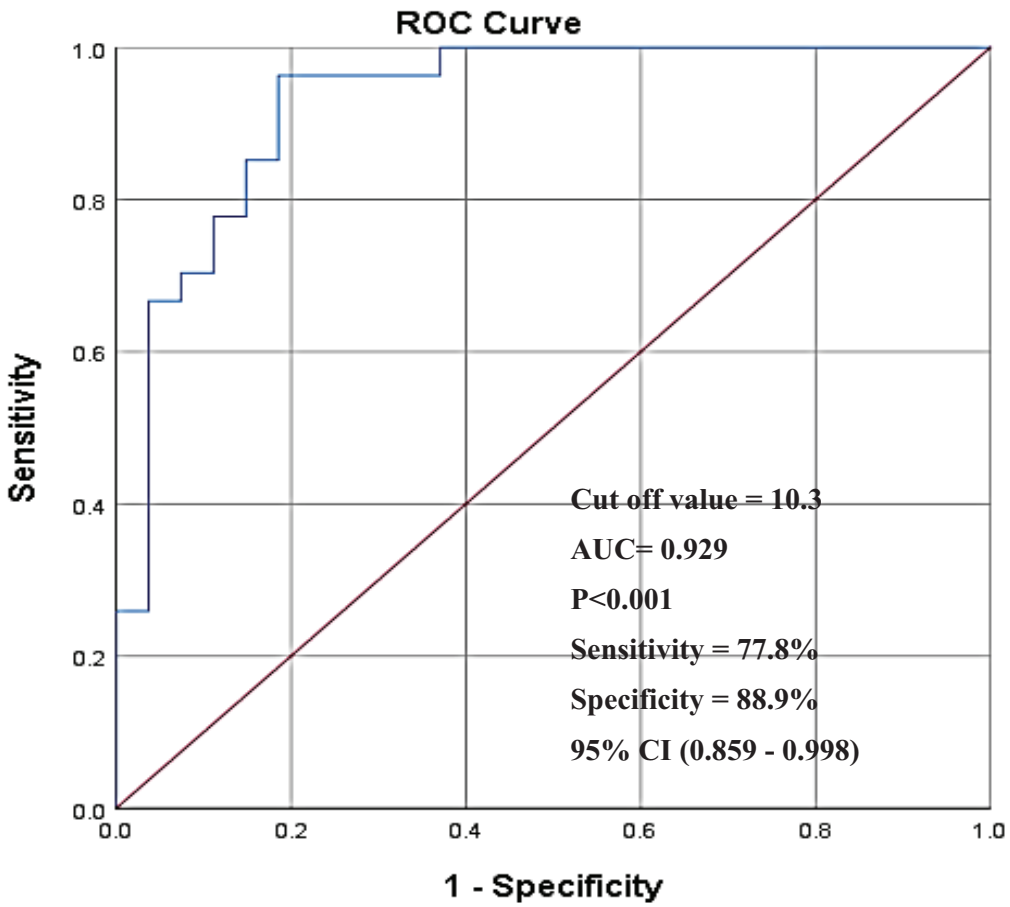

FIGURE 7 | ROC curve analysis of EDN in ALS.

that damages the normal neuron, like autoimmune diseases (14). Previous studies showed that EDN was found to be elevated in patients with ALS (7).

Few studies regarding ALS were done in Bangladesh. In a study conducted, it was found that MND was homogenously distributed with no known etiology, the ALS variety was the highest (61%), the male-to-female ratio was 1.79:1, and then mean ± SD age for ALS was 53 ± 7.18 (15). Other Bangladeshi researchers stated that serum ferritin, uric acid, and serum homocysteine were found to be elevated in patients with ALS, indicating their association with ALS (16).

In the present study, respondents were taken from all age groups irrespective of sex and religion. The mean age of ALS patients was 43.3 ± 14.9 years, with an age range of 18–67 years, and the mean age of the controls was 38.0 ± 8.9 years, with an age range of 18–55 years. A previous study showed the mean age of ALS patients and healthy controls was 54.7 ± 9.7 and 51.8 ± 9.6 years, respectively (17). In our study the mean age of ALS patients was low in comparison to other studies performed in different population groups. Out of 27 cases, 18 (66.7%) were male and 9 (33.3%) were female, and the male-female ratio was 2:1. Among the control group, 20 (74.1%) were male and 7 (25.9%) were female, and the male-female ratio was 2.85:1. A previous study in Hong Kong showed a total case of 84 with a male preponderance of 1.98:1 (18). We found a similar male preponderance with the above-mentioned study.

On assessment of the severity state of disease, 44.4% of ALS patients were in mild state, 40.7% in moderate state, 7.4% in severe state, and 7.4% in an advanced state of severity at the time of assessment. This finding was consistent with a previous study where the median ALSFRS-R score was in a moderate state of severity (19). Severity state among the types of ALS shows all severe and advanced cases were in definite ALS (mean 31.5 ± 7.5, median 33). Among probable ALS, 80% were in a mild state and 20% were in a moderate state (mean 41.7 ± 2.4, median 42.5). All patients with possible ALS were in a mild state (mean 43.0 ± 1.7 and median 44.0). Overall reflecting the tempo of disease progression.

EDN was elevated in the sera of patients with ALS. In our study, the mean EDN level in ALS patients (13.5 ± 4.05) was higher than the control group (5.72 ± 3.25) (p-value < 0.001). The EDN level was significantly increased by 2.36-fold in the sera of ALS patients as compared to the control group. These findings were consistent with a previous study where serum EDN was increased by 2.17-fold in the sera of ALS patients ($p < 0.005$) (7).

Serum EDN was compared among the types of ALS and the control group. EDN level was significantly higher in definite, probable, and possible ALS than in the control group ($EDN_{Definite}$ – 13.60 ± 3.84, EDN_{CG} – 5.72 ± 3.25; $p < 0.001$, $EDN_{Probable}$ – 14.43 ± 4.27, EDN_{CG} – 5.72 ± 3.25; $p < 0.001$, and $EDN_{Possible}$ – 9.97 ± 3.84, EDN_{CG} – 5.72 ± 3.25; $p < 0.05$). The previous study by Liu et al. (7) did not show any comparison among the types of ALS and the control group.

In our study, no significant positive correlation was observed between serum EDN level and ALFRS-R ($r = +0.225$, $p > 0.05$). Thus, our findings indicate that neuroinflammation resulting from increased levels of EDN occurs at an early stage of ALS, and there is no correlation with the ALSFRS-R score. Previously, it was shown that no statistically significant correlation exists between ALSFRS-R and the duration of the disease (7).

In this study, we aimed to assess the diagnostic accuracy of EDN as an indicator for ALS. For this purpose, we conducted a ROC curve analysis. The ROC curve from our study demonstrated that EDN effectively differentiated ALS from the control group, with a cut-off value of 10.3 ng/ml [AUC – 0.929, $p < 0.001$, sensitivity – 77.8%, specificity – 88.9%, positive predictive value 87.50%, negative predictive value 80.00%, and accuracy 83.33%]. A previous study in 2013 reported an area under the curve (AUC) value of 0.8262 for EDN, with 77.27% sensitivity and 84.09% specificity, which aligns closely with our findings (7). The higher level of EDN in ALS may be a damaging factor, signal, or immune response; however, the mechanisms are not clear. The current study design did not allow us to conclude a causal relationship between elevated EDN levels and ALS. However, the higher serum levels of EDN in ALS patients compared to the control group may suggest a neuroinflammatory process in the central nervous system. This could potentially facilitate the early diagnosis of ALS. Ultimately, diagnosing ALS relies on clinical evaluations accompanied by imaging and neurophysiological tests. Nevertheless, the presence of elevated EDN levels might help predict probable or possible ALS cases early in the disease's progression, enabling timely intervention.

Conclusion

We observed a significant association of EDN with ALS. EDN was significantly increased in ALS patients than the control group. An EDN level of 10.3 ng/ml reliably differentiated ALS from control with a sensitivity and specificity of 77.27% and 84.09%, respectively. So, EDN can help in early diagnosis of ALS as well as initiation of early therapeutic trials.

Results

Statistic	Value	95% CI
Sensitivity	77.78%	57.74%–91.38%
Specificity	88.89%	70.84%–97.65%
Positive predictive value	87.50%	70.27%–95.40%
Negative predictive value	80.00%	66.11%–89.13%
Accuracy	83.33%	70.71%–92.08%

Author contributions

MBAM, SMA, NA, and SK: Conception and design of study; SK, MH, SS, and AC: Acquisition of data; SK, MAAM, and SMA: Analysis and/or interpretation of data; SK, MAAM, and MH: Drafting the manuscript; MBAM, SMA, NA, and MAAM: Critical review/revision.

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Conflicts of interest

All authors declare no conflicts of interest.

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