VASCULAR ENDOTHELIAL GROWTH FACTOR AND AMYOTROPHIC LATERAL SCLEROSIS: THE INTERPLAY WITH EXERCISE AND NONINVASIVE VENTILATION

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ABSTRACT: Introduction: We evaluated plasma vascular endothelial growth factor (VEGF) levels in patients with amyotrophic lateral sclerosis (ALS) with reference to the effects of respiratory failure, noninvasive ventilation (NIV), and exercise. Methods: We studied plasma VEGF levels in 83 ALS patients, 20 healthy controls, and 10 patients with other disorders. There were 4 groups of ALS patients: G1, 27 patients without respiratory problems; G2, 14 patients stabilized on nocturnal NIV; G3, 30 patients presenting with respiratory failure; G4, 12 patients on an aerobic exercise protocol. Results: VEGF plasma levels did not differ significantly between ALS patients and controls, or between ALS groups. In G3, the mean VEGF levels increased 75% during NIV. In G4, the mean VEGF level increased by 300% during the exercise program. VEGF levels did not change during the course of the disease. Conclusions: VEGF levels in ALS depend on changes in ventilation and exercise but are probably not affected by the disease process itself.


Approximately 90–95% of patients with amyotrophic lateral sclerosis (ALS) present as a sporadic disorder, whereas 5–10% of cases have a clear family history, and are classified as familial ALS.1 In addition to Mendelian inheritance, other genetic factors have been associated with an increased risk of ALS.3 One such example is vascular endothelial growth factor (VEGF). VEGF regulates angiogenesis and, in the central nervous system, plays several roles, including neuroprotection. Haplotypes of genetic variations in the VEGF promoter/leader sequence have been reported to increase the risk of sporadic ALS in some European populations,2 but not in others.3 Furthermore, lower VEGF plasma levels have been associated with the genotypes that confer the greatest risk for ALS. In an analysis of association studies of VEGF in ALS that included more than 7,000 homozygous carriers of the 2578AA risk allele, there was a significantly increased risk for ALS.4

There has been much interest in VEGF levels in cerebrospinal fluid (CSF), serum, and plasma in ALS, but these studies have been inconclusive.5,6 We sought to determine whether hypoxia, noninvasive ventilation (NIV), and physical activity might be possible confounders of plasma VEGF levels in ALS. We anticipated that VEGF expression in ALS might be modulated by these factors.

MATERIALS AND METHODS
ALS Patients. We studied 83 patients aged 18–85 years who were diagnosed with probable or definite ALS according to the revised El Escorial criteria and the electrodiagnostic criteria set out in the Awaji guidelines.7,8 All gave informed consent as required by our local Ethics Committee. All patients were followed in the Neuromuscular Unit of the Department of Neurosciences (Hospital de Santa Maria, Lisbon). We excluded patients with other medical conditions such as diabetes, cancer, heart failure, or lung disorders. No patient was on tube feedings or taking any nutritional supplements other than vitamins. At the time of blood sampling disease severity was scored using the ALS-FRS (Amyotrophic Lateral Sclerosis Functional Rating Scale).9 We used the respiratory subscore of the ALS-FRS-R to quantify respiratory symptoms. Forced vital capacity (FVC) and arterial blood gas measurements were determined,10 and mean nocturnal O2 saturation was measured by pulse-oximetry nocturnal oximetry as described previously.11

There were 4 groups of ALS patients. Group 1 (G1) consisted of 27 patients (32.5%) without clinical (respiratory subscore = 12) or laboratory features [FVC ≥80% predicted value and mean O2 saturation on percutaneous nocturnal oximetry (PNO) ≥95%] of respiratory distress. Group 2 (G2) contained 14 patients (17%) with chronic

Abbreviations: ALS, amyotrophic lateral sclerosis; ALS-FRS, amyotrophic lateral sclerosis functional rating scale; CSF, cerebrospinal fluid; ELISA, enzyme-linked immunosorbent assay; FVC, forced vital capacity; NIV, noninvasive ventilation; VEGF, vascular endothelial growth factor

Key words: amyotrophic lateral sclerosis; biomarker; exercise; noninvasive ventilation; plasma VEGF levels

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respiratory insufficiency (respiratory subscore <12 and FVC <80% predicted value) who were stable on nocturnal NIV (mean O₂ saturation on PNO ≥95%), although sometimes requiring additional hours of daytime ventilation. Group 3 (G3) consisted of 30 patients (36%) with rapidly progressive type 2 respiratory failure (PaO₂ <60 mmHg and PaCO₂ >50 mmHg) as an early manifestation of ALS (respiratory fatigue as the presenting symptom and respiratory insufficiency as the most critical functional limitation), in whom we measured the plasma VEGF level on the day of NIV initiation and later, during NIV. In Group 4 (G4) there were 12 (14.5%) additional patients with (6) and without (6) symptomatic respiratory involvement in whom plasma VEGF levels were tested before and during an aerobic exercise protocol, consisting of a twice-weekly multimuscular exercise on a treadmill, with progressive resistance on a ramp angled 20% lower than that determined by the cardio-pulmonary exercise stress test (CPET) performed just before exercise protocol initiation. CPET was performed according to current guidelines. Exercise was limited by subjects’ fatigue as evaluated by the Borg perceived scale (7/10), heart rate (75% of the predicted value) and peak oxygen uptake at 55–65% of the maximum predicted peak oxygen uptake to not exceed a moderate level on the Borg perceived exertion scale, but still producing a physiological response. Patients with respiratory symptoms performed the exercise protocol on NIV.

Healthy Controls. Twenty healthy subjects matched for age and gender with the ALS population were investigated as a comparison group.

Patients with Other Neuromuscular Disorders. We also investigated 10 patients with other neuromuscular disorders without ventilatory difficulty (3 with progressive muscular atrophy, 3 with chronic inflammatory demyelinating polyneuropathy, 2 with a nonprogressive monomelic form of motor neuron disease, 1 with primary lateral sclerosis, and 1 with neuroacanthocytosis), matched for age and gender.

VEGF Determination: VEGF Enzyme-Linked Immunosorbent Assay. Blood was collected in a tube containing ethylene-diamino-tetra-acetic acid (EDTA) and was centrifuged at 1,000 g for 10 min. The supernatant plasma was conserved at −80°C. The first described and the most important member of the VEGF family is VEGF-A. In this study, VEGF-A levels in plasma were determined with a chemiluminescent immunoassay method using a commercial enzyme-linked immunosorbent assay (ELISA) assay (QuantiGlo®; R&D Systems) in accordance with the manufacturer’s instructions. A calibration curve was derived using the following VEGF-A concentrations: 0, 6.4, 32, 160, 800, 4,000, and 20,000 pg/ml. A cubic-spline curve fit was applied to calculate the VEGF-A concentrations of the samples by interpolation; the correlation coefficient varied between 0.9995 and 1 for the different assays. Luminescence measurements were done using a Turner Biosystems Modulus Microplate Reader with a 1.0 min lag time and 0.5 s/well read time. All ELISA measurements were performed in duplicate, and the average was used. All values determined were greater than the lower detection limit of the calibration curve. We were aware of the precautions necessary regarding techniques of sample collection, including the use of plasma rather than serum to avoid release of VEGF from platelets, and the potential impact of centrifugation and temperature on VEGF levels.

Data Analysis. Data analysis was performed using Graph-Pad Prism® (GraphPad Software, Inc.). We applied the D’Agostino & Pearson omnibus test of normal distribution in the data. A one-way ANOVA followed by the Tukey test for multiple comparisons was applied in a post hoc analysis of the data from the different groups. An unpaired t-test, assuming equal variances, was used to test for differences between variables in the ALS subgroups. A paired t-test was applied to test for differences between 2 VEGF measurements in each ALS subgroup. The Pearson product-moment correlation coefficient was used to analyze the linear dependence between tested variables. We considered P-values <0.05 to be significant statistically.

RESULTS

The 20 healthy controls and 10 patients with other neuromuscular disorders showed similar VEGF levels (mean, 28.7 pg/ml; SD 10.7 vs. mean 26.1 pg/ml; SD 10.7, respectively, P = 0.54). We therefore merged these 2 groups to form a larger control group of 30 subjects. The results from all groups studied are summarized in Table 1. Age and gender distributions were similar between groups. Disease duration was not different statistically among the 4 ALS groups. The ALS-FRS was significantly lower for the groups that required NIV (G2 and G3) than for the remaining ALS groups (Table 1), indicating more severe impairment. As expected, the respiratory subscore and FVC were reduced significantly in G2 and G3 compared with the other 2 groups (Table 1), but there was no difference between G2 and G3. The VEGF level was higher in ALS patients than controls, but this difference did not reach statistical significance (P = 0.052), and there was no significant difference between the different groups of ALS patients.
Comparison of the G4 – ALS selected for G3 – ALS with severe disease.

G4 8.8 0.96), pCO2 (r 0.18; P 0.05). There was no significant correlation between VEGF levels and age (r 0.38), disease duration (r 0.285; P 0.025).

Within the ALS groups, the only significant correlation was a negative correlation between VEGF level and FVC in ALS G2, consisting of patients on long-term noninvasive ventilation (r −0.51; P = 0.025).

VEGF levels were measured in G3 before and during NIV and in G4 before and during exercise. In G3, only 10 of the 30 patients could be studied before and during NIV initiation (4.9 months; SD ± 3.9; range, 2–14 months) due to their tenuous clinical condition and the requirement for stable, normal nocturnal oximetry at the time of the second VEGF determination. In these 10 patients there was a striking (75%) increase in the mean VEGF (Table 2 and Fig. 1). Figure 2 represents subsequent VEGF level determinations after NIV initiation in patients in G3; the mean values remained higher. Of the ALS patients in G1 and G2, who were in a stable state (ventilated in G2), VEGF levels were determined twice in 9 patients (mean interval 1.9 months; SD ± 0.9; range, 0.7–3.3 months). There was a nonsignificant trend for VEGF levels to increase (Table 2).

In G4, 6 of the 12 patients were assessed before and during their exercise protocol (3 with and 3 without NIV on exercise). The remaining 6 failed to complete the protocol. In the 6 exercised patients, the mean VEGF increased 300% during exercise (Table 2, mean interval between evaluations 9.1 months; SD ± 6.2; range, 3–16 months). Individual results of VEGF levels in the 6 exercised patients in this group are shown in Figures 1B and 3. In 2 patients VEGF did not increase during exercise. One was very spastic and progressed rapidly, and the other was on NIV before exercise and followed the exercise protocol irregularly; neither patient surpassed predicted values of 50% for heart rate and peak oxygen uptake. One showed a fall in plasma VEGF level at the time of major functional decline from a respiratory infection after a significant increment of the VEGF level in the initial phase. Three patients continued exercise for a longer time; 1 used NIV at the time of

### Table 1. Demography and VEGF plasma concentrations (pg/ml) in the different groups.*

<table>
<thead>
<tr>
<th>ALS</th>
<th>n</th>
<th>M/F</th>
<th>Age (years)</th>
<th>Disease duration (years)</th>
<th>ALS-FRS</th>
<th>Respiratory subscore</th>
<th>FVC (% predicted value)</th>
<th>VEGF level (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 – stable ALS without ventilatory compromise</td>
<td>83</td>
<td>56/27</td>
<td>63.0 ± 13.9</td>
<td>2.5 ± 2.9</td>
<td>26.4 ± 7.5</td>
<td>9.8 ± 2.2</td>
<td>80.9 ± 20.5</td>
<td>42 ± 30</td>
</tr>
<tr>
<td>G2 – stable ALS, on NIV</td>
<td>27</td>
<td>15/12</td>
<td>61.8 ± 12.9</td>
<td>3.7 ± 4.2</td>
<td>29.9 ± 6.1</td>
<td>11.3 ± 1.6</td>
<td>94.6 ± 18.5</td>
<td>44 ± 30</td>
</tr>
<tr>
<td>G3 – ALS with severe respiratory distress</td>
<td>14</td>
<td>9/5</td>
<td>64.3 ± 12.5</td>
<td>2.4 ± 1.9</td>
<td>23.2 ± 9.1</td>
<td>8.3 ± 2.4</td>
<td>64.2 ± 15.2</td>
<td>48 ± 48</td>
</tr>
<tr>
<td>G4 – ALS selected for exercise program</td>
<td>30</td>
<td>22/8</td>
<td>62.2 ± 15.9</td>
<td>1.8 ± 2.1</td>
<td>23.4 ± 6.8</td>
<td>9.1 ± 1.7</td>
<td>71.3 ± 15.3</td>
<td>36 ± 20</td>
</tr>
<tr>
<td>Controls</td>
<td>12</td>
<td>10/2</td>
<td>66.5 ± 13.4</td>
<td>1.8 ± 1.2</td>
<td>31.4 ± 4.4</td>
<td>10.5 ± 2.5</td>
<td>89.1 ± 18.9</td>
<td>40 ± 22</td>
</tr>
</tbody>
</table>

Comparison of the different groups- P-value

In the total ALS population, we found no significant difference in VEGF levels between men and women (P = 0.58) or bulbar-onset vs. spinal-onset patients (P = 0.07). There was no significant correlation between VEGF levels and age (r = 0.10; P = 0.38), disease duration (r −0.15; P = 0.17), ALS-FRS (r 0.08; P = 0.48), ALS-FRS-R (r −0.19; P = 0.10), FVC (r −0.05; P = 0.74), pO2 (r 0.01; P = 0.96), pCO2 (r 0.18; P = 0.25), or mean O2 saturation on PNO (r −0.05; P = 0.77). Within the ALS groups, the only significant correlation was a negative correlation between VEGF level and FVC in ALS G2, consisting of patients on long-term noninvasive ventilation (r −0.51; P = 0.025).

### Table 2. Changes in VEGF plasma concentrations (pg/ml) in ALS patients.*

<table>
<thead>
<tr>
<th>ALS groups</th>
<th>Time between evaluations (months)</th>
<th>n</th>
<th>Before</th>
<th>After</th>
<th>Slope (pg/ml/month)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1+G2</td>
<td>1.9 ± 0.9</td>
<td>9</td>
<td>40 ± 41</td>
<td>45 ± 39</td>
<td>2.2</td>
<td>0.1722</td>
</tr>
<tr>
<td>G3</td>
<td>4.9 ± 3.9</td>
<td>10</td>
<td>36 ± 20</td>
<td>63 ± 34</td>
<td>5.5</td>
<td>0.0117*</td>
</tr>
<tr>
<td>G4</td>
<td>8.8 ± 6.2</td>
<td>6</td>
<td>40 ± 23</td>
<td>120 ± 73</td>
<td>9.1</td>
<td>0.0263*</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SD; n, number of subjects; VEGF, vascular endothelial growth factor.
†Comparison before and after intervention (P < 0.05).
exercise initiation. All 3 later developed major functional declines, and 2 were wheelchair confined at the time of the last VEGF determination (Fig. 3). We calculated the mean rate of VEGF increase/month in G1, G2, G3, and G4, and we noted striking differences between the groups (Table 2), especially in the newly ventilated (G3) and exercise (G4) groups.

**DISCUSSION**

VEGF is an important circulating factor in the neovascularization response to hypoxia, in certain cancers and metastases, and in POEMS (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes) syndrome. VEGF levels are increased in acute ischemic stroke. VEGF activity is also important in the pathogenesis of diabetic neuropathy and diabetic retinopathy. Studies in the SOD1G93A mouse model of ALS have suggested that VEGF has a neuroprotective function to increase survival of affected animals and delay clinical disease onset. There are only a few reports of VEGF levels in CSF, serum, and plasma in humans with ALS, and these have given conflicting results. VEGF levels are much higher in serum than in plasma and even lower in CSF, and measurements in serum are potentially contaminated by leakage of VEGF from platelets.

Serum levels of VEGF in ALS have been reported as unaltered or higher than normal by different investigators. Lambrechts et al. found that plasma VEGF levels were around 50% lower in people with ALS than in their spouses, especially in those carrying at-risk VEGF genotypes. However, in other studies, plasma levels of VEGF were within normal limits in ALS patients and were without significant change in patients with hypoxemia. Devos et al. studied CSF VEGF levels (incorrectly stated in their study as pg/L rather than pg/ml) in 24 patients with ALS; they reported lower CSF levels in the early phase of the disease when compared with other disease controls but similar to levels recorded in healthy subjects. In contrast, Gupta et al. found that VEGF levels were increased in CSF and serum in ALS in

**FIGURE 1.** (A) Individual and mean values of VEGF levels before and after initiation of NIV in G3. There was a 75% increase in the VEGF mean level. (B) Individual and mean values of VEGF levels before and after initiation of exercise in G4. Data were available from 6 of 12 patients selected initially who completed the protocol. There was a 300% increase in the mean VEGF level during the period of observation (see individual results in Fig. 2 and text).

**FIGURE 2.** VEGF level during NIV in patients in G3. The dashed line indicates the onset of NIV. The first sample was collected before NIV but on the same day as NIV onset. The VEGF level increased in the patients followed for several months, but stabilized in 1 subject (see text).

**FIGURE 3.** VEGF level (pg/ml) in 6 patients in G4, before and during exercise. The dashed line indicates onset of exercise. In 4 of the 6, the VEGF level increased significantly after initiating exercise, but returned to baseline when exercise was discontinued due to increasing weakness (see text).
general, and they increased further with hypoxia. In long duration ALS, Ilzecka\textsuperscript{25} found that the VEGF level in CSF was increased. The VEGF level in CSF is not clearly associated with hypoxia in ALS, as a lack of upregulation of CSF VEGF during hypoxemia has been reported, when compared with hypoxicemic controls.\textsuperscript{26}

We found that the levels of plasma VEGF in our ALS population were similar statistically to both age and gender-matched healthy controls and to other neurogenic disorders. There was no relationship between plasma levels and gender, region of onset, or ALS-FRS, as described elsewhere.\textsuperscript{17,21,23,26} In addition, we did not find a negative correlation between FVC, PaO\textsubscript{2}, or PCO\textsubscript{2}, and VEGF level, meaning that hypoxia was not an effective stimulant of VEGF production in our patients with ALS. This latter observation is consistent with similar findings reported elsewhere.\textsuperscript{17,21,23,26} Indeed, Moreau and co-workers showed that hypoxia inducible factor-1 was not activated by hypoxia in monocytes from ALS patients.\textsuperscript{27}

However, unexpectedly, we found that ALS patients in a critical phase of uncontrolled respiratory distress showed a 75% increase in VEGF level only after NIV was established, and the increase remained in 2 patients in G3 studied repeatedly during a period of more than 2 years (Fig. 2). We suggest that this finding suggests that correction of hypoxia leads to higher activation of the hypoxia element response in the VEGF promoter by the hypoxia inducible factor-1. There is a suggestion of a rebound response after hypoxia correction with NIV (Fig. 2). The plasma VEGF levels remained similar to baseline levels in 9 patients not treated with NIV or engaged in the exercise program that was followed 2 months later. In 6 ALS patients undergoing exercise in G4, the plasma VEGF level increased by 300%, although in 2 of them there was no change (Fig. 3). In 4 of the 6 patients in G4 the increase in plasma VEGF level was not maintained later in the disorder, when they could no longer continue exercising. Increased expression of VEGF mRNA with exercise has been demonstrated in muscle of normal exercising mice.\textsuperscript{28}

Our findings show that VEGF levels are not consistently abnormal in ALS, but suggest that there is a specific dysregulation of the physiological response to hypoxia. Moreover, our results indicate that several external factors can modulate VEGF expression, such as initiation of NIV in patients with marked respiratory involvement, and physical activity. The potential positive impact of this modulation requires further investigation. These results do not invalidate the suggested role of genomic polymorphisms described by previous investigators to be associated with ALS, but they indicate the need for caution in interpreting plasma and other VEGF levels in the disease.

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REFERENCES

1. Costa J, Gomes C, de Carvalho M. Diagnosis, pathogenesis and thera-

2. Lambrecht D, Storkebaum E, Morimoto M, Del-Fevero J, Desmet F, Marklund SL, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motor neurons against ischae-


6. Storkebaum E, Lambrecht D, Carmeliet P. VEGF: once regarded as a specific angiogenic factor, now implicated in neuroprotection. Bio-
essays 2004;26:945–954.

7. Brooks BR, Miller RG, Sash C, Munsat TL, for the World Federa-


9. Cedarbaum JM, Stambler N. Performance of the Amyotrophic Later-
lateral Sclerosis Functional Rating Scale (ALSFRS) in multicenter clini-

ophysiol 2004;26:943–954.


16. Nobile-Orazio E, Terenghi F, Gannotta C, Gallia F, Nozza A. Serum VEGF levels in POEMS syndrome and in immune-mediated neuropa-

17. Lee SC, Lee KY, Kim VJ, Kim SH, Koh SH, Lee YJ. Serum VEGF levels in acute ischaemic strokes are correlated with long-term prog-


