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Electrical and Viscoelastic Parameters of Erythrocytes as a Part of Diagnostic Models for Differentiating Fatty Liver Disease of Mixed Genesis from Non-Alcoholic and Alcohol-Related Fatty Liver Disease

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Aim: creation of diagnostic models including electrical, viscoelastic parameters of erythrocytes to distinguish fatty liver disease of mixed etiology (metabolic + alcoholic) from non-alcoholic and alcoholic fatty liver disease.
Materials and methods. We examined 46 men with non-alcoholic fatty liver disease (NAFLD), 43 men with alcoholic fatty liver disease (AFLD), as well as 54 men with fatty liver disease (FLD) of mixed genesis (metabolic + alcohol-related); average age of the patients included in the study made 48.4 ± 9.6 years. The diagnosis was established on the basis of liver ultrasound findings and FLI liver steatosis index with a fibrosis grade of F1 or less (FibroScan® 502, Echosens, France). The electrical and viscoelastic parameters of erythrocytes were investigated by the diagnostic technique of dielectrophoresis using an electrooptical cell detection system.
Results. The most significant parameters for differentiating fatty liver disease of mixed genesis (metabolic + alcoholic) from NAFLD using the Volcano plot have turned out to be cell polarizability at a frequency of 10^6 Hz ($p = 6.49 \times 10^{-5}$), erythrocyte cell membrane capacity ($p = 0.00077$), relative polarizability ($p = 0.001$), the levels of which were higher in patients with NAFLD. On the contrary, the index of red blood cells destruction at 10^5 Hz was higher in FLD of the mixed genesis ($p = 0.047$) and the crossover frequency was shifted to the high frequency range more than in NAFLD ($p = 0.0005$). The discriminant analysis has additionally revealed the significance of the degree of erythrocyte deformation at 5×10^5 Hz in distinguishing between mixed-genesis FLD and NAFLD. In differentiating FLD of mixed genesis from NAFLD, a diagnostic model incorporating the above red blood cells parameters has provided an AUC of 0.829 (confidential interval: 0.742–0.916), sensitivity of 80.9 %, and specificity of 83.3 %.
Two indicators of red blood cells have been established that statistically significantly distinguish the mixed-genesis FLD from the AFLD (Volcano plot); these are the index of red blood cells destruction at a frequency of 5×10^5 Hz, which was higher with AFLD ($p = 0.0007$), and the capacity of cell membranes, the value of which prevailed in mixed-genesis FLD ($p = 0.011$). When distinguishing the mixed-genesis FLD from the AFLD, the combined model with the inclusion of three parameters of red blood cells, namely the index of red blood cells destruction at a frequency of 5×10^5 Hz, the capacity of erythrocyte membranes, and polarizability at a frequency of 10^6 Hz, has shown the highest levels of diagnostic accuracy, namely AUC = 0.751 (confidential interval: 0.611–0.908) with a sensitivity of 79.5 %, specificity of 74.7 %.
Conclusion. The electrical and viscoelastic parameters of erythrocytes studied using the diagnostic technique of dielectrophoresis should be considered as promising biomarkers for the diagnosis of diffuse liver disease.
Keywords: fatty liver disease, genesis, diagnostic models, erythrocytes, red blood cells, dielectrophoresis
Conflict of interest: the authors declare no conflict of interest.
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Электрические и вязкоупругие параметры эритроцитов в составе диагностических моделей для дифференцирования жировой болезни печени смешанного генеза от неалкогольной и алкогольной жировой болезни печени

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Цель исследования: создание диагностических моделей, включающих электрические, вязкоупругие параметры эритроцитов, для различения жировой болезни печени смешанной этиологии (метаболическая + алкогольная) от неалкогольной и алкогольной жировой болезни печени.

Материалы и методы. Обследованы 46 мужчин с неалкогольной жировой болезнью печени (НАЖБП), 43 мужчины с алкогольной жировой болезнью печени (АЖБП) и 54 мужчины с жировой болезнью печени (ЖБП) смешанного генеза (метаболический + алкогольный); средний возраст обследованных составил 48,4 ± 9,6 года. Диагноз был выставлен на основании данных ультразвукового исследования печени и индекса стеатоза печени FLI со степенью фиброза не более F1 (FibroScan® 502, Echosens, Франция). Электрические, вязкоупругие параметры эритроцитов исследованы методом диэлектрофореза с помощью электрооптической системы детекции клеток.

Результаты. Наиболее значимыми для дифференцирования ЖБП смешанного генеза (метаболический + алкогольный) от НАЖБП при использовании метода Volcano plot оказались поляризуемость клеток на частоте 10^6 Гц ($p = 6{,}49 \times 10^{-5}$), емкость клеточной мембраны эритроцитов ($p = 0{,}00077$) и относительная поляризуемость ($p = 0{,}001$), уровни которых были выше у пациентов с НАЖБП. Напротив, индекс деструкции эритроцитов на частоте 10^5 Гц оказался выше при смешанном генезе ЖБП ($p = 0{,}047$), а равновесная частота смещена в высокочастотный диапазон по сравнению с показателями при НАЖБП ($p = 0{,}0005$). Дискриминантный анализ дополнительно выявил значимость степени деформации эритроцитов на частоте 5×10^5 Гц в различении ЖБП смешанного генеза и НАЖБП. Диагностическая модель при дифференцировании ЖБП смешанного генеза от НАЖБП, включающая вышеописанные параметры эритроцитов, обеспечила AUC 0,829 (доверительный интервал (ДИ): 0,742–0,916), чувствительность — 80,9 %, специфичность — 83,3 %.

Установлены два показателя эритроцитов, статистически значимо отличающие жировую болезнь печени смешанного генеза от АЖБП (Volcano plot), — это индекс деструкции на частоте 5×10^5 Гц, который был выше при АЖБП ($p = 0{,}0007$), и емкость мембран клеток, величина которой преобладала при ЖБП смешанного генеза ($p = 0{,}011$). При различении ЖБП смешанного генеза от АЖБП комбинированная модель с включением трех параметров эритроцитов — индекса деструкции на частоте 5×10^5 Гц, емкости мембран эритроцитов и поляризуемости на частоте 10^6 Гц — показала наиболее высокие уровни диагностической точности: AUC = 0,751 (ДИ: 0,611–0,908) с чувствительностью 79,5 %, специфичностью 74,7 %.

Заключение. Электрические и вязкоупругие параметры эритроцитов, изученные с помощью метода диэлектрофореза, следует рассматривать как перспективные биомаркеры для диагностики диффузной патологии печени.

Ключевые слова: жировая болезнь печени, генез, диагностические модели, эритроциты, диэлектрофорез

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver diseases worldwide, reaching 25–30 % [1]. Since its first description in 1980, NAFLD has been considered a form distinct from alcohol-related fatty liver disease (AFLD), despite the fact that both diseases share common pathophysiological mechanisms, genetic and epigenetic factors and often coexist [2]. Both diseases are characterised by a wide range of histological features, ranging from isolated steatosis to steatohepatitis and cirrhosis [3]. The distinction between NAFLD and alcohol-related FLD is currently

based on the amount of alcohol consumed, which is nominally established [4, 5]. Given the synergistic effect between alcohol consumption, obesity and metabolic dysfunction, it is likely that alcohol consumption serves as a significant risk factor for liver disease progression in NAFLD and metabolic syndrome [6–8].

According to F. Idalsoaga et al., there are patients with alcohol-related liver disease in clinical practice having metabolic cofactors (alcohol-related fatty liver disease with metabolic syndrome) and patients with NAFLD who consume alcohol, which is contributing to the pathological process (metabolically associated fatty liver disease with alcoholic component). These patient populations tend to the opposite poles of those with non-alcoholic liver disease without alcohol contribution and alcoholic one without manifestations of metabolic syndrome [9]. Establishing the predominant etiological factor in patients with FLD is a challenging clinical task, due to the similarity of histological picture when performing liver biopsy, changes when using imaging methods [3], overlapping metabolic profiles [2], unidirectional changes in biomarkers included in diagnostic panels (Ash-Fibro Test, Nash-Fibro Test algorithms as part of the FibroMax test, FM) [9, 10].

The term "steatotic liver disease" (SLD, or "hepatic steatosis") proposed in 2023 includes pathology with different etiological factors for the development of steatosis (cardiometabolic, alcohol, hepatitis viruses, drugs and others), including steatotic liver disease associated with metabolic dysfunction, metabolically associated alcoholic disease [11], which indicates the relevance of this problem.

Our previous studies have shown the capability of using the electrical and viscoelastic parameters of erythrocytes studied diagnostic technique of dielectrophoresis in establishing the etiology of FLD [12–15].

The aim of this study is as follows: to create diagnostic models including electrical, viscoelastic parameters of erythrocytes for distinguishing FLD of mixed etiology (metabolic + alcohol-related) from non-alcoholic and alcohol-related fatty liver disease.

Materials and methods

We have examined 46 men (average age — 48.6 ± 8.7 years) with NAFLD; 43 men with alcohol-related fatty liver disease (average age — 47.6 ± 10.1 years) and 54 men (average age — 49.1 ± 9.9 years) with fatty liver disease of mixed genesis (metabolic + alcohol-related).

Inclusion criteria for the study: male gender; age between 25 and 65 years; a verified diagnosis of "fatty liver disease" (FLD) according to generally accepted criteria based on the results of ultrasound examination of the abdominal cavity organs; degree of liver fibrosis which is not higher than the 1st degree according to indirect elastometry (FibroScan® 502, Echosens, France); abstinence from alcohol for 7–10 days; signing an informed consent to participate in the study.

Exclusion criteria: female gender; age younger than 25 and older than 65 years; FLD associated with viral, medically induced, autoimmune, genetically determined liver diseases, parenteral nutrition; early withdrawal periods (less than 7–10 days); degrees of liver fibrosis exceeding the first one, according to indirect elastometry; clinically significant severe concomitant pathology in the acute stage.

The NAFLD has been diagnosed in accordance with the criteria of clinical recommendations [16]. The diagnosis of NAFLD has been confirmed by the index of NAFLD liver fat score [17]. Most of the patients (73.9 %) have not consumed alcohol at all or have occasionally (3 to 4 times a year) used low doses of alcohol (on average 17.8 ± 8.5 g per day equivalent to pure ethanol). The data of the questionnaires and biochemical parameters made it possible to exclude the alcoholic etiology of FLD.

The alcoholic genesis of steatosis has been established according to the data of reliably confirmed presence of systematic alcohol consumption at present, and in the past medical history (using CAGE questionnaire and AUDIT test) in combination with the results of clinical and instrumental examination. Mixed etiology (metabolic + alcohol-related) has been diagnosed in case of regular alcohol consumption in patients with manifestations of metabolic syndrome according to the Recommendations of Experts of the Russian Scientific Society of Cardiologists [18].

Other liver pathology as a cause of steatosis has been excluded.

Signs of metabolic syndrome have been detected in patients with NAFLD and FLD of mixed genesis, which have been assessed according to the following criteria: the main criterion — central (abdominal) type of obesity, waist circumference is equal exceeds 94 cm; additional criteria are blood pressure > 130/85 mmHg or treatment of arterial hypertension with medical drugs; increased triglyceride levels (≥ 1.7 mmol/L); decrease in the level of high-density lipoprotein cholesterol (HDL-C) (<1.0 mmol/L); content enhancement of low-density lipoprotein cholesterol (LDL-C) > 3.0 mmol/L; fasting plasma glucose concentration ≥ 6.1 or 7.8 mmol/L — 2 hours after glucose tolerance test. Metabolic syndrome has been considered as reliable when three criteria were present: one main and two additional criteria [18].

As a comparison group, 38 men (average age — 47.9 ± 14.3 years) have been selected; they had a healthy lifestyle, and consumed alcohol not more than once a month in doses not exceeding 20 g per day in terms of pure ethanol, without any

manifestation of pathology of internal organs and onset of metabolic syndrome.

All examined persons have undergone a study of biochemical parameters, including liver function tests, lipid profile parameters; liver steatosis index called FLI (Fatty Liver Index) [16], NAFLD liver fat score [19] and CARO [20] have been determined. The degree of liver fibrosis severity has been determined using the technique of indirect elastometry of the liver on FibroScan® 502 device (Echosens, France) (from F0 to F4 according to METAVIR scale). It has not exceeded F1 in patients with FLD, but it has corresponded to F0 degree in the comparison group.

Electrical and viscoelastic parameters of erythrocytes have been studied in all examined patients using the diagnostic technique of dielectrophoresis in a non-uniform alternating electric field by means of an electrooptical cell detection system [13]: mean cell diameter (μm), fractions of discocytes, spherocytes, deformed cells (%), polarizability of cells at different frequencies of the range (m^3), relative polarizability (ratio of the index value at 10^6 Hz to 10^5 Hz), summarized index of rigidity (N·m), viscosity (Pa·s), electrical conductivity of membranes (Sm/m), indices of red blood cells (RBC) destruction (at different frequencies of the range) (%) and RBC aggregation (relative units), amplitude of RBC deformation at 10^6 Hz (m), degree of cell deformation at 5×10^5 Hz (%), RBC membrane capacitance (F), velocity of RBC motion to the electrodes (μm/s), position of crossover frequency (Hz), value of dipole moment (Cl·m). The original CELLFIND software package has been used for cell image recognition and computer data processing. The reproducibility error of the technique is equal to 7–12 %.

Statistical data processing has been performed using IBM SPSS Statistics v. 26.0 (IBM Corp., USA). In case of normal distribution (Kolmogorov – Smirnov test), the mean (M) and standard deviation (SD) have been calculated. When comparing two normally distributed samples, the Student's t-test has been used. In the absence of a normal distribution, a median (*Me*), and 25th and 75th percentiles (*Me* (25 %; 75 %)) have been calculated, and the reliability of the differences in the values has been assessed using non-parametric test criteria (e.g., the Mann – Whitney U-test, the Kraskell – Wallis test), the Pearson's chi-squared test has been applied. The critical significance level of the null hypothesis (p) has been assumed to be equal to 0.05. The relationships between the traits have been assessed by calculating Pearson's linear correlation coefficient and Spearman's rank correlation coefficient.

The orthogonal partial least squares discriminant analysis (OPLS-DA) has allowed us to identify the differences, the unpaired *t*-test (comparison of parameter levels of patient groups in pair) and the Volcano plot and a system of machine learning algorithm named Random Forest have been used, using MATLAB software (R2019a, Math Works) and the R programming language [21]. A ROC analysis has also been performed.

Results

The presence of hepatic steatosis in all examined patients has been confirmed by values of the FLI liver steatosis index exceeding 60 (with a probability of more than 78 %) [16], and the NAFLD liver fat score exceeding –0.64 (with sensitivity 86 %, specificity 71 %) [17]. All patients with NAFLD and mixed-genesis FLD have shown evidence of the metabolic syndrome, such as abdominal obesity, arterial hypertension, hypertriglyceridemia, hypercholesterolemia, we have also found statistically significant increase in insulin, fasting blood glucose, and uric acid. The CARO index value of less than 0.33 indicates the presence of insulin resistance in all patients with NAFLD and a mixed-genesis FLD [20].

Patients with FLD of an alcohol-related and mixed genesis had an AUDIT test score of ≥ 8 points and positive answers to 3–4 questions on the CAGE questionnaire. Most of the patients observed with alcohol-related FLD have consumed alcohol regularly (more than 2–3 times per week) over a period of 8 to 22 years, the single dose of alcohol consumed has amounted to 128.5 ± 80.8 g and the weekly dose was 653.7 ± 473.2 g expressed as pure ethanol. 65.1 % of patients have indicated a preference for strong alcoholic drinks (vodka, cognac, whiskey), 30.2 % of persons have consumed alcoholic drinks of various strengths, including strong alcohol. Patients with mixed-genesis FLD (metabolic + alcohol-related) have also consumed alcohol on a regular basis at a frequency of ≥ 1 time per week with a preference for strong alcoholic drinks in ~60 % of cases, but with lower single and weekly alcohol doses (108.2 ± 65.3 and 219.8 ± 120.7 g, respectively, expressed as pure ethanol).

The activity of most hepatic enzymes (transaminases, GGTP, alkaline phosphatase), the content of total bilirubin and serum iron, reflecting liver damage, were higher in patients with FLD than in the comparison group, staying within the reference values or with minimal deviations from them, which did not exclude the presence of steatohepatitis [22]. Activity of AST and GGTP, de Ritis ratio, direct bilirubin and serum iron content have been higher in patients with alcoholic genesis of FLD compared to other groups, which indirectly confirms the severity of toxic effects of high doses of ethanol on hepatocytes [23]. The content of total protein and albumin in patients with FLD has been comparable with the values of the male comparison group. This

fact evidences a preservation of protein-synthetic function of the liver. Dyslipidaemia has been detected in all groups of patients with FLD, the highest levels of total cholesterol and triglycerides have been found in the group with mixed genesis of the disease; HDL-C in patients with alcoholic FLD was higher than in NAFLD, which probably reflects the presence of regular alcohol consumption [23]. The liver stiffness investigated by indirect elastometry has tended to increase in patients with NAFLD, mixed-genesis FLD compared to a group of healthy men [24]. Liver density has turned out to be significantly higher in patients with alcoholic genesis of FLD than in the control group ($p = 0.028$). This confirms that ethanol is a relevant factor for fibrogenesis [23].

Patients with FLD have differed from the comparison group by a lower proportion of discocytic and a higher proportion of spherocytic, deformed forms of erythrocytes. The RBCs of patients with FLD have indicated lower values of amplitude of RBC deformation, membrane capacity, velocity of RBC motion to the electrodes, dipole moment value, polarizability at high electric field frequencies of 10^6 and 0.5×10^6 Hz, and relative polarizability compared to the control group, and, in contrast, higher levels of summarized indices for viscosity, rigidity, electrical conductivity, RBC aggregation and destruction at all electric field frequencies, and polarizability at low field frequencies of 0.1×10^6 and 0.05×10^6 Hz ($p < 0.000001$). A shift of the crossov frequency into the high frequency range (more than 0.5×10^6 Hz) has been detected in patients with FLD. The most pronounced deviations in the parameters of erythrocytes compared with healthy individuals have been found in patients with alcohol-related and mixed-genesis FLD [12–15].

In the alcoholic genesis of FLD, the index of RBC destruction has turned out to be statistically significantly higher at a frequency of 5×10^5 Hz ($p = 0.016$), the crossover frequency was more shifted to the high-frequency range ($p = 2.13 \times 10^{-6}$). On the contrary, the cell membrane capacity ($p = 1.21 \times 10^{-11}$), the degree of change in the amplitude of RBC deformation at a frequency of 5×10^5 Hz ($p = 2.38 \times 10^{-8}$), the polarizability of cells at a frequency of 10^6 Hz ($p = 9.38 \times 10^{-8}$), the velocity of RBC motion to the electrodes ($p = 4.32 \times 10^{-6}$), the magnitude of the dipole moment ($p = 1.66 \times 10^{-5}$), and the relative polarizability ($p = 2.35 \times 10^{-5}$) were lower in AFLD compared with NAFLD [15].

A normalization of electrical and viscoelastic indices of erythrocytes has been performed on the median (centred on the mean value) to create a differential diagnostic model in the pair "FLD of mixed etiology vs. NAFLD" (Fig. 1).

To identify differences in the normalized levels of erythrocyte parameters in patients with FLD of mixed etiology and NAFLD, a discriminant analysis (OPLS-DA) has revealed a list of erythrocyte parameters that differ significantly between the groups depending on the RBC levels (Fig. 2A).

The use of the Volcano plot method (unpaired statistics) has ensured the establishment of electrical and viscoelastic parameters of erythrocytes that are most significant for distinguishing patients with non-alcoholic and mixed-genesis FLD (Table 1).

The polarizability of cells at a frequency of 10^6 Hz ($p = 6.49 \times 10^{-5}$), crossover frequency position ($p = 0.0005$), the capacity of the cell membrane ($p = 0.00077$), relative polarizability ($p = 0.001$), the index of destruction of erythrocytes at a frequency of 10^5 Hz have turned out to be the most significant for distinguishing NAFLD and FLD of mixed etiology ($p = 0.047$). At the same time, the polarizability of cells at a frequency of 10^6 Hz, relative polarizability, and cell membrane capacity have turned out to be higher in NAFLD compared with the mixed-genesis FLD. In contrast, the crossover frequency in mixed-genesis FLD was shifted to the high frequency range, and cell hemolysis was more expressed at 10^5 Hz than in NAFLD.

Figure 3A shows the ranking of the studied electrical, viscoelastic parameters of erythrocytes according to their contribution to the distinction between FLD with non-alcoholic and mixed etiology. The contribution to the distinction has been provided by such characteristics as the proportion of deformed cells, electrical conductivity, index of RBC aggregation, which are higher with mixed genesis of FLD and the degree of deformation of erythrocytes at a frequency of 5×10^5 Hz, the magnitude of the dipole moment, which, on the contrary, are higher with NAFLD.

The associations of some parameters of erythrocytes significant for the differential diagnosis of FLD have been studied, such as membrane capacity and polarizability at a frequency of 10^6 Hz. The strongest direct ($r \geq 0.5$) connections have been found between the capacity of erythrocyte membranes and the magnitude of the dipole moment ($p = 0.002$), the degree of deformation at a frequency of 5×10^5 Hz, the amplitude of RBC deformation at a frequency of 10^6 Hz ($p = 0.0001$), the relative polarizability ($p = 0.006$), the velocity of RBC motion to electrodes ($p < 0.0001$), and a strong feedback has been established with the equilibrium frequency position ($p = 0.0015$). The polarizability at a frequency of 10^6 Hz was directly and strongly associated with the polarizability at a frequency of 5×10^5 Hz ($p = 0.007$), as well as with the velocity of RBC motion to the electrodes ($p = 0.0038$), the degree of cell deformation at a frequency of 5×10^5 Hz ($p = 0.001$) and vice versa — with the

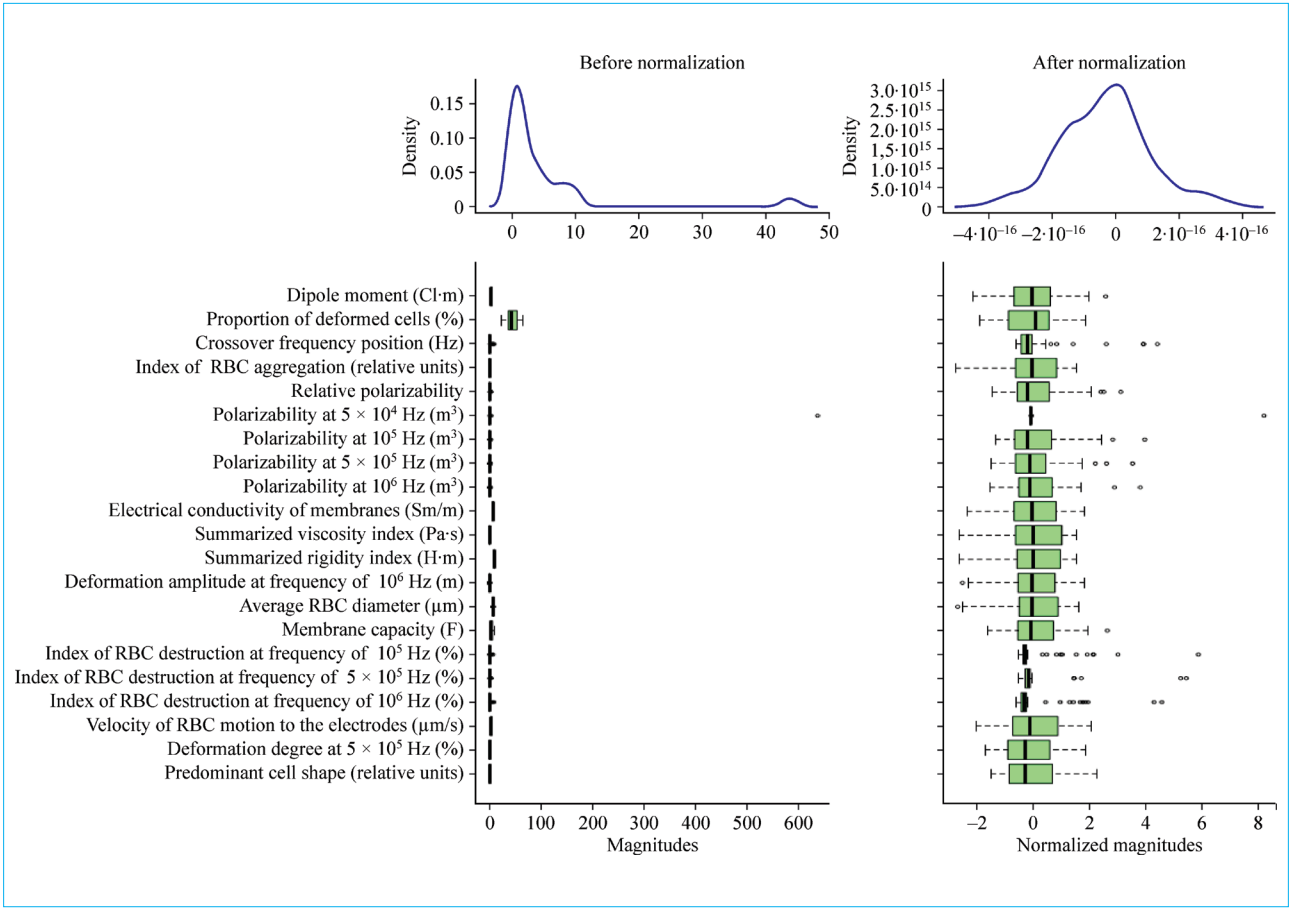

Figure 1. Normalization of the values of electrical and viscoelastic parameters of erythrocytes in groups of patients with FLD of non-alcoholic and mixed etiology (on the left — parameter values before normalization, on the right — after normalization)

Рисунок 1. Нормализация величин электрических и вязкоупругих параметров эритроцитов в группах пациентов с ЖБП неалкогольной и смешанной этиологии (слева — значения параметров до нормализации, справа — после нормализации)

Before normalization — До нормализации; Summarized rigidity index (N·m) — Обобщенный показатель жесткости (Н·м); After normalization — После нормализации; Deformation amplitude at frequency of 10^6 Hz (m) — Амплитуда деформации на частоте 10^6 Гц (м); Dipole moment (Cl·m) — Дипольный момент (Кл·м); Average RBC diameter (μm) — Средний диаметр эритроцита (мкм); Proportion of deformed cells (%) — Доля деформированных клеток (%); Membrane capacity (F) — Емкость мембран (Ф); Density — Плотность; Crossover frequency position (Hz) — Положение равновесной частоты (Гц); Index of RBC destruction at frequency of … Hz (%) — Индекс деструкции на частоте … Гц (%); Index of RBC aggregation (relative units) — Индекс агрегации (усл. ед.); Velocity of RBC motion to the electrodes (μm/s) — Скорость движения клеток к электродам (мкм/с); Relative polarizability — Относительная поляризуемость; Deformation degree at 5 × 10^5 Hz (%) — Степень деформации на частоте 5 × 10^5 Гц (%); Polarizability at … Hz (m^3) — Поляризуемость на частоте … Гц ($м^3$); Predominant cell shape (relative units) — Преобладающая форма клеток (усл. ед.); Electrical conductivity of membranes (Sm/m) — Электропроводность мембран (См/м); Magnitudes — Величины; Summarized viscosity index (Pa·s) — Обобщенный показатель вязкости (Па·с); Normalized magnitudes — Нормализованные величины.

index of RBC destruction at a frequency of 10^5 Hz ($p = 0.004$).

The cluster analysis performed (Fig. 4) has allowed us to identify three main clusters, which include most of the patients of the studied groups. It is likely that different clusters included patients with FLD with varying severity of necroinflammatory changes in liver tissue.

The ROC analyses performed for individual RBC parameters to distinguish patients with NAFLD from mixed-genesis NAFLD has demonstrated sufficient levels of diagnostic accuracy: for membrane capacity — AUC = 0.759, sensitivity 66.7 %, specificity 79.5 %; for degree of cell deformation at 5 × 10^5 Hz — AUC = 0.807, sensitivity 70 %, specificity 84.6 %; for cell polarizability at 10^6 Hz —

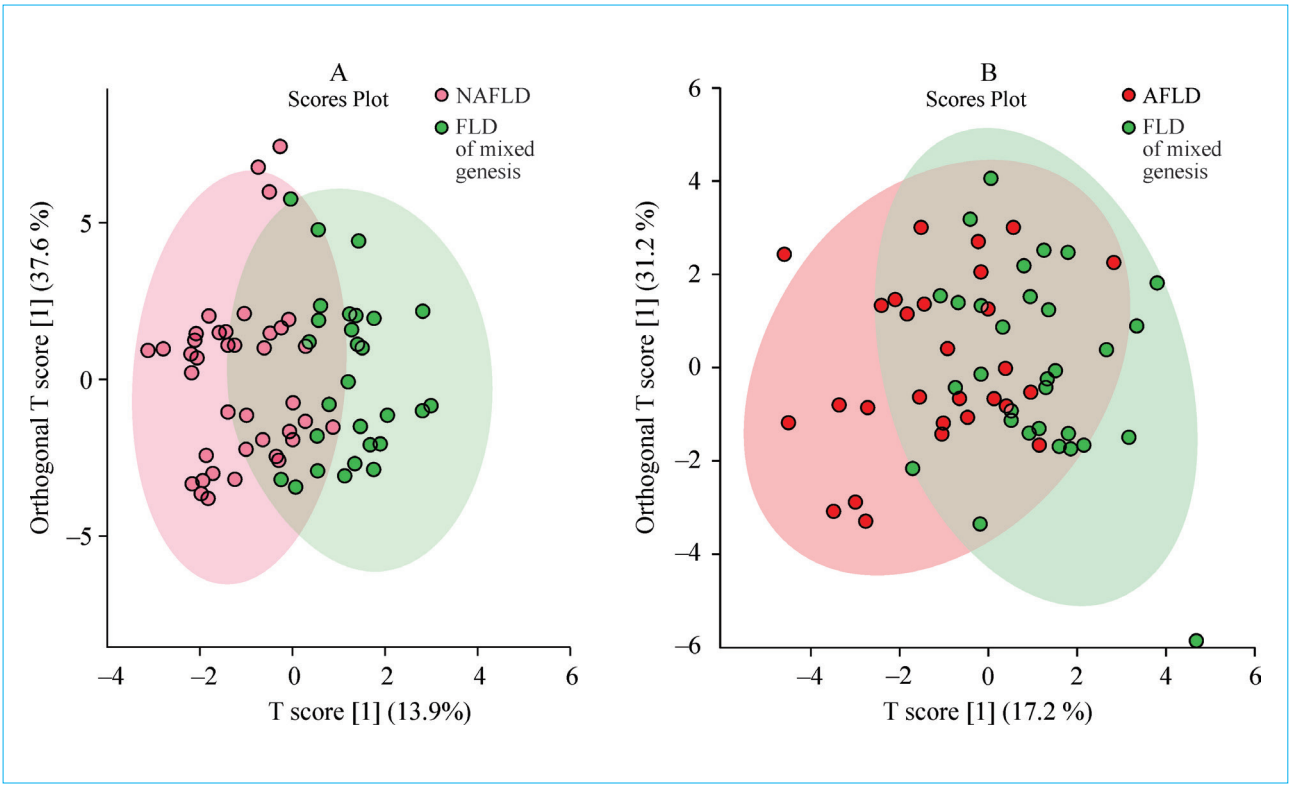

Figure 2. Discriminant analysis for distinguishing the levels of electrical, viscoelastic parameters of erythrocytes in patients with FLD of mixed etiology: A — from non-alcoholic origin of FLD (pink cloud, pink dots — levels of erythrocyte parameters in the NAFLD group; green cloud, green dots — in the group of patients with FLD mixed genesis); B — from the alcoholic origin of FLD (red cloud, red dots — levels of erythrocyte parameters in the alcoholic FLD group; green cloud, green dots — in the group of patients with FLD of mixed genesis)

Рисунок 2. Дискриминантный анализ для различения уровней электрических, вязкоупругих параметров эритроцитов пациентов с ЖБП смешанной этиологии: А — от неалкогольного генеза ЖБП (розовое облако, розовые точки — уровни параметров эритроцитов в группе НАЖБП; зеленое облако, зеленые точки — в группе пациентов с ЖБП смешанного генеза); В — от алкогольного генеза ЖБП (красное облако, красные точки — уровни параметров эритроцитов в группе алкогольной ЖБП; зеленое облако, зеленые точки — в группе пациентов с ЖБП смешанного генеза)

Table 1. Electrical and viscoelastic parameters of erythrocytes, studied by the Volcano plot method (unpaired statistics), in patients with FLD of various etiologies (NAFLD vs. FLD of mixed etiology) — markers for differentiation

Таблица 1. Электрические и вязкоупругие параметры эритроцитов, исследованные методом Volcano plot (непарная статистика), у пациентов ЖБП различной этиологии (НАЖБП против ЖБП смешанной этиологии) — маркеры для дифференцирования

Electrical and viscoelastic parameters of erythrocytes ***Электрические и вязкоупругие параметры эритроцитов***	**Factor of change** ***Кратность изменений*** **(FC)**	**log2(FC)**	***p* values** ***Значения p*** **(raw.*p* val)**	**−log10(*p*)**
Polarizability at frequency 10^6 Hz, m^3 *Поляризуемость на частоте 10^6 Гц, $м^3$*	1.6423	0.71569	6.49E-05	4.188
Crossover frequency position, Hz *Положение равновесной частоты, Гц*	0.30763	−1.7007	0.000509	3.2932
Cell membrane capacity, F *Емкость клеточной мембраны, Ф*	1.6034	0.68115	0.00077	3.1137
Relative polarizability *Относительная поляризуемость*	1.5037	0.58854	0.001094	2.9609
Destruction index at 10^5 Hz frequency (%) *Индекс деструкции на частоте 10^5 Гц (%)*	0.54131	−0.88547	0.047761	1.5092

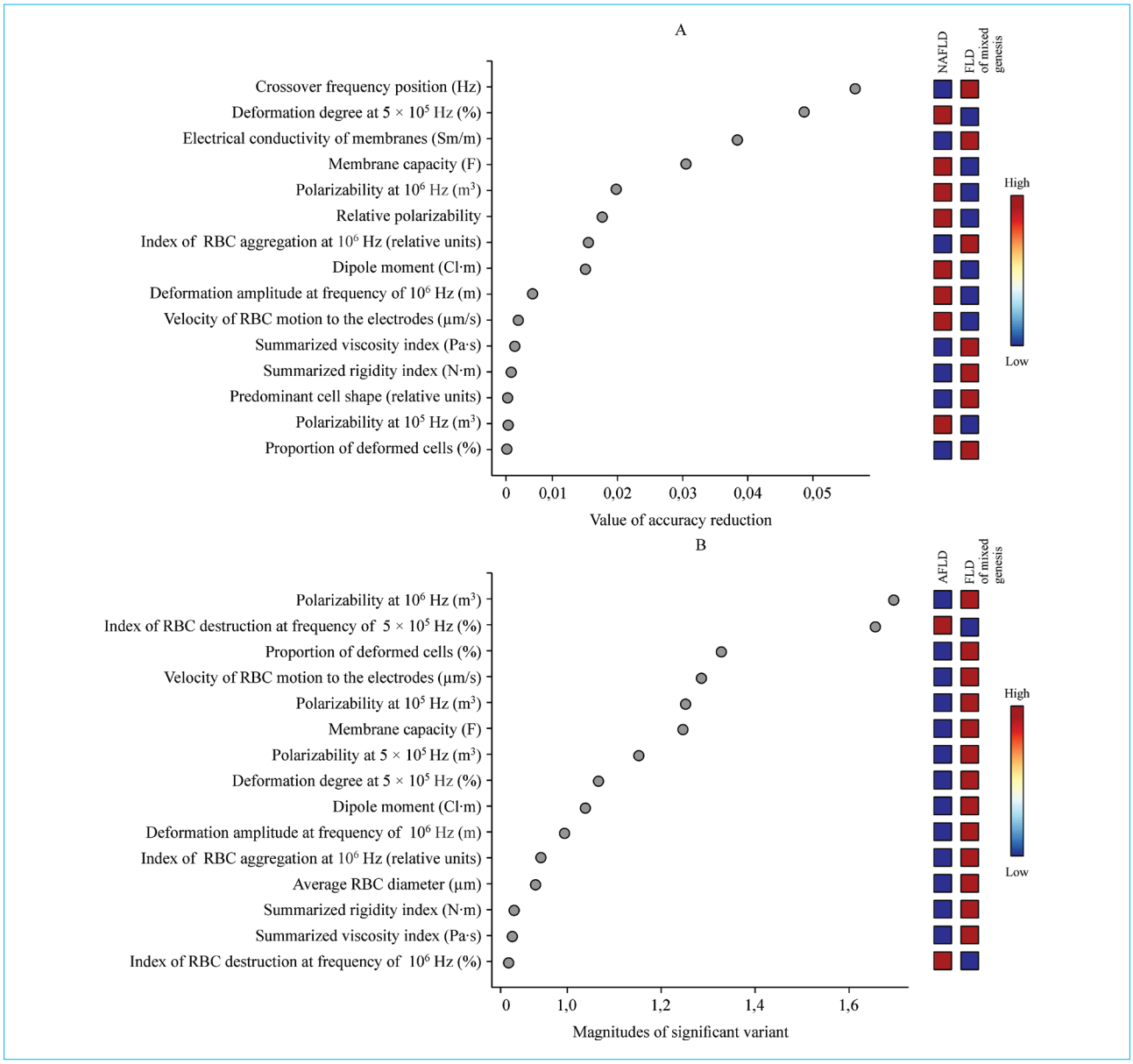

Figure 3. Ranking of electrical, viscoelastic parameters of erythrocytes according to their contribution to differentiation between FLD of mixed origin: A — from non-alcoholic FLD, Б — from alcoholic FLD

Рисунок 3. Ранжирование электрических, вязкоупругих параметров эритроцитов по вкладу в дифференцирование между ЖБП смешанного генеза: А — от неалкогольной ЖБП, Б — от алкогольной ЖБП

Crossover frequency position (Hz) — Положение равновесной частоты (Гц); Summarized viscosity index (Pa·s) — Обобщенный показатель вязкости (Па·с); Deformation degree at 5 × 10^5 Hz (%) — Степень деформации на частоте 5 × 10^5 Гц (%); Summarized rigidity index (N·m) — Обобщенный показатель жесткости (Н·м); Electrical conductivity of membranes (Sm/m) — Электропроводность мембран (См/м); Predominant cell shape (relative units) — Преобладающая форма клеток (усл. ед.); Membrane capacity (F) — Емкость мембран (Ф); Proportion of deformed cells (%) — Доля деформированных клеток (%); Polarizability at … Hz (m^3) — Поляризуемость на частоте … Гц ($м^3$); Index of RBC destruction at frequency of … Hz (%) — Индекс деструкции на частоте … Гц (%); Relative polarizability — Относительная поляризуемость; Average RBC diameter (μm) — Средний диаметр эритроцита (мкм); Index of RBC aggregation at 10^6 Hz (relative units) — Индекс агрегации на частоте 10^6 Гц (усл. ед.); Value of accuracy reduction — Значение снижения точности; Dipole moment (Cl·m) — Дипольный момент (Кл·м); Values of significant variants — Величины значимых вариантов; Deformation amplitude at frequency of 10^6 Hz (m) — Амплитуда деформации на частоте 10^6 Гц (м); High/Low — Высоко/Низко; FLD of mixed genesis — ЖБП смешанного генеза; Velocity of RBC motion to the electrodes (μm/s) — Скорость движения клеток к электродам (мкм/с); NAFLD — НАЖБП; AFLD — АЖБП.

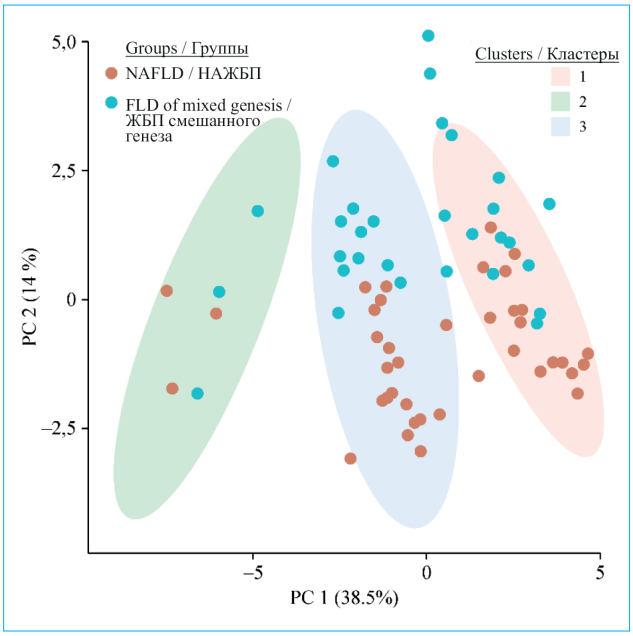

Figure 4. Cluster analysis (K-means clustering) of electrical and viscoelastic parameters of erythrocytes in patients with NAFLD and FLD of mixed genesis

Рисунок 4. Кластерный анализ (K-means clustering) электрических и вязкоупругих параметров эритроцитов у пациентов с НАЖБП и ЖБП смешанного генеза

AUC = 0.784, sensitivity 80 %, specificity 69.2 %; for crossover frequency position — AUC = 0.748, sensitivity 63.3 %, specificity 87.2 %. The combined model has turned out to be optimal in terms of sensitivity and specificity with a minimum number of erythrocyte indices, including the following parameters: the degree of deformation of erythrocytes at a frequency of 5×10^5 Hz, cell polarizability at a frequency of 10^6 Hz and membrane capacity, providing an AUC of 0.829 (CI: 0.742–0.916), sensitivity of 80.9 %, specificity of 83.3 %.

When considering the pair of "FLD of mixed genesis vs. alcohol-related FLD", procedures for normalization of parameter levels have consistently been implemented, after which a discriminant analysis has been performed (Fig. 2B), a Volcano plot study (Table 2), and the parameters of erythrocytes have been ranked according to the degree of their contribution to the distinction of the mixed-genesis FLD from the alcohol-related FLD (Fig. 3B).

It can be seen on Figure 2B that there is a smaller number of differing levels of erythrocyte parameters in this pair of "FLD of mixed genesis vs. alcohol-related FLD" than in "FLD of mixed genesis vs. NAFLD", which has been confirmed by the data of the Volcano plot (Table 2). Only two indicators of RBCs have been established that statistically significantly distinguish mixed-genesis FLD from AFLD — these are the index of RBC destruction at a frequency of 5×10^5 Hz, which was higher with AFLD ($p = 0.0007$), and the capacity of cell membranes, which has turned out to be higher than with mixed-genesis FLD ($p = 0.011$).

The ranking of the contribution of electrical and viscoelastic parameters of erythrocytes to the distinction between mixed-genesis FLD and alcohol-related FLD has demonstrated the significance of polarizability at 10^6 Hz, the proportion of deformed cells, and the velocity of RBC motion to the electrodes, which were higher in patients with mixed-genesis FLD than in alcohol-related FLD (Fig. 3B).

The ROC analysis (Fig. 5B) has showed lower levels of diagnostic accuracy in distinguishing alcohol-related FLD and mixed-genesis FLD compared with the pair "NAFLD vs. FLD of mixed genesis". Thus, the use of individual erythrocyte indices in the pair "Alcohol-related FLD vs. FLD of mixed genesis" has provided either sufficient levels of sensitivity with low specificity — for the index of RBC destruction at 5×10^5 Hz (AUC = 0.718, sensitivity 86.7 %, specificity 59.3 %), for the proportion of deformed cells (AUC = 0.668, sensitivity 77.3 %, specificity 59.3 %); or sufficient specificity with low sensitivity — for polarizability at 10^6 Hz (AUC = 0.705, sensitivity 63.3 %, specificity 74.1 %), for membrane capacity (AUC = 0.636, sensitivity 58.1 %, specificity 74.1 %).

Table 2. Electrical and viscoelastic parameters of erythrocytes, studied by the Volcano plot method (unpaired statistics), in patients with FLD of various etiologies (alcoholic FLD versus FLD of mixed origin) — markers for differentiation

Таблица 2. Электрические и вязкоупругие параметры эритроцитов, исследованные методом Volcano plot (непарная статистика), у пациентов ЖБП различной этиологии (алкогольная ЖБП против ЖБП смешанного генеза) — маркеры для дифференцирования

Electrical and viscoelastic parameters of erythrocytes ***Электрические и вязкоупругие параметры эритроцитов***	**Factor of change** ***Кратность изменений*** **(FC)**	**log2(FC)**	***p* values** ***Значения p*** **(raw.*p* val)**	**–log10(*p*)**
Destruction index at the frequency of 5×10^5 Hz (%) *Индекс деструкции на частоте 5×10^5 Гц (%)*	3.6763	1.8787	0.000715	3.1453
Cell membrane capacity, F *Емкость клеточной мембраны, Ф*	0.5565	–0.84775	0.011818	1.9274

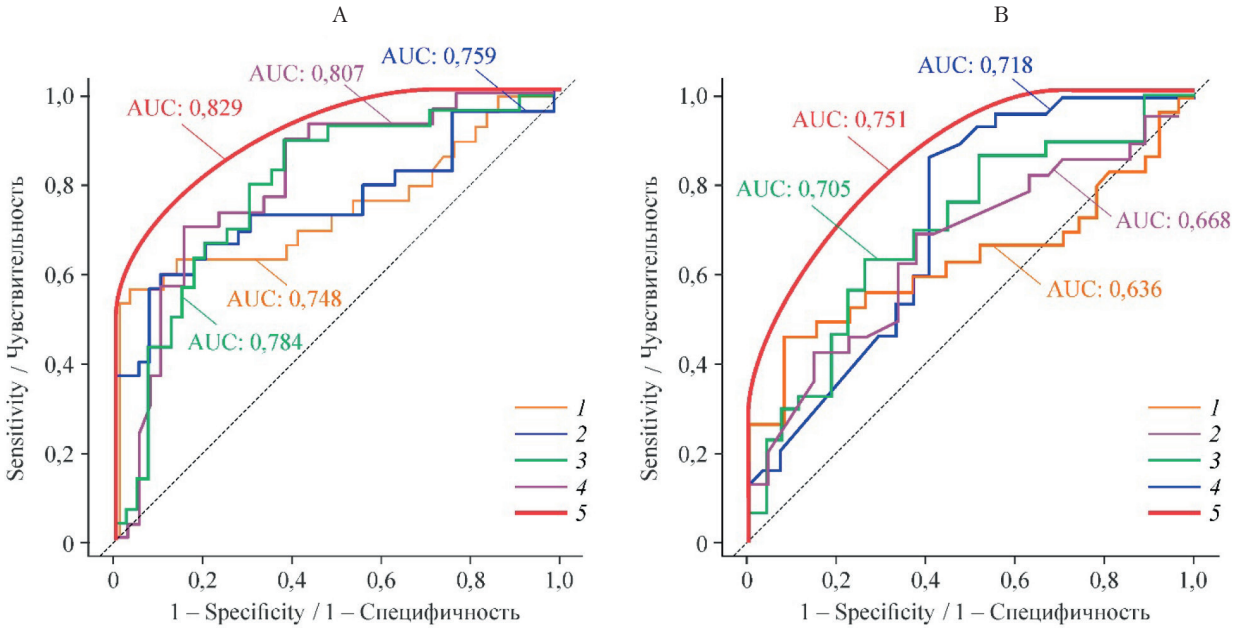

Figure 5. ROC curves for electrical and viscoelastic parameters of erythrocytes in distinguishing patients with FLD of mixed origin: A — from NAFLD (lines: *1* — position of crossover frequency (Hz); *2* — membrane capacitance (F); *3* — polarizability of cells at a frequency of 10^6 Hz (m^3); *4* — degree of deformation of erythrocytes at a frequency of 5×10^5 Hz (%); *5* — combined model, including three parameters: the degree of deformation of erythrocytes at a frequency of 5×10^5 Hz, cell polarizability at a frequency of 10^6 Hz and membrane capacitance); B — from AFLD (lines: *1* — membrane capacity (F); *2* — proportion of deformed cells (%); *3* — polarizability of cells at a frequency of 10^6 Hz (m^3); *4* — index of destruction of erythrocytes at a frequency of 5×10^5 Hz (%); *5* — combined model, including three parameters: erythrocyte destruction index at a frequency of 5×10^5 Hz, cell polarizability at a frequency of 10^6 Hz and membrane capacitance)

Рисунок 5. ROC-кривые для электрических и вязкоупругих параметров эритроцитов в различении пациентов с ЖБП смешанного генеза: А — от НАЖБП (линии: *1* — положение равновесной частоты (Гц); *2* — емкость мембран (Ф); *3* — поляризуемость клеток на частоте 10^6 Гц ($м^3$); *4* — степень деформации эритроцитов на частоте 5×10^5 Гц (%); *5* — комбинированная модель, включающая три параметра: степень деформации эритроцитов на частоте 5×10^5 Гц, поляризуемость клеток на частоте 10^6 Гц и емкость мембран); В — от АЖБП (линии: *1* — емкость мембран (Ф); *2* — доля деформированных клеток (%); *3* — поляризуемость клеток на частоте 10^6 Гц ($м^3$); *4* — индекс деструкции эритроцитов на частоте 5×10^5 Гц (%); *5* — комбинированная модель, включающая три параметра: индекс деструкции эритроцитов на частоте 5×10^5 Гц, поляризуемость клеток на частоте 10^6 Гц и емкость мембран)

The most optimal model in terms of sensitivity and specificity with a minimum list of indicators has turned out to be a model consisting of three characteristics — the index of RBC destruction at a frequency of 5×10^5 Hz, membrane capacity, and polarizability at a frequency of 10^6 Hz — AUC = 0.751 (CI: 0.611–0.908), sensitivity 79.5 %, specificity 74.7 % (Fig. 5B).

The correlation analysis has revealed associations of the most significant parameters of erythrocytes and indicators of alcohol consumption patterns for distinguishing the mixed-genesis FLD from alcohol-related FLD.

Correlations of erythrocyte parameters with some manifestations of metabolic syndrome have been established. Here are the most significant values. The capacity of RBC membranes was inversely correlated with waist circumference ($r = -0.419$, $p = 0.02$); the index of RBC destruction at a frequency of 10^5 Hz was directly associated with BMI ($r = 0.417$, $p = 0.022$), and the degree of obesity ($r = 0.399$, $p = 0.029$). The index of RBC destruction at a frequency of 5×10^5 Hz was directly correlated with the level of uric acid ($r = 0.475$, $p = 0.0001$). The destruction of erythrocytes at a low frequency of 10^5 Hz was also associated with uric acid ($r = 0.557$, $p = 0.0001$). Inverse associations of polarizability indices with fasting glucose and uric acid levels have been revealed ($r = -0.591$, $p = 0.0001$ and $r = -0.514$, $p = 0.0001$, respectively). The index of RBC destruction at a frequency of 5×10^5 Hz was associated

with polarizability at a frequency of 10^6 Hz and relative polarizability ($r = -0.480$, $p = 0.003$ and $r = -0.518$, $p = 0.0001$, respectively); the index of RBC destruction at a frequency of 10^5 Hz was associated with polarizability at a frequency of 10^6 Hz ($r = -0.422$, $p = 0.023$).

Discussion

Our previous studies have shown the peculiarities of erythrocyte parameters in patients with the alcohol-related and non-alcoholic FLD [12–15], but it has still remained unclear how the NAFLD and alcohol-related FLD differ from the mixed-genesis FLD in terms of electrical and viscoelastic parameters.

The use of dielectrophoresis method for this purpose is due to the possibility of single-step obtaining information on more than 20 parameters of erythrocytes, reflecting both the state of cell membranes and cytoplasm in early terms, before the appearance of abnormalities in the traditionally used hemogram [13].

Electrical and viscoelastic indices of erythrocytes associated with their resistance, membrane state, surface charge and ability to deform have turned out to be the most significant parameters for differentiating FLD types. For a pair of "NAFLD vs. mixed-genesis FLD", five parameters have turned out to be essential for distinguishing, as follows. The polarizability at a frequency of 10^6 Hz, the relative polarizability, and the membrane capacity were higher for patients having NAFLD. In contrast, an increased index of RBC destruction at 10^5 Hz and a more pronounced shift of crossover frequency into the high-frequency range was associated with FLD of mixed genesis. A subsequent analysis, taking into account the ranking of the influence of erythrocyte parameters on the distinction between the mixed-genesis FLD and NAFDL, has revealed the significance of the degree of erythrocyte deformation at 5×10^5 Hz, which has been incorporated afterwards into the diagnostic model. According to the Volcano plot, only two parameters are significant for the distinction in the pair "Mixed-genesis FLD vs. alcohol-related FLD", namely a high index of RBC destruction at a frequency of 5×10^5 Hz in the alcohol-related FLD and a higher membrane capacity in the mixed-genesis FLD. A subsequent analysis has revealed a contribution to the difference in this pair and the polarizability of cells at a frequency of 10^6 Hz.

There are known data on increased hemolysis of erythrocytes associated with alcohol intoxication [25, 26], hyperglycemia and hyperinsulinemia [27, 28], cytolysis syndrome in diffuse liver diseases [29, 30], dyslipidemia [31, 32], as well as on elevated uric acid levels [33]. The presence of associations between the above-described factors and the index of RBC destruction has been revealed. It should be noted that the degree of hemolysis at a low frequency of 10^5 Hz was one of the differentiating markers of the mixed-genesis FLD and NAFLD, reflecting a chronic toxic effect of ethanol on cells in the combined metabolic and alcoholic genesis of FLD, which was missing in patients with NAFLD. The shift of the crossover frequency into the high-frequency range has also reflected a result of long-term exposure of cells to high doses of ethanol, which had been shown earlier [12] and determined the role of this index in differentiating the groups with the NAFLD and mixed-genesis FLD. In the pair "mixed-genesis FLD vs. alcohol-related FLD", the index of RBC destruction was also significant at high frequency of electric field equal to 5×10^5 Hz. In this case, the alcohol factor is present in both groups, but single and weekly doses of ethanol intake for the alcohol-related FLD were higher than for the mixed-genesis FLD. The dose-dependent effect of ethanol on the severity of hemolysis has been shown in experimental studies of A. Bertola et al. [34], C. Zheng et al. [35], as well as in a prospective study made by S. Mueller et al. [36]. In a large cohort of alcohol abusers ($n = 439$), macroscopic signs of hemolysis have been found in 10 % of all studied samples [35]. According to a study by L.M. Chi et al., ethanol induces the formation of membrane pores with a diameter of approximately 13 A, which may be associated with disruption of the structure of RBC membrane cytoskeletal proteins [37], subsequently leading to hemolytic anemia [25, 26, 38]. Free hemes can cause a variety of prooxidant and proinflammatory effects [39], and an increase in the serum level of the hemolysis marker CD163 [35].

The index of RBC destruction in studies using dielectrophoresis is closely related to cell polarizability, which reflects cell viability, biological activity and is closely combined with the electrical parameters of the cell [40, 41], the sialic acid content in the membrane, an electrophoretic mobility of cells and indices of RBC aggregation [42]. Experiments in silico show, that cells from individuals consuming alcohol systematically are more fragile in response to phenylhydrazine as the hemolytic agent [35], and this is consistent with our findings of reduced levels of the polarizability at 10^6 Hz and relative polarizability. This circumstance has determined the role of the polarizability at high frequencies (10^6 Hz) and relative polarizability as biomarkers for distinguishing the mixed-genesis FLD from the NAFLD and alcohol-related FLD.

The ability of an erythrocyte to deform is due to the liquid nature of the cellular contents, the elasticity of the erythrocyte membrane and the relative excess of the membrane surface area relative to the intraerythrocyte volume and the state of hemoglobin [43, 44]. In patients with metabolically associated

alcohol-related FLD, RBC changes are exacerbated by the effects of prolonged exposure to ethanol and its metabolites, as reflected in the degree of change in amplitude of RBC deformation at 5×10^5 Hz in the mixed-genesis FLD and NAFLD [45–48]. Hyperglycemia and potentially elevated levels of HbA1c increase the summarized index of cell rigidity and inversely affect its ability to deform [49], affecting the summarized index of cell viscosity and cell deformability.

Changes in the membrane capacity parameters were more pronounced in the mixed-genesis FLD than in NAFLD. At the same time, lower values of membrane capacity have been found in patients with alcoholic etiology of fatty liver disease in the pair "Mixed-genesis FLD vs. AFLD". The presence of two etiological factors, namely the metabolic and alcoholic, have suggested more pronounced changes in this indicator: low values of membrane capacity have been found in patients with alcoholic etiology of FLD. However, the ethanol doses leading to structural changes in cell membranes were significantly higher in the cases of alcoholic genesis of FLD, probably with a greater severity of shifts confirmed by the experiments of C. Zheng et al. on dose-dependent effects of ethanol [35], accompanied by revealed changes in the cholesterol/phospholipid ratio, and phospholipid fractions, increased density of protein bands 3, 4.2, 4.9, of actin and glycophorins [50], with changes in the fatty acid profile of erythrocyte membranes [51], which has influenced the index of the erythrocyte membrane capacity in AFLD to a greater extent.

Conclusion

Thus, the most significant parameters for differentiating the FLD of mixed genesis (metabolic + alcohol-related) from NAFLD using the Volcano plot have turned out to be cell polarizability at a frequency of 10^6 Hz ($p = 6.49 \times 10^{-5}$), the RBC cell membrane capacity ($p = 0.00077$), the relative polarizability ($p = 0.001$), the levels of which were higher in patients with NAFLD. The index of RBC destruction at 10^5 Hz was higher in mixed-genesis FLD ($p = 0.047$) and the crossover frequency was shifted to the high frequency range more than in NAFLD ($p = 0.0005$). The discriminant analysis has additionally revealed the significance of the degree of erythrocyte deformation at 5×10^5 Hz in distinguishing between mixed-genesis FLD and NAFLD. A diagnostic model including the degree of erythrocyte deformation at 5×10^5 Hz, cell polarizability at 10^6 Hz, and membrane capacity has provided an AUC of 0.829 (CI: 0.742–0.916), sensitivity of 80.9 %, and specificity of 83.3 % in differentiating FLD of mixed genesis from NAFLD.

Two indicators of RBCs have been established that statistically significantly distinguish mixed-genesis FLD from alcohol-related FLD (Volcano plot); those are the index of RBC destruction at a frequency of 5×10^5 Hz, which was higher with alcohol-related FLD ($p = 0.0007$), and the capacity of cell membranes, the value of which prevailed with the mixed-genesis FLD ($p = 0.011$). When distinguishing mixed-genesis FLD from alcohol-related FLD, the combined model with the inclusion of three parameters of RBCs, namely the index of RBC destruction at a frequency of 5×10^5 Hz, the capacity of erythrocyte membranes, and polarizability at a frequency of 10^6 Hz has shown the highest levels of diagnostic accuracy – AUC = 0.751 (CI: 0.611–0.908) with a sensitivity of 79.5 %, specificity of 74.7 %.

The electrical and viscoelastic parameters of erythrocytes studied using the diagnostic technique of dielectrophoresis should be considered as promising biomarkers for the diagnosis of diffuse liver disease in further studies.

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