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# *Morphobiochemical parallels in evaluating the condition of the spinal soft tissue vascular flow in patients with neurofibromatosis type I*

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**Purpose** To compare and analyze structural changes in the vascular bed of the spine skin, paravertebral muscles in the zone of surgical intervention (at the apex of the spine deformity), as well as serum concentrations of vascular endothelial growth factors and their receptors in patients with kyphoscoliosis due to neurofibromatosis type I (NF-I). **Material and methods** The work is based on the results of examining a continuous cohort of patients with NF-I (n = 12). During the examination the concentrations of vascular endothelial growth factors (VEGF, VEGF-A, FGF-basic) and receptors for these factors (VEGF-R2, VEGF-R3) were determined in blood serum using the immunoenzyme analysis. Histological techniques of light and scanning electron microscopy were used for studying the biopsy material of the spine skin and paravertebral muscles obtained in the projection of the spine deformity apex during surgical correction of kyphoscoliosis. **Results** Concentration of VEGF and VEGF-A was 8 % (p = 0.00908) and 417 % (p = 0.00392) higher than reference values, respectively. VEGF-R2 receptor was 215 % (p = 0.00622) and exceeded the level of reference values. On the contrary, VEGF-R3 receptor concentration was 58.1 % (p = 0.00415), lower than the relative reference values. FGF-basic growth factor in blood serum was within control values (p = 0.05613). Changes in the histostructure of the vascular walls of large- and medium-size arteries and veins, fibrosis and obliteration of some vessel lumens, hyperemia of venous and capillary microvessels, formation of inflammatory infiltrates in adventitia of large- and medium-size vessels and in perivascular spaces, hemorrhages were revealed in bioptates of the spine skin. Pathological changes in muscles manifested themselves as fibrosis and constriction (up to complete obliteration) of the lumens of arterial vessels, increase in the diameter of thin-walled venous vessels, increase in their permeability leading to widespread extensive hemorrhages. **Conclusion** Increased concentration of vascular endothelial growth factors and their imbalance with receptors in patients with NF-I are combined with pathohistological structural changes in the vascular system of the spine skin and paravertebral muscles due to the inflammatory process present. The obtained results should be taken into account both in surgical treatment of this complex disease manifestations, and when planning treatment and rehabilitation measures in general.

**Keywords**: neurofibromatosis type I (NF-I), spinal deformities, skin, paravertebral muscles, vascular bed, vascular endothelial growth factors (VEGF, VEGF-A, FGF-basic), receptors (VEGF-R2, VEGF-R3)

## INTRODUCTION

Neurofibromatosis type I (NF-I) is a multisystem disorder affecting the musculoskeletal [1–4], cardiovascular [5–8], endocrine [9], central and peripheral nervous systems [10, 11].

According to the literature, vasculopathy is a recognized manifestation of NF-I [5–8, 12], which can occur in various areas of the body [5–7, 12, 13]. Manifestations of vasculopathy include renovascular stenosis with concomitant hypertension, cerebrovascular occlusion, aortic coarctation, visceral ischemia, and small artery aneurysms [8, 14–18].

The prevalence of symptomatic peripheral vascular anomalies in patients with type NF-I is about 1 %, while no associations were found between vasculopathy and the patient's clinical status [12]. The etiology of vascular pathology due to NF-I remains insufficiently described [12, 14, 16, 19–22]. Little attention has been paid to the analysis of the vascular bed of soft tissues, in particular, the skin and spinal muscles, which is especially important for NF-I patients with spinal deformities [23, 24].

Purpose We aimed to compare and analyze the morphological and biochemical parallels by studying structural changes in the vascular bed of the spine skin, paravertebral muscles in the zone of surgical intervention (at the apex of the spine deformity), as well as serum concentrations of vascular endothelial growth factors and their receptors in patients with kyphoscoliosis due to neurofibromatosis type I.

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## MATERIAL AND METHODS

This study in humans was approved by the ethics committee of the Center (protocol No. 7 (32) of 12.24.2012). It was carried out in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association (amended in 2013). Patients over 18 years of age as well as the parents of minor age patients or their legal representatives have signed informed voluntary consents to conduct diagnostic tests and publish data without identification.

The subjects were a continuous cohort of 12 patients with neurofibromatosis type I (7 men and 5 women) aged from seven to 24 years (mean age, 14.5 ± 1.5 years). Vertebral syndrome was represented by a curvature in the cervicothoracic spine in two cases and curves in the thoracolumbar spine in 10. Spinal deformity varied from 50 to 140° (mean, 98.0 ± 12.2°) according to Cobb. All patients included in the group had normal excretory function.

Serum concentration of vascular endothelial growth factors (VEGF, VEGF-A, FGF-basic) and receptors for these factors (VEGF-R2, VEGF-R3) was measured using the equipment from Thermo Fisher Scientific (USA): Multiscan FC detector, Shaker-401, WellWash automatic plate washer. The analysis was conducted in accordance with the methodology from the guidelines to the eBioscience kits (VEGF-A, VEGF-R2, VEGF-R3), R&D Systems (FGF-basic), Invitrogen (VEGF).

The values of concentrations of vascular endothelial growth factors and their receptors obtained during examination of patients were compared with the values of the reference group of the same age and gender. The reference group consisted of 104 somatically healthy individuals aged from seven to 24 years (50 males, 54 females). The healthy people age groups and gender are presented Table 1.

Table 1

Age groups and gender of healthy individuals

| Age group | Gender | Age (years) | Number |
|---|---|---|---|
| Junior school age | boys | 8–12 | 19 |
| | girls | 7–11 | 21 |
| Senior school age | boys | 13–17 | 23 |
| | girls | 12–16 | 19 |
| Transient to adult age group | boys | 18–24 | 9 |
| | girls | 17–24 | 13 |

The study did not include persons with allergic, somatic and neuropsychiatric diseases, pregnant women, obese and low-weight persons, as well as with concomitant diseases such as peptic ulcer, chronic cholecystitis and pancreatitis, chronic obstructive pulmonary disease, infectious diseases.

To assess the structural changes in the vascular bed of spine soft tissues in patients with kyphoscoliosis due to NF-I, a histological examination of biopsy samples of the spine skin and paravertebral muscles taken in the projection of the deformity apex of the main curve (between Th5 and Th10 vertebrae) on the convex side was performed while approaching the posterior spinal column during the procedure of surgical correction of kyphoscoliosis.

A skin biopsy sample of approximately 10 × 6 × 6 mm was divided into two parts. The first part of the biopsy specimen (approximate size 5 × 3 × 3 mm) was fixed in a 10 % solution of neutral formalin, followed by dehydration and pouring into paraffin. Histological sections were stained with hematoxylin and eosin, according to Unna-Taenzer. Morphological studies of the skin by light microscopy were performed using an AxioScope.A1 stereo microscope with an integrated AxioCam digital camera (Carl Zeiss Micro Imaging GmbH, Germany). The second fragment of the skin biopsy sample (approximate size 5 × 3 × 3 mm) was impregnated with camphene (3,3-dimethyl-2-methylenebicyclo-[1,2,2]-heptane) and dried in air under dustless conditions, and then sprayed with a conductive layer in an IB 6 ionizer (Japan) and was examined by scanning electron microscopy using a JSM-840 microscope (JEOL, Japan).

Paravertebral muscle biopsy samples of approximately 10 × 5 × 5 mm were taken from the multifidus muscle and fixed in 10 % neutral formalin, followed by histological conduction and paraffin embedding. The sections obtained were stained with hematoxylin and eosin according to Van Gieson and the trichromic method according to Masson. The preparations were examined using an AxioScope.A1 stereo microscope and an integrated AxioCam digital camera (Carl Zeiss Micro Imaging GmbH, Germany).

Statistical data were processed using the Microsoft EXCEL-2010 and the AtteStat application software [25]. The research results were processed by the method of variation statistics used for small samples with the adoption of a significance level of $p \leqslant 0.05$. The significance of differences between two unrelated samples was determined using the Wilcoxon, Dunn, and Mann-Whitney tests for independent samples [26].

## RESULTS

Analysis of serum concentrations of vascular endothelial growth factors (VEGF, VEGF-A, FGF-basic) and receptors for these factors (VEGF-R2, VEGF-R3) in patients with NF-I showed a significant imbalance of growth factors and their receptors (Table 2). The concentration of VEGF was higher than the reference values by only 8 % (p = 0.009023), but VEGF-A exceeded the normal level by 417 % (p = 0.003948). The content of the VEGF-R2 receptor in the blood serum of patients was 215 % (p = 0.00622) higher than that of the control group.

The concentrations of the VEGF-R3 receptor were reduced by 58.1 % (p = 0.00415) while the concentration of FGF-basic growth factor in the blood serum was within the reference values (p = 0.05613) in NF-I patients.

Structural changes in the vascular bed were revealed in biopsy specimens of the spine skin at the apex of deformity in all patients. Hyperemia of most venous and capillary microvessels was seen. Fibrosis of the lumens of separate vessels was observed. Histostructural changes in the walls of large and medium-sized arteries were revealed, expressed as disorders of the ratio of muscle to connective tissue components, spatial orientation of cells and fibers, and partial destruction of the internal elastic membrane (Fig. 1 a). In the adventitial layer of such vessels, signs of inflammation were observed. Vessels with destruction and fibrosis of all layers, as well as complete fibrosis or obliteration of their lumens were found in the reticular layer and subcutaneous fatty tissue (Fig. 1 b).

Table 2

Concentration of VEGF and VEGF-A, FGF-basic and receptors VEGF-R2, VEGF-R3 in serum of NF-I patients (M ± m, n – number of cases)

| Growth factor name | Unit | Reference group (n = 104) | NF-I group before treatment (n = 12) |
|---|---|---|---|
| VEGF | Ng/mm | 0.165 ± 0.03 | 0.178 ± 0.04* |
| VEGF-R2/KDR | Ng/mm | 11.3 ± 0.75 | 35.7 ± 1.30* |
| VEGF-A | Ng/mm | 0.12 ± 0.03 | 0.62 ± 0.05* |
| VEGF-R3/FLT-4 | Ng/mm | 102.01 ± 11 | 42.75 ± 4.7* |
| FGF-basic | Ng/mm | 8.98 ± 1.33 | 8.975 ± 0.85 |

Note: * – significance of differences with reference values according to the criteria of Wilcoxon, Mann-Whitney and Dunn, p ⩽ 0.05; ng/ml (nanograms of the substance in 1 ml of blood serum)

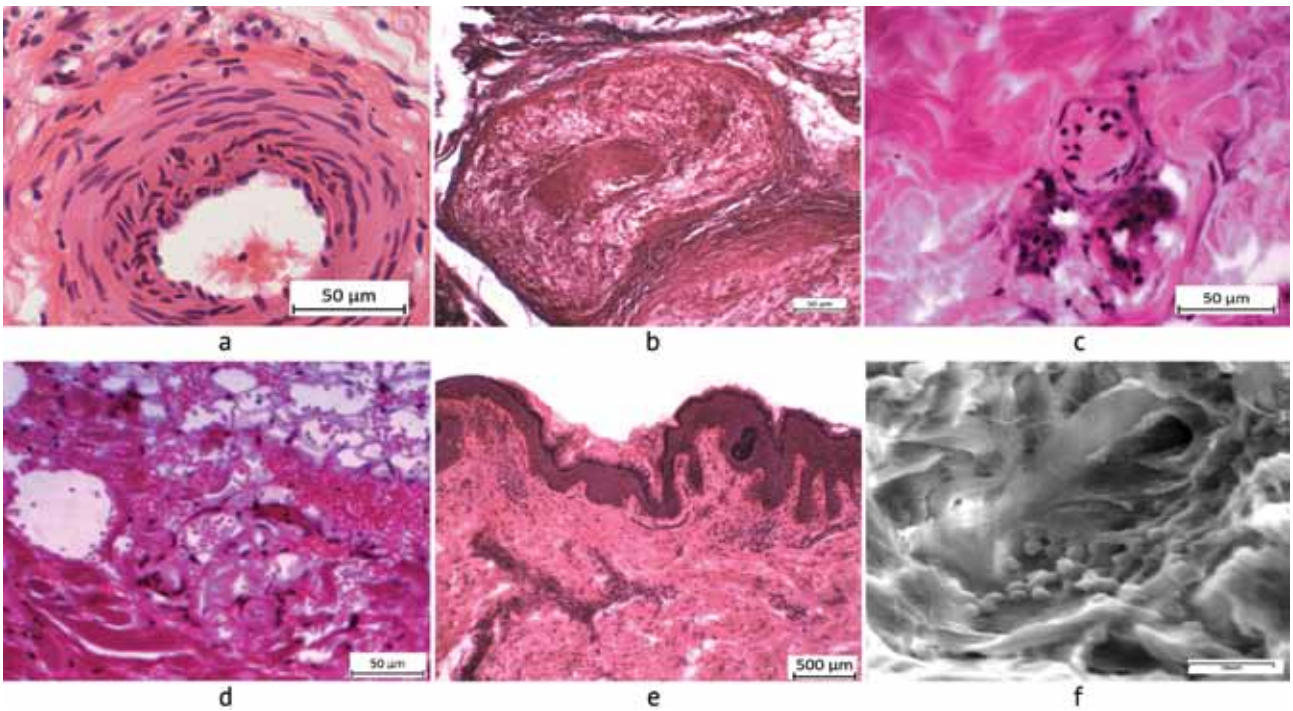


**Fig. 1** Biopsy of the spine skin at the top of the deformity of the patient with NF-I: ***a*** change in the histostructure of the vascular walls and fibrosis of the lumen of the artery; ***b*** destruction of all layers and complete closure of the lumen of the artery; ***c*** "melting" of connective tissue and muscle components of the outer and middle layers of veins; ***d*** dilated thin-walled microvessels with foci of extensive hemorrhages in the intervascular spaces; ***e, f*** presence of inflammatory infiltrate along the course of microvessels in the papillary and reticular layers of the dermis. Staining: a, c, d, e hematoxylin and eosin; b – orcein according to Tenser /Unn; e scanning electron microscopy. Magnification: ***a–d*** – 400 ×; ***e*** – 100 ×; ***f*** – 3300 ×

The effect of "melting" of the connective tissue and muscle components of the outer and middle layers was noted in some veins due to inflammation of the vascular wall (Fig. 1 c). Most of the vessels of the venous type were in the state of vasodilation. Dilated sinusoidal capillaries and thin-walled venules with foci of extensive hemorrhages in the intervascular spaces due to constriction of arterial vessels were revealed in some areas (Fig. 1 d).

In the perivascular spaces along the course of microvessels in the papillary and reticular layers of the dermis, inflammatory infiltrates were also found (Fig. 1 e, f).

In biopsies of the multifidus muscle, the arterial vessels frequently had narrowed lumens, a substantially fibrosed middle membrane with disoriented smooth muscle cells and an enlarged adventitia membrane, represented by loose connective tissue containing fibroblasts, histiocytes, and other cell and fibrous structures (Fig. 2 a). Many vessels had closed lumen and a fibrosed adventitial layer (Fig. 2 b). Mosaic-like patterns were also observed: single preserved myocytes, including atrophic ones, on the background of increased endomysial space, alternated with adipocyte fields with vascular bundles immersed in them (Fig. 2 b).

The vessels of the venous type had thinned walls and widened lumens filled with blood, which, along with fibrosed and often almost closed vessels of the arterial type, caused effusion of blood cells and extensive hemorrhages (Fig. 2 a, c). In the enlarged perimysium space, spiral-shaped connective tissue fibers were visualized oon the background of hemorrhages (Fig. 2 d).

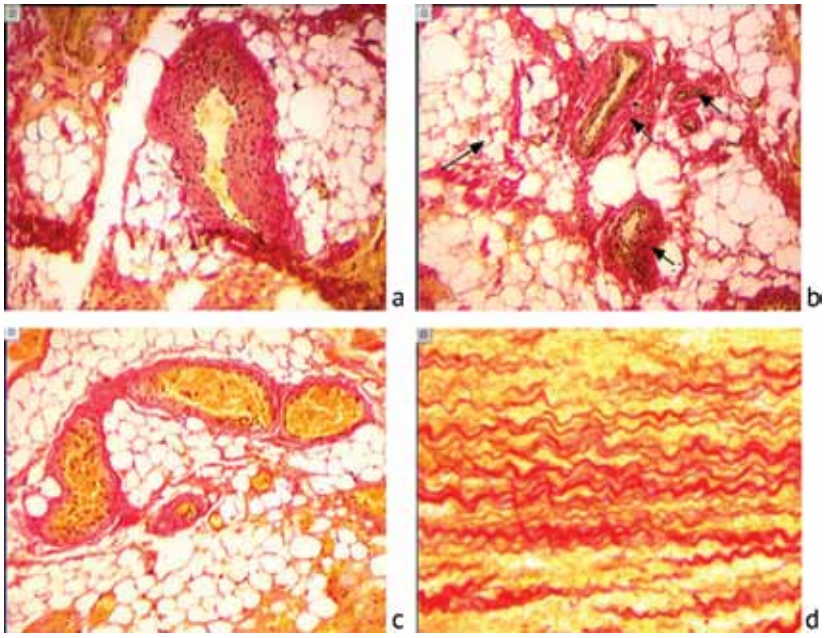


**Fig. 2** Biopsy of multifidus muscle at the apex of deformation of patients with NF-I: ***a*** artery with fibrosed middle membrane (in the center); ***b*** muscle fibers replaced by adipocytes (arrow), vessels with narrowed lumens and adventitial fibrosis (short arrows); ***c*** vessels of the venous bed dilated, filled with elements of blood along with massive fatty degeneration of muscle fibers; ***d*** connective tissue fibers in perimisium and extensive hemorrhages. Van Gieson stain; magnification: ***a, b, c*** – 79 ×, ***d*** – 200 ×

## DISCUSSION

Neurofibromatosis is a hereditary disease affecting the skin, nervous tissue, bone and soft tissue structures [3, 27]. Kyphoscoliotic spinal deformities develop in 25 to 50 % of patients with NF-I [1-3], the main feature of which is early manifestation and rapid progression [4, 28]. Irreversible cardiopulmonary and neurological disorders develop, and only timely surgical intervention is able to prevent them [29]. With the use of the methods of posterior thoracoplasty, morphofunctional features of the soft tissues in the surgical intervention area should be considered for the prognosis of wound healing and planning of postoperative rehabilitation measures. In patients with spinal deformities due to NF-I, the condition of the soft tissues of the spine at the top of the deformity has a number of hypoplastic and degenerative structural changes associated with impaired innervation and blood supply to the skin and muscles [23].

It is known that vasculopathy is one of the clinical features of NF-I, which is renovascular stenosis with concomitant hypertension, cerebrovascular occlusion, coarctation of the aorta, visceral ischemia and aneurysms of small arteries [8, 14-18]. The prevalence of symptomatic

peripheral vascular anomalies in patients with type NF-I is about 1 %, while no associations were found between vasculopathy and the patient's clinical status [12].

The etiology of vascular disorders in NF-I remains insufficiently discussed [12, 14, 16, 20, 22]. Internal lesions of the arterial walls (formation of neointima and vascular occlusion) [19] are an important manifestation of NF-I. Arterial stenosis of non-tumor manifestations of NF-I may have serious or even fatal consequences [6, 7, 20].

NF-I is known to result from mutations in the tumor suppressor gene NF1, which encodes neurofibromin (a tumor growth suppressor protein). Neurofibromin regulates the transmission of Ras-mitogenic signal to the cell nucleus by proteins. It is expressed in endothelial and smooth muscle cells of blood vessels, and NF-I vasculopathy may be the result of a change in the function of neurofibromin in these cells and a decrease in its expression [14, 16].

According to B.K. Stansfield et al., who studied experimental models and patients, the normal number of endothelial colony-forming cells in the peripheral blood is determined in NF-I, but these cells show increased Ras-ERK activation and increased proliferation and migration in response to environmental changes. Hyperproliferation is demonstrated by each type of cell, which includes the vascular wall (endothelium, smooth muscle cells, pericytes) [20]. Also, these researchers suggested that the Ras-ERK pathway in neurofibromine-deficient macrophages is responsible for increased neointima formation [21].

In our study, we compared the structural changes in the vascular bed of the skin and spine muscles in the area of surgical intervention (at the top of the spinal deformity) and serum concentrations of vascular endothelial growth factors (VEGF and VEGF-A, FGF-basic) and their receptors (VEGF-R2, VEGF-R3) in NF-I patients.

VEGF, a heterodimeric glycoprotein growth factor, is known to be responsible for revascularization and angiogenesis [30, 31]. VEGF is a mitogen for vascular epithelial cells, capable of increasing vascular permeability [32]. It was shown that an increase in the level of VEGF in the blood indicates its increased secretion [33, 34].

According to the results of our study, it was found that in patients with NF-I, all metabolic processes ran under an increased content of angiogenic growth factors VEGF, VEGF-A and their VEGF-R2 receptor in the blood serum, which not only contributes to an increase in vascularization, but also indicates the presence of persistent inflammation in the tissues, which is a precursor of morphological changes and leads to the progression of connective tissue degeneration [35].

An increased concentration of VEGF-A factor stimulates the release of pro-inflammatory factors (cytokines) and cannot be associated with the VEGF-R3 receptor [36]. This fact may lead to an increase in bone marrow vascularization and the release of monocytes and macrophages. Prolonged inflammation results in fibrosis, increased vascular permeability, and formation of inflammatory infiltrates [37].

Our study showed that an imbalance of vascular endothelial growth factors and their receptors in patients with NF-I can lead to pathological structural changes in the vascular bed of the soft tissues of the spine: changes in the histostructure and fibrosis of the vascular walls of large and medium-sized arteries and veins, fibrosis and obliteration of their lumens, and in some cases, complete degeneration of vessels, expansion and plethora of thin-walled vessels of the venous type with an increased permeability, causing hemorrhage.

In our opinion, such changes are due to the inflammatory process, as evidenced by the presence of leukocyte infiltrate in the adventitial layer of large and medium vessels of the skin, in the perivascular spaces along the course of microvessels. The inflammatory nature of vasopathy in patients with NF-I is confirmed by the findings of other authors. So, E.A. Lasater et al. conducted the peripheral blood analysis in patients with NF-I who had no obvious clinical signs of vascular pathology and revealed an increased concentration of inflammatory cells and cytokines. It indicates to genetic and cell prerequisites for vascular inflammation in NF-I patients [19].

## CONCLUSION

Patients with neurofibromatosis type I have an increased concentration of VEGF and VEGF-A and their imbalance with receptors VEGF-R2, VEGF-R3 which are combined with histopathological structural changes in the vascular bed of the skin and paravertebral muscles of the spine. Biopsies of the skin of the spine show changes in the histostructure of the vascular walls of large and medium-sized arteries and veins, fibrosis and obliteration of the lumens of separate vessels, hyperemia of skin microvessels of the capillary and venous types, formation of inflammatory infiltrates in the adventitia of large and medium vessels and in the perivascular spaces, presence of hemorrhages. In paravertebral muscles, pathological

changes are manifested by fibrosis and narrowing of the lumen of the vessels of the arterial type, an increase in the diameter of thin-walled vessels of the venous type and their increased permeability, causing widespread hemorrhages. The changes detected, most likely, are due to an inflammatory process, which must be taken into account both in the surgical treatment of the manifestations of this complex disease, and by planning treatment and rehabilitation measures in general. More attention should be paid to vascular and anti-inflammatory therapy in the postoperative period to create conditions for wound healing and reduce the number of wound complications, and also consider the inflammatory nature of the pathology of the vascular bed of soft tissues (skin and muscles) while developing therapeutic and diagnostic interventions.

*The authors declare no conflict of interest.*

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