

# Could tear endothelin-1 levels be associated with disability in multiple sclerosis?

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## Abstract

**Background:** Endothelin-1 (ET-1) is a potent vasoconstrictor substance mainly secreted by endothelial cells. Increased ET-1 levels in plasma or cerebrospinal fluid (CSF) have been identified in multiple sclerosis (MS). We aimed to analyze tear ET-1 levels; visual evoked potential (VEP) and disability scores in patients with MS and in healthy controls. **Methods:** Adult patients (18-65 year-old) diagnosed with MS according to the McDonald criteria, and healthy controls were recruited for the study. Demographic features, VEP, and tear ET-1 levels were evaluated. Disability in the MS group was assessed and grouped by EDSS score (<3 vs. ≥3). The EDSS score in healthy controls was zero. **Results:** Both in the total number of patients overall and in the patients with EDSS score <3, tear ET-1 levels were higher in the MS group than that in the controls ( $p<0.001$ ). In the MS group, the ET-1 level was higher in the patients with EDSS score ≥3 than in those with EDSS score <3 ( $p<0.001$ ). The tear ET-1 level was positively correlated with age and EDSS score in the MS group ( $p<0.001$ ). **Conclusion:** To the best of our knowledge, there is no study in the literature that measures tear ET-1 levels in MS and examines their relationship with EDSS score and VEP. Higher tear ET-1 levels seem to be associated with disability and abnormal VEP in MS. Tear ET-1 measurement may be a simple new noninvasive marker indicating the disability in the patients with MS in the future.

**Keywords:** Endothelin, multiple sclerosis, EDSS, VEP, endothelin 1.

## INTRODUCTION

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system characterized by inflammation and neurodegeneration.<sup>1</sup> Symptoms of vascular dysregulation, such as migraine, may be observed in MS.<sup>2</sup> Cerebral hypoperfusion has been shown to be associated with chronic hypoxia, the formation of focal lesions, axonal degeneration, and fatigue or cognitive impairment, and can be seen by imaging methods in MS.<sup>3-5</sup>

Recent research contributes to the pathogenesis of MS with various mechanisms including cardiovascular factors such as endothelin-1 (ET-1).<sup>6</sup> ET-1 is a potent vasoconstrictor substance mainly secreted by endothelial cells, and distributed in neurons, the eyes, and glia in CNS.<sup>7</sup> ET-1 has been implicated in the pathogenesis of various diseases such as hypertension, rheumatoid arthritis, and migraine.<sup>8</sup> ET-1 has been implicated also in MS, and in particular, contributes to reduced cerebral blood flow (CBF).<sup>5</sup> ET-1 levels have been

shown to be elevated in the plasma or cerebrospinal fluid (CSF) of patients with MS.<sup>3,5,8-11</sup> In one study, the ratio of the ET-1 level of the internal jugular vein to that of the peripheral vein was higher in MS patients compared to that in the control group. It suggests that ET-1 is released from brain into the circulatory system.<sup>10</sup> In that study, administration of the ET-1 receptor antagonist, bosentan, led to an increase in CBF, and ET-1 staining was positive in reactive astrocytes in MS plaques on postmortem examination.<sup>10</sup>

Expanded Disease Severity Status (EDSS) is a clinical index of disability, and includes all functional systems affected in MS.<sup>12</sup> The number of studies investigating the association of ET-1 with EDSS score is limited. Chang *et al.* showed that elevated ET-1 levels in MS were found to be correlated with EDSS.<sup>9</sup>

ET-1 is a potent vasoconstrictor of the vasculature of the eye, and plays a role in the pathogenesis of diabetic retinopathy or glaucoma.<sup>8,13</sup> In one study indicating elevated plasma ET-1 levels in MS, blood flow was shown

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to be altered in extraocular vessels.<sup>8</sup> ET-1 has been implicated also in the development of optic neuritis (ON), which may be a presenting symptom in about 25% of patients with MS.<sup>14</sup>

Studies analyzing the ET-1 levels in body fluids are limited. To our knowledge, there is no previous study regarding the association of tear ET-1 levels with EDSS score or visual evoked potential (VEP) in MS. We aimed to analyze tear ET-1 levels, VEP, and EDSS scores in patients with MS and in healthy controls.

## METHODS

### *Study design*

This prospective study was conducted in Çanakkale Onsekiz Mart University Hospital, and approved by the local Ethics Committee of Çanakkale Onsekiz Mart University with an approval number of Date 03.11.2021 Decision No:2021-08. The study was conducted in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent was obtained from all participants. Anonymity of data was maintained in our study.

The adult patients with MS followed-up in Neurology Clinics in Çanakkale Onsekiz Mart University Medicine Faculty Hospital between November 2021 and February 2022 were analyzed prospectively. An MS diagnosis was established according to McDonald criteria.<sup>15</sup> Those patients with a history of relapse or given systemic steroid therapy during the previous 3 months were excluded. All patients with MS were given disease-modifying therapies for MS. Those for whom data were missing, or who were older than 65 or younger than 18 were excluded. Those with a history of malignancy or with active malignancy, ocular infections, known ophthalmological disease, or with an EDSS score of >6.5, those taking any medication or with systemic diseases affecting ET-1 activity were also excluded.<sup>16</sup> Healthy controls were selected from the subjects who had no previous diagnosis of neurological, autoimmune or other systemic or chronic diseases.

### *Data collection*

Demographic parameters (age, sex), clinical parameters such as disease duration, and drugs used for MS were recorded by using electronic and written patient files.

### *Laboratory measures*

The Schirmer test was performed to collect tears both from the patients with MS and the controls. Sterile Whatman paper was placed on the lower eyelid of one eye, and participants were instructed to close their eyes for 5 minutes. We used Whatman papers with a diameter of 125 mm. The diameter of tears which were absorbed on Whatman papers was ranged in between 10-30 mm. To ensure the reliability of the study, we equalized the volume of tears obtained both from the patients and the controls by cutting the Whatman papers by 10 mm. A phosphate buffer (0.05 M) with a pH:7.4 was prepared to make homogenate. Tear-absorbed Whatman papers were placed in Eppendorf tubes containing 1 mL of phosphate buffer. Using a shaker incubator at 4°C for 12 hours ensured that the tears passed completely through to the buffer. Then, the samples were preserved at -80°C in a refrigerator until analysis.

The ET-1 level was measured by enzyme-linked immunosorbent assay (ELISA) (Human Endothelin-1 ELISA Kit (USCN Life Science Inc.-CEA482Hu)). The assay sensitivity limit was lower than 2.71 pg/mL. Absorbance of the samples was measured with a microplate reader at a wavelength of 450 nm against a standard of 6.17-500 pg/mL. By using the equation of the curve obtained from the standard graphics, ET-1 concentrations in relation to each tear sample were defined as pg/mL. Each assay was measured in duplicate for each sample at the same time.

### *Expanded Disability Severity Scale (EDSS)*

The level of clinical disability was assessed by the EDSS score, which had been presented in a previous report.<sup>17</sup>

### *Visual evoked potential (VEP)*

We recorded VEP as described previously.<sup>18</sup> Electrodes were placed over the occipital cortex of the subject, and the subject was asked to look at a screen on which a checkboard pattern was displayed. VEP was evaluated from the same eye as that from which the tear sample had been obtained. We also determined whether conduction was impaired or not (abnormal VEP).

The patients with MS were further grouped according to their EDSS score: EDSS <3 and EDSS ≥3. The EDSS score for all participants in the control group was 0. Demographic parameters, abnormal VEP (present vs. absent), EDSS scores

(<3 vs. ≥3) and tear ET-1 levels were compared between the MS and control groups. Tear ET-1 levels were also compared according to EDSS score.

### Statistical analysis

Data obtained in the study were analysed statistically using SPSS 27.0 (IBM Corporation, Armonk, New York, United States). The conformity of the data to normal distribution was evaluated using the Shapiro-Wilk test. When comparing two independent groups of quantitative data according to each other, the Mann-Whitney U test was used with Monte-Carlo results. When comparing categorical variables with each other, the Pearson Chi-Square with Monte Carlo simulation technique was used. Comparison of column ratios with each other was expressed by Benjamini-Hochberg corrected p values. To analyze the correlation of variables with each other, Spearman's rho test was used. The Binary Multiple Logistic Regression test with Backward and Enter methods was used to predict the presence of MS. Quantitative variables were stated as median (percentile 25[q1]/percentile 75[q3]) values, and categorical variables as number (n) and percentage (%) in the tables.

Variables were evaluated at a 95% confidence level, and a value of p<0.05 was accepted as statistically significant.

## RESULTS

In total, 91 individuals, with a median age of 32 (q1/q3=25/40), of whom 57.1% (n=52) were female were included in the study. Age and sex did not differ between the control and MS groups. Abnormal VEP was present in 71.7% (n=43) of the patients with MS, a figure that was significantly higher in the MS group than that in the controls (p<0.001). Abnormal VEP was absent in the control group. The ratio of the subjects with EDSS score ≥3 was 0% in control group, 46.7% (n=28) in MS group (p<0.001). Tear ET-1 levels were higher in the MS group than in the control group (p<0.001). When comparing the participants with EDSS score <3, tear ET-1 levels were higher in the MS Group than in the control group (p<0.001). The EDSS score was higher in the MS group than in the control group (p<0.001) (Table 1).

Tear ET-1 levels were 15.27 pg/mL (12.56 / 19.17) in those with EDSS ≥3, and 9.56 pg/mL (6.23 / 12.38) in those with EDSS<3 (p<0.001);

**Table 1: Demographic and clinical parameters of the participants**

	Total (n=91)	Control (n=31)	MS (n=60)	p
<b>Age (year), median (q1/q3)</b>	32 (25 / 40)	29 (22 / 42)	34 (27 / 39.5)	0.210 <sup>u</sup>
<b>Sex, n(%)</b>				0.999 <sup>c</sup>
<b>Female</b>	52 (57.1)	18 (58.1)	34 (56.7)	
<b>Male</b>	39 (42.9)	13 (41.9)	26 (43.3)	
<b>Abnormal VEP, n(%)</b>				<b>&lt;0.001<sup>c</sup></b>
<b>Absent</b>	45 (52.7)	28 (90.3)	17 (28.3)	
<b>Present</b>	46 (47.3)	3 (9.7)	43 (71.7)	
<b>EDSS, n(%)</b>				<b>&lt;0.001<sup>c</sup></b>
<b>&lt;3</b>	63 (69.2)	31 (100.0)	32 (53.3)	
<b>≥3</b>	28 (30.8)	0 (0.0)	28 (46.7)	
<b>Tear ET-1 level (pg/mL), median (q1/q3)</b>	8.93 (4.46 / 14.24)	3.31 (2.61 / 5.06)	12.12 (8.72 / 15.67)	<b>&lt;0.001<sup>u</sup></b>
<b>EDSS score, median (q1/q3)</b>	1.5 (0 / 3)	0 (0 / 0)	2.5 (1.5 / 3)	<b>&lt;0.001<sup>u</sup></b>
	<b>EDSS score &lt;3</b>			
	<b>Total (n=63)</b>	<b>Control (n=31)</b>	<b>MS (n=32)</b>	<b>p</b>
<b>Tear ET-1 level (pg/mL), median (q1/q3)</b>	5.55 (3.31 / 10.05)	3.31 (2.61 / 5.06)	9.56 (6.23 / 12.38)	<b>&lt;0.001<sup>u</sup></b>

<sup>u</sup> Mann Whitney U Test (Monte Carlo), <sup>c</sup> Pearson Chi-Square Test (Monte Carlo), Q1:25th percentile, Q3:75th percentile.

**Table 2: Correlation of parameters in the control and patient groups**

		Total		Control		MS	
		r	p	r	p	r	p
Age (year)	* Tear ET-1 level (pg/mL)	0.339	<b>0.001</b>	-0.176	0.344	0.679	<b>&lt;0.001</b>
Age (year)	* EDSS score	0.449	<b>&lt;0.001</b>	-	-	0.872	<b>&lt;0.001</b>
Tear ET-1 level (pg/mL)	* EDSS score	0.886	<b>&lt;0.001</b>	-	-	0.762	<b>&lt;0.001</b>

Spearman's rho Test, r: Correlation Coefficient

and 13.49 pg/mL (10.55 / 17.39) in those with abnormal VEP, 8.51 pg/mL (6.28 / 12.65) in those without abnormal VEP ( $p < 0.001$ ). The ratio of the presence of abnormal VEP (present/absent) was higher in those with EDSS  $\geq 3$  (90.3/9.7%) than in those with EDSS  $< 3$  (56.3/43.8%) ( $p < 0.001$ ) (not shown on the tables).

Tear ET-1 levels were positively correlated with age and EDSS score in the MS group ( $p < 0.001$ ). The EDSS score was positively correlated with age in the MS group ( $p < 0.001$ ) (Table 2).

Tear ET-1 level and VEP were positive predictors for MS (Odds Ratio: 2.845,  $p < 0.001$ ; Odds Ratio: 13.092,  $p = 0.042$ ; respectively). In the patients with EDSS score  $< 3$  also, VEP and tear ET-1 levels were significant predictors for MS (Table 3).

Tear ET-1 levels were higher in the MS than in the control group, and higher in those with more disability. Tear ET-1 level was correlated with age and disability score in MS. Higher tear ET-1 levels and abnormal VEP predicted MS.

## DISCUSSION

Neurological disability caused by MS may

affect patients to varying degrees with respect to symptoms and signs, the number of relapses, residual disability between relapses or worsening disability.<sup>19,20</sup> Worsening of disability with time is probably the most important factor from the standpoint of an individual suffering from MS. Patient-determined Disease Steps (PDDS) evaluates disability by patient-determined outcome.<sup>21</sup> However, to evaluate disability, we used the EDSS score based on a clinical assessment by a physician. We considered that EDSS might be more appropriate in such a clinical study.

We found that tear ET-1 level was elevated in the MS group, and was associated with age and EDSS score in the MS group. In one study, plasma ET-1 level was shown to increase in MS, but was not correlated with age or EDSS score.<sup>11</sup> The number of patients with MS was relatively lower in that study. Several studies in the literature revealed that an increased plasma ET-1 level was similar to and might be an indicator of an increased level of ET-1 in CSF in patients with MS.<sup>3,5,8-11</sup> Elevated plasma ET-1 level is not a specific feature for MS, it may be observed in various situations, such as

**Table 3: Logistic regression showing the predictors for MS**

	p	Odds Ratio	95% C.I., for OR	
			Lower	Upper
<b>Total (n=91)</b>				
Age (year)	0.556	1.032	0.930	1.145
Sex	0.087	6.251	0.768	50.903
Abnormal VEP (present)	<b>0.042</b>	13.092	1.098	156.139
Tear ET-1 level (pg/mL)	<b>&lt;0.001</b>	2.845	1.609	5.031
<b>EDSS score &lt;3 (n=63)</b>				
Age (year)	0.537	1.034	0.931	1.148
Sex	0.095	5.953	0.732	48.447
Abnormal VEP (present)	<b>0.047</b>	12.358	1.034	147.744
Tear ET-1 level (pg/mL)	<b>0.001</b>	2.766	1.543	4.961

Multiple Logistic Regression (Method; Enter), C.I.: Confidence interval OR: Odds Ratio

autoimmune conditions or diabetic neuropathy.<sup>16</sup> It was shown that increased plasma ET-1 level was associated with decreased neural conduction in diabetic neuropathy.<sup>22</sup> Via binding to the endothelin A receptor, ET-1 released in cerebral circulation from reactive astrocytes located in the plaques exerts its vasoconstrictor effect.<sup>23</sup> Hence, it may play an important role in decreased CBF occurring in MS.<sup>5</sup> Reduced CBF could be elevated to the level of that in healthy subjects with oral administration of the ET-1 receptor antagonist, bosentan, in patients with MS.<sup>10</sup> In another study in which ET-1 level was not measured in patients with MS, bosentan treatment did not provide an increase in CBF.<sup>24</sup> Besides, impaired CBF was shown not to influence EDSS scores in MS.<sup>25</sup> However, our findings suggest that EDSS score was in positive correlation with tear ET-1 levels. Therefore, the other possible mechanisms through which ET-1 has a role in the pathogenesis of MS, and the associations between ET-1 levels in tears, and in CSF or plasma, remain to be elucidated.

To our knowledge, the number of studies regarding the measurement of ET-1 in tears is limited. Median tear ET-1 concentration measured in the control group in our study was also higher than that in plasma or CSF of controls reported in previous studies.<sup>3,8-11</sup> In a study analyzing the ET-1 levels in tears in patients with glaucoma, the mean ET-1 level was  $2.74 \pm 0.99$  pg/mL in healthy controls, which is similar to our findings.<sup>26</sup> In a rabbit study, the concentration of ET-like immunoreactivity was found to be  $13.85 \pm 3.94$  pg/mL and 15-fold higher than that in plasma.<sup>27</sup> Endothelium may secrete ET-1 in various tissues. To eliminate local factors in stimulating ET-1 production in the eye, we excluded subjects with any known ophthalmological diseases. Possible mechanisms of a higher concentration of ET-1 in tears than plasma or CSF remain to be explained. Measurement of ET-1 in higher concentrations in tears may indicate that its use is valuable in studies regarding ET-1. Further studies are suggested.

We showed that elevated tear ET-1 levels were correlated with EDSS score, and would suggest that it might be a useful marker in the evaluation of disease progression. In one study comparing chronic inflammatory demyelinating polyneuropathy, Alzheimer's disease, acute inflammatory demyelinating polyneuropathy and MS, plasma ET-1 levels was found to be higher in CIDP and MS compared to other diseases and healthy controls.<sup>9</sup> They showed a positive correlation between plasma ET-1 level and EDSS scores in MS patients ( $p=0.001$ ,  $\rho=0.518$ ). In

some studies, plasma ET-1 level in MS was shown not to be correlated with EDSS score, and not associated with different stages or forms of MS. The disease duration was associated with plasma ET-1 level in one study, not in another.<sup>11,28</sup> We could not analyze the forms of the disease in our study. Different from their findings, we found that tear ET-1 level was positively correlated with age. It might be a result of alterations in the physiology of lacrimation or endothelial functioning with aging.

ON is one of the most frequent manifestations of MS. In one study investigating ET-1 levels in aggressive and non-aggressive types of ON in MS, serum ET-1 levels did not differ between two types, but ET-1 level in CSF was significantly higher in the aggressive subtype.<sup>14</sup> It suggests that the measurement of plasma ET-1 may not point to the activity of ET-1 in CSF or other body fluids.

In the case of hypoxia or inflammation, cells other than vascular endothelial cells may produce ET-1 and increase plasma ET-1 levels.<sup>29</sup> Some immune factors, such as TNF alpha and IL-1, may stimulate ET-1 production; and ET-1 may enhance the inflammatory reaction.<sup>30</sup> It was proposed that the general and local inflammatory status of the patients with MS may affect ET-1 levels in plasma or other body fluids. In one study analyzing inflammation in MS, plasma levels of cytokines were found to be similar to those in MS and control subjects.<sup>3</sup> They also showed that plasma ET-1 level was higher in the MS group than in the controls, but ET-1 level was not found to be associated with cytokine levels. It suggests that changes in the levels of ET-1 and peripheral immune markers are independent of each other. In one study, the ratio of plasma ET-1 level collected from the internal jugular vein to that in the peripheral vein was found to be higher in the MS group compared to that of those in the control group.<sup>10</sup> Immunohistochemical staining in postmortem analysis in that study showed that reactive astrocytes in MS plaques were the principal source of ET-1. These findings suggest ET-1 to be of central origin in patients with MS. The impact of centrally produced ET-1 on ET-1 concentration in tears should be investigated in subjects by measuring ET-1 and cytokines in tears, and in central and peripheral vessels. An in-vitro study suggests that ET-1 produced by astrocytic cell lines would be suppressed by some medications.<sup>31</sup> If supported by future studies, tear ET-1 may be used in the follow-up of patients with MS under treatment targeting ET-1 suppression. Therefore, the normal range of tear ET-1 level in

MS and healthy subjects should be clearly defined.

In the existence of ON, VEP was found to be abnormal in >90% of the patients with MS (34). However, it was abnormal in >50% of those without ON.<sup>32</sup> To our knowledge, no investigation regarding the association between ET-1 level and abnormal VEP in MS has been conducted. We showed that the ratio of abnormal VEP was 71.7% in the MS group, and found abnormal VEP to be a strong predictor for MS both in overall group and in the patients with EDSS score <3. Intravitreal injection of ET-1 in rabbits led to abnormal VEP.<sup>33</sup> In two other animal studies, ET-1 injected Intravitreally was shown to decrease VEP amplitude, which was partially reversed by nicardipine or iganidipine.<sup>34,35</sup> We detected higher tear ET-1 levels in patients with abnormal VEP in the MS group than in those without abnormal VEP. Given the studies indicating the role of ET-1 both in the development of ON and its severity in MS, and a high frequency of ON in MS either at diagnosis or in the course of the disease, our findings may suggest that tear ET-1 may be a useful marker in the diagnosis of MS. In a previous study, abnormal VEP was found to be correlated with higher EDSS in MS.<sup>36</sup> Some studies analyzed the association of summation of scores of VEP, sensory or brainstem auditory evoked potentials with EDSS in MS.<sup>37,38</sup> As suggested by previous reports, we also showed a higher frequency of abnormal VEP in those patients with higher EDSS scores.

In conclusion, tear ET-1 level was associated with age and disability in MS. Tear ET-1 level and VEP predicted MS. Tear ET-1 was found to be higher in patients with abnormal VEP in MS. The collection of tears to detect ET-1 levels is a simple, cheap and noninvasive method, which may provide an important clue for diagnosis, provide information about disability, and identify abnormal VEP in MS. Moreover, the monitoring of the disease by tear ET-1 levels in the future may be an option. Further studies analyzing ET-1 levels simultaneously in plasma, CSF and tears should look to identify differences in their clinical importance in MS. Then, the measurement of tear ET-1 levels may emerge as a new component in clinical practice, and become part of accepted guidelines.

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## DISCLOSURE

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