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Fluorescence spectroscopy of blood plasma and cerebrospinal fluid for the diagnosis of cerebral gliomas

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This study investigated the capabilities of fluorescence spectroscopy of blood plasma and cerebrospinal fluid in diagnosing cerebral gliomas. Key findings included a decreased ratio of tyrosine to tryptophan autofluorescence in the blood plasma of glioma patients compared to a control group with traumatic brain injuries. Furthermore, upon excitation at 320 nm, the plasma fluorescence spectra of Grade IV glioma patients exhibited a long-wavelength shift relative to lower-grade tumors. Additionally, analysis of cerebrospinal fluid fluorescence decay kinetics at 280 nm excitation successfully differentiated glioma patients from the control cohort.

Keywords: autofluorescence spectroscopy, time resolved fluorescence spectroscopy, blood plasma, cerebrospinal fluid.

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1. Introduction

Oncological diseases of the brain are among the most difficult for early diagnosis and choice of treatment tactics, characterized by an aggressive course and poor prognosis. The incidence of gliomas is about 80 % of malignant brain tumors [1]. Approximately 48 % of all gliomas are diagnosed with the most aggressive form of the tumor, called glioblastoma multiforme (GB) (G4) [2]. The prognosis for patients with initially diagnosed GB is currently disappointing, and the median survival is just over one year on average [3]. The main problem lies in the late diagnosis, when the prognosis of the cure is unfavorable [4]. It is important to detect a malignant tumor at an early stage. However, currently used imaging methods such as magnetic resonance imaging [5], computed tomography [6], positron emission tomography [7] are ineffective with a small tumor size and cannot be used for early diagnosis. At the same time, when a cancerous tumor occurs and develops, the molecular composition of body fluids significantly changes [8]. For example, it has been shown that when gliomas develop, various biomarkers are released into blood, cerebrospinal fluid (CSF), saliva, and other body fluids [9]. These can be tumor cells, circulating deoxyribonucleic (DNA) and ribonucleic acids (RNA), microRNAs, exosomes containing the genetic material of the tumor, various proteins and metabolites. Biomarkers can be detected in body fluids even before diagnostic signs of cancer appear. The highest concentrations of biomarkers were found in CSF.

Optical spectroscopy methods make it possible to study body fluids without any additional time-consuming sample preparation procedures. In this case, endogenous contrast can be used as an indicative feature, i.e. the signal initially present in the molecules of biofluids, for example, vibrational spectra or autofluorescence. It is known that vibrational spectroscopy methods such as infrared [10,11] and terahertz [12] spectroscopy, as well as Raman spectroscopy [13] are used for the diagnosis of gliomas, differentiation of glioblastoma from traumatic brain injury [14], and monitoring the effectiveness of surgical tumor removal based on serum or blood plasma analysis [15]. At the same time, the possibilities of the biofluid autofluorescence signal for the diagnosis of cerebral gliomas remain poorly explored.

Autofluorescence spectroscopy of biofluids is an effective method for diagnosing diseases attributable to the presence of selectively excitable endogenous fluorophores, the fluorescent signal of which is sensitive to the course of pathological processes. The blood plasma is one of the most promising objects for diagnosis, as it contains a large number of fluorophore markers released into the blood during the development of pathology. Due to the high speed of measurement and its minimally invasive nature, fluorescence spectroscopy of blood plasma has significant potential for use in clinical practice. A number of studies have demonstrated the successful use of blood plasma fluorescence spectroscopy for the diagnosis of oncological diseases [16–18]. When considering diseases

of the central nervous system (CNS), CSF is of great clinical importance, since it contains higher concentrations of specific biomarkers that make it possible to establish a diagnosis at an early stage of pathology development. Unlike blood plasma spectroscopy, the study of the optical properties of CSF is an invasive technique, which limits its use. Molecular biology methods have identified 14 metabolites found in CSF that are significantly associated with the risk of developing glioblastoma. These metabolites belong to various biochemical classes, including lipids, vitamins, amino acids, nucleotides, and a number of others, which highlights the multifactorial nature of glioblastoma development and progression [19]. The optical properties of blood plasma and CSF were studied in this paper to examine the possibility of using biofluid spectroscopy in the diagnosis of brain cancer.

2. Materials and methods

2.1. Description of samples

The study was conducted in accordance with the principles of the Helsinki Declaration. Each patient signed an informed consent form, and the clinical data was anonymized. The Ethics Commission of the Novosibirsk Scientific Research Institute of Traumatology and Orthopedics named after Y.L.Tsivyan approved the research protocol (Authorization #004/22-1, January 17, 2022). Peripheral blood samples were collected in EDTA vacutainers (SarstedtAG&Co. KG, Numbrecht, Germany). Plasma was separated by centrifugation at 2800 g for 15 minutes at a temperature of $+4\,^{\circ}$C. Blood plasma samples were frozen and stored at a temperature of $-80\,^{\circ}$ C until the day of analysis. The optical properties of 19 blood plasma samples were studied. The control group of samples included three blood plasma samples obtained from patients with traumatic brain injuries who had no history of cancer, including glioma. The remaining 16 samples were obtained from patients with a histologically confirmed diagnosis with correlation with the degree of malignancy.: 1 sample with $G1$, 4 samples with $G2$, 3 samples with $G3$, 8 samples with $G4$. The optical characteristics of 17 CSF samples were also measured, among which 9 samples belonged to the control group of patients, i.e., to the group of patients with traumatic brain injuries who did not have a history of cancer, and 8 samples were obtained from patients with a confirmed cancer diagnosis in the absence of traumatic brain injuries.

2.2. Measurement of absorption and fluorescence spectra

The absorption spectra of blood plasma and CSF were measured in the range of 280–380 nm and 320–700 nm using a Lambda 25 spectrophotometer (Perkin-Elmer, USA). The blood plasma fluorescence spectra were measured using Fluoromax-4 fluorimeters (Horiba Jobin Yvon, France) and FLUO (SolarLaserSystems, Belarus). Blood plasma samples were diluted 75 times in phosphate-salt buffer (pH 7.4) for measuring the fluorescence in the wavelength range of 320–420 nm, they were dilluted 850 times for measurements in the ultraviolet range (UV). CSF samples were diluted 12 times in a phosphate-salt buffer for fluorescence measurements.

2.3. Measurement of fluorescence attenuation kinetics

The kinetics of CSF fluorescence attenuation were measured using the time-correlated single photon counting (TCSPC) method. An EPL Series laser (Edinburgh Instruments, UK) with a wavelength of 280 nm, a pulse duration of 880 ps and a pulse repetition frequency of 10 MHz was used as an exciting radiation source. The fluorescence signal was detected in 16 channels in the spectral range of 300–490 nm using a photoelectron multiplier (PML-16-1- C, Becker& Hickl, Germany) and the single photon counting module (SPC-130EM, Becker& Hickl, Germany).

The obtained kinetics were approximated by a model with two exponents:

$$I_{fl} = \sum_{i=1}^{2} a_i e^{-t/\tau_i}. \quad (1)$$

CSF samples were diluted 12 times in a phosphate-salt buffer for measurements.

3. Results and discussion

3.1. Absorption and fluorescence of blood plasma in the near ultraviolet and visible ranges for patients with gliomas and the control group

Representative absorption spectra of blood plasma samples are shown in Fig. 1, *a*. A non-negative matrix factorization (NMF, non-negative matrix factorization) [20] procedure was performed to identify the main plasma chromophores and determine their contributions to the absorption spectrum of the sample. Three components were identified in the absorption spectra as a result of this procedure (Fig. 1, *b*). The NMF-2 component has characteristic hemoglobin maxima in the regions of 405 and 500–600 nm. The NMF-3 component has a pronounced peak in the region of 460 nm and can be attributed to bilirubin [21]. The NMF-1 component is characterized in the region of > 450 nm by broadband absorption, smoothly decreasing with wavelength, and can be attributed to the products of oxidation and glycation of plasma proteins [18]. It was found that the amplitude of the NMF-2 component corresponding to hemoglobin is greater for patients with cancer compared with the control group (Fig. 1, *with*). The statistical significance of the NMF-2 amplitude differences for patients with the disease and the control group was

estimated using the Mann-Whitney-Wilcoxon criterion — parameter p-value was 0.008. Thus, it was shown that in the studied samples, the efficiency of hemolysis in the preparation of blood plasma is higher for patients with gliomas compared with the control group.

Blood plasma fluorescence spectra were measured at excitations in the range of 320−420 nm in increments of 10 nm. When excited in the 320 nm region, a shift of the maximum emission spectrum of samples from $G4$ to the long-wavelength region is observed relative to samples from $G2$ and $G3$ (Fig. 2, $a-b$). t-criterion was used to estimate the statistical significance of the differences in the wavelengths of the position of the peaks of the emission spectra of the $G4$ samples and the other groups and it was shown that p-value < 0.003. The shift in the maximum position was less pronounced for the spectra obtained at other excitation wavelengths.

3.2. Fluorescence signal of blood plasma in the ultraviolet range for patients with gliomas and the control group

We also measured the fluorescence spectra of blood plasma when excited in the UV range in the range of 280−295 nm. The fluorescent signal of blood plasma in the UV region is determined by the contribution of aromatic amino acid residues tyrosine and tryptophan in protein molecules, mainly albumin. The ratio of the fluorescence intensities of tryptophan and tyrosine is sensitive to changes in protein conformation, so it can serve as a marker of the disease [18,22]. The contributions of tyrosine and tryptophan to the UV autofluorescence of the samples were determined according to the procedure described in Ref. [16,19] (Fig. 3, a). At the same time, an increase in the ratio of tyrosine integral signals to tryptophan (Tyr/Trp) was found for the control group of patients relative to samples with gliomas (Fig. 3, b). The Mann-Whitney-Wilcoxon criterion was used to estimate the statistical significance of the differences in the values of Tyr/Trp — for patients with $G4$ and the control group —the parameter p-value was 0.04.

3.3. Absorption spectra and time-resolved fluorescence spectroscopy of CSF samples in the UV range for patients with gliomas and the control group

Next, CSF absorption spectra were measured. It was found that the value of optical density in the range of 280 nm for CSF of patients with gliomas exceeds that for the control group of samples (Fig. 4). The Mann-Whitney-Wilcoxon criterion was used to evaluate the statistical significance of differences in CSF optical density values in the 280 nm region for patients with cancer and the control group, and it was found that p-value < 0.04.

In addition, the kinetics of attenuation of CSF fluorescence with subnanosecond time resolution were measured when excited in the region of 280 nm with emission in the region of 300−490 nm in increments of 12 nm. The kinetics of fluorescence attenuation were approximated using a model with two exponents (1) with fixed components $\tau_1 = 2$ ns and $\tau_2 = 6.5$ ns for all emission channels. Fig. 5, a shows the values of the parameter a_2 approximating the kinetics of fluorescence attenuation during excitation in the region of 280 nm and emission in the region of 345 nm for two groups of patients. As you can see, the values of the parameter a_2 are lower for CSF samples from the control group. As a result of applying the Mann-Whitney-Wilcoxon criterion, statistically significant differences were found between the amplitude of a_2 for the kinetics of fluorescence attenuation of samples of the two groups during emission in the region of 345 nm. As can be seen in Fig. 5, b, the greatest differences in the amplitudes of a_2 are observed for emissions in the region of 350 nm.

3.4. Differences in the optical parameters of blood plasma and CSF in patients with gliomas of varying degrees of malignancy

The optical absorption spectroscopy of blood plasma was used to detect the increase in hemoglobin concentration in samples obtained from patients with cancer relative to the control group (Fig. 1, c). There is currently no clear answer to the hemoglobin content in the blood plasma of patients with glioma and there is no correlation between this indicator and survival [23]. As a result of measuring the fluorescent signal of blood plasma in the range of 320−420 nm, it was found that when the fluorescent signal was excited in the region of 320 nm, the emission spectra in the case of patients with gliomas $G4$ were shifted to the long-wavelength region relative to the fluorescence spectra of samples from patients with tumors of lesser malignancy (Fig. 2). The fluorescent signal of blood plasma is determined in the region of 320 nm by the signal of the cofactor nicotinamide adenine dinucleotide (NADH) and the oxidation products of plasma proteins [24,25]. Consequently, the shift in the maximum plasma emission observed for the group of patients with $G4$ upon excitation in the 320 nm region may be associated with a change in the ratio of concentrations/quantum yields of fluorescence of the NADH coenzyme and protein oxidation products. Thus, fluorescence spectroscopy of blood plasma under excitation in the 320 nm region makes it possible to identify patients with high-grade glioma $G4$. An increase in absorption in the 280 nm CSF region of patients with gliomas was also found relative to the control group (Fig. 4), which indicates an increase in the concentration of total protein. An increase in protein concentration in CSF was noted in glioblastoma, while a number of protein molecules can act as specific markers of glioma [26,27]. An important diagnostic marker of the course of the disease are changes in the conformation of proteins in the body's biofluids [28,29]. The ultraviolet fluorescence of protein molecules occurs attributable to the presence of aromatic acid residues in them, mainly tyrosine

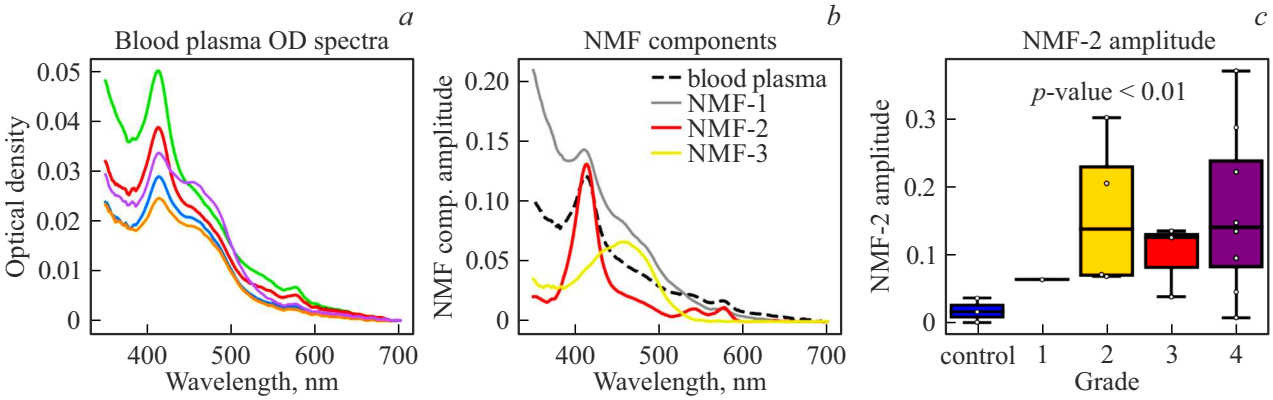

Figure 1. (*a*) Representative absorption spectra of blood plasma. (*b*) The spectra of the absorption components of blood plasma obtained as a result of their non-negative matrix factorization (NMF). (*c*) The amplitude of the NMF-2 component depends on the malignancy of the blood plasma donor patient's disease.

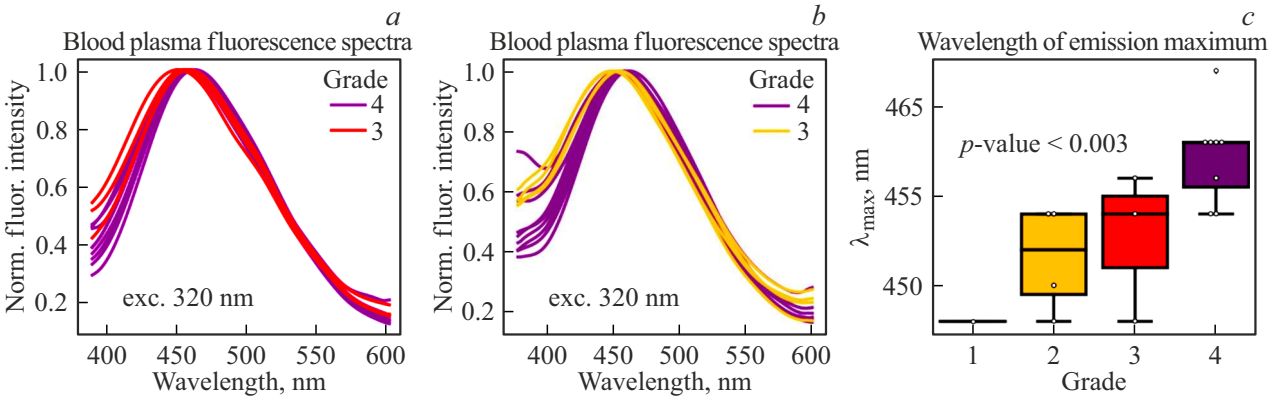

Figure 2. Plasma fluorescence spectra upon excitation in the 320 nm region for patients with malignancy 4 and 3 (*a*) and 4 and 2 (*b*). (*c*) The wavelength of the maximum emission spectrum upon excitation is in the region of 320 nm, depending on the malignancy of the patient's tumor (glioma).

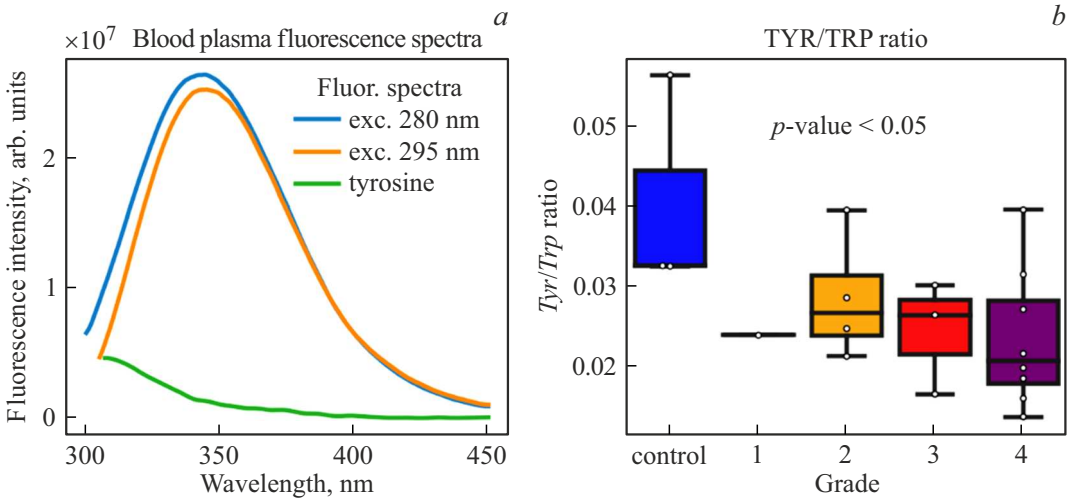

Figure 3. (*a*) Identification of tyrosine and tryptophan fluorescence spectra in blood plasma upon excitation at wavelengths 280 and 295 nm. (*b*) The ratio of integral tyrosine and tryptophan fluorescence signals (Tyr/Trp) for patients with different tumor malignancy.

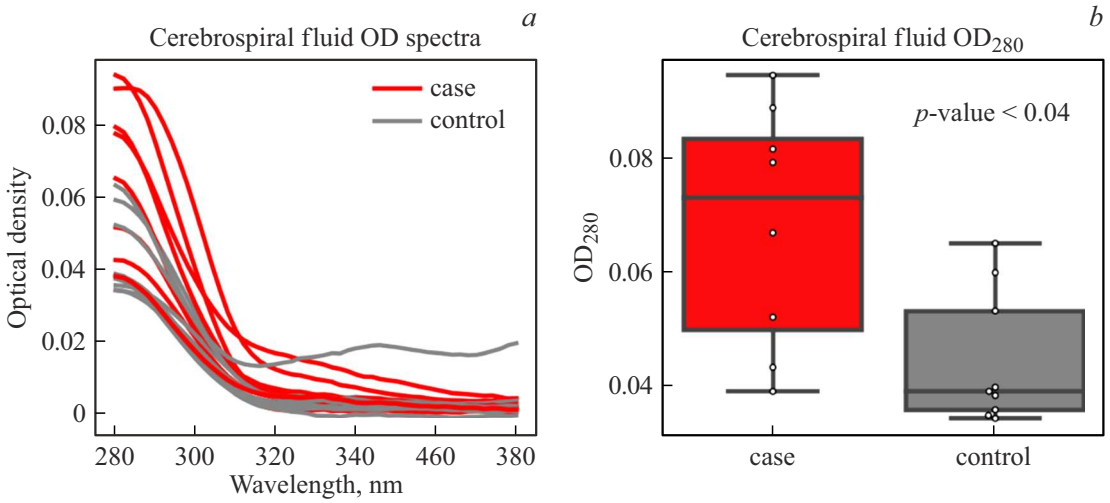

Figure 4. (*a*) CSF absorption spectra for patients with gliomas (red curves) and the control group. (*b*) The values of the absorbance of CSF in the range of 280 nm.

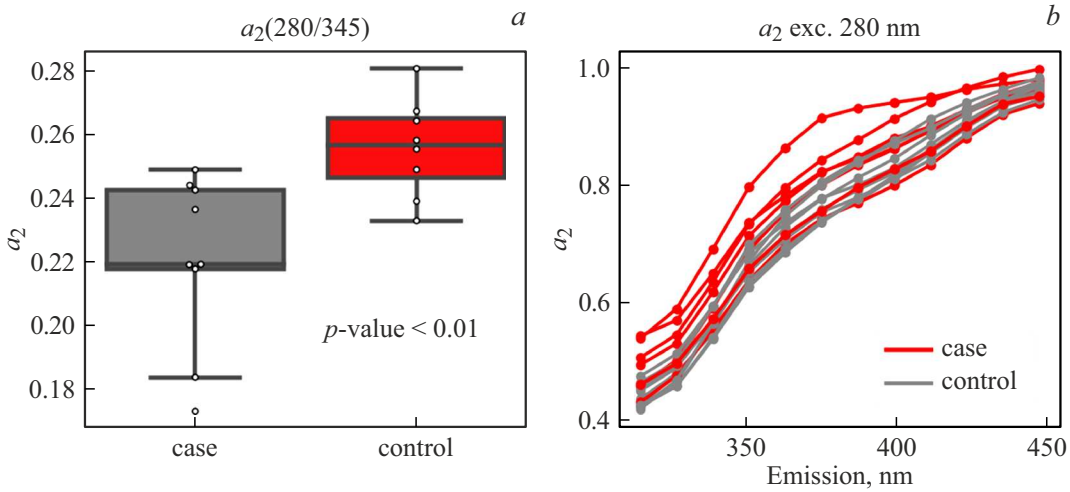

Figure 5. (*a*) Values of the approximation parameter a_2 for patients with gliomas and the control group. (*b*) The values of the approximation parameter are a_2 depending on the emission wavelength.

and tryptophan, whose fluorescent signal is sensitive to the parameters of the microenvironment. Thus, a change in the ratio of tyrosine and tryptophan integral signals is a marker of the course of pathological processes. As a result of this study, a decrease in the Tyr/Trp ratio was found among patients with gliomas relative to the control group (Fig. 3,b), which may indicate conformational changes in plasma proteins during the development of oncology. In addition to stationary fluorescence spectroscopy of protein molecules in the UV spectral region, the time-resolved fluorescent signal is also of great interest [30–32]. The fluorescent CSF signal with subnanosecond time resolution was studied in this paper for two groups of patients. The measured kinetics of CSF fluorescence attenuation were approximated by a model with two exponents with fixed components of the fluorescence lifetime of $\tau_1 = 2$ ns and $\tau_2 = 6.5$ ns, which is close to the values characteristic of the fluorescent albumin signal [33]. Differences in the parameters of approximation of the kinetics of CSF fluorescence attenuation for the two groups of patients were observed (Fig. 5, *b*), which may indicate a change in protein conformation and/or a change in the ratio of their concentrations in CSF of patients with brain cancer.

Conclusion

The possibility of using fluorescence spectroscopy of blood plasma and CSF for the diagnosis of cerebral gliomas has been tested. The results of the study showed that, based on the stationary fluorescent signal of blood plasma and the kinetics of attenuation of the fluorescent signal of CSF in the UV range, samples of patients with gliomas and patients with traumatic brain injuries can be separated. In

addition, using fluorescence spectroscopy of blood plasma under excitation in the 320 nm region, it was possible to differentiate patients with a high degree of malignancy ($G4$) from patients with a lower degree. Further studies with an increased sample of patients and the addition of other optical techniques are of significant interest and may contribute to the introduction of blood plasma fluorescence spectroscopy for the early diagnosis of cerebral gliomas into clinical practice.

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Conflict of interest

he authors declare that they have no conflict of interest.

Compliance with ethical standards

The study was conducted in accordance with the principles of the Helsinki Declaration. Informed voluntary consent was obtained from each patient who participated in the study, and clinical data was anonymized. The Ethics Commission of the Novosibirsk Scientific Research Institute of Traumatology and Orthopedics named after Y.L.Tsivyan approved the research protocol (Authorization #004/22-1, January 17, 2022).

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