

## FN1 mRNA expression of fibronectin 1 and distribution of fibronectin-associated leukocytes in humans with chronic diffuse liver diseases

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### Article info

Received 24.04.2020

Received in revised form

22.05.2020

Accepted 23.05.2020

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**Dolhikh, H. V., Maslak, H. S., Chernenko, G. P., Minchenko, O. H., & Dolhikh, A. O. (2020). FN1 mRNA expression of fibronectin 1 and distribution of fibronectin-associated leukocytes in humans with chronic diffuse liver diseases. *Regulatory Mechanisms in Biosystems*, 11(2), 200–206. doi:10.15421/022029**

Chronic diffuse liver diseases are characterized by continuous progression of fibrosis, ultimately leading to cirrhosis with the following loss of the normal functioning of this organ due to excessive accumulation of the components of extracellular matrix. To find new, more available diagnostic markers of detecting disorders in the liver, we used methods of antifungal cytofluorometry and quantitative real-time polymerase chain reaction. Intensity of exposure of fibronectin and plasmatic membrane of lymphocytes in the group of patients with chronic diffuse diseases compared with the control group of practically healthy donors decreased both inside and on the surface of the cells respectively by 45.3% and 16.2%. Similar tendency towards decrease was observed during the assays of the level of the exposure of fibronectin on the surface and inside the blood granulocytes: by 25.0% and 36.5%, respectively. In the blood of the patients suffering from chronic diffuse diseases, compared with the control group, there was determined reliable increase in percentage of lymphocytes and granulocytes which contain topical fibronectin, by 32.3% and 2.78 times, correspondingly. The level of monocytes (as a percentage) with cell-associated fibronectin and fibronectin localized inside, by contrast, reliably decreased in 2.07 and 4.50 times, respectively. Analysis of the expression of FN1 in lymphocytes of blood of the studied groups using quantitative real-time polymerase chain reaction revealed decrease in the level of FN1 mRNA expression by 34.0% in the group of ill patients compared with the control group. We determined excellent diagnostic informativeness of the parameters of the level of exposure of fibronectin inside and on the surface of granulocytes and prognostic accuracy of the classifier from these parameters at the level of 100% using the method of support vector machine, SVM. High levels of diagnostic informativeness were recorded for the tests of all types of analyzed leukocytes with cell-associated fibronectin, and the classifiers based on the pair combinations of the tests with cell-associated fibronectin and fibronectin localized within the cells provide high diagnostic accuracy of the prognosis. Because the mentioned indicators are highly-sensitive tests, they can be proposed for early diagnostics and evaluation of the effectiveness of the conducted therapy of chronic diffuse liver diseases, which would allow reducing the use of paracentetic trepanobiopsy, a painful and risky procedure, which still remains the main type of diagnostic.

**Keywords:** fibronectin; FN1 expression; lymphocytes; monocytes; granulocytes.

### Introduction

Chronic diffuse liver diseases are characterized by continuous progression of fibrosis, resulting in cirrhosis and following impairment of this organ. Liver fibrosis is the essential link in the development of pathological process in the tissue of the liver, and its level is quite a sensitive non-specific marker of pathological changes under the effect of different etiologic factors. Fibrogenesis includes the participation of Kupffer cells (fixed macrophages), activated perisinusoidal cells, which transform into myofibroblasts and synthesize extra-cellular matrix which fills the space of Disse, leading to the phenomenon of “capillarization of sinusoids”, and then to necrosis of hepatocytes, replacement of hepatocytes with the connective tissue and, as a result, disorder in the architectonics of the liver – cirrhosis. Perisinusoidal cells, microfibroblasts and cells of the bone marrow come in close interaction with hepatocytes and immune cells to cause scars as response to impairment of the liver (Dolhikh et al., 2018). An important outcome of this interaction is also an extremely complex process of activation, including a number of significant cellular changes. The most distinctive sign of the activation is increase in the production of extracellular matrix (ECM), includ-

ing collagen of I, III, IV types, proteoglycans, laminin and fibronectin. At the same time, release of anti-inflammatory cytokines, enzymes which ruin the matrix occurs. Therefore, an important role in the fibrogenic process belongs to the components of the ECM, as well as collagen, fibronectin, enzymes, etc., and cellular immune system (Chundru et al., 2014). Fibronectin (FN) is involved in numerous processes related to wound healing, blood coagulation, migration of cells, phagocytosis, embryonic development and malignant transformation. Soluble plasma form of this glycoprotein synthesizes, mainly by hepatocytes. In the healthy liver, plasmatic FN is the most distributed protein of the extracellular matrix, whereas non-soluble cellular fibronectin is normally present in very low concentrations. It localizes mostly in the ECM which surrounds the cells and forms bundles connected with microvilli of hepatocytes in the space of Disse. Despite the fact that during progression of fibrosis, usually, a marked increase in the level of plasmatic Fn is observed, the content of this protein decreases during cirrhosis. Presumably the increase in the level of the soluble form of FN during early fibrosis of the liver contributes to the action of the protein as a chemotactic factor for production of collagen, whereas decrease in the level of fibronectin in the patients with liver cirrhosis is related to the liver dysfunction (Attallah et al., 2013).

The diagnostic importance of fibronectin and its role during chronic diffuse liver diseases are still unrevealed. According to one perspective, in the process of fibrogenesis, FN functions as a pro-fibrogenic protein: it deposits at the early stages of the development of fibrogenesis, contributes to the organization of the extracellular matrix, helps it to bind cells, conduces to the activation and proliferation of the perisinusoidal cells, induces collagen gene and helps in deposition of other components of the ECM (Birukawa et al., 2014). According to the opposite data, fibronectin protects against excessive liver fibrosis, modulating the accessibility and sensitivity of the perisinusoidal cells to cytokine TGF- $\beta$ , especially due to the interaction with the integrin receptor through RGD-binding fragment (sequence of arginine-glycine-aspartic acid) (Altrock et al., 2015).

Chronic inflammation and fibrosis are directly related, because the interaction between the immune cells, local fibroblasts and tissue macrophages in the places of the formation of scars is the result of a damaged liver. After the processes which operate the inflammation and fibrosis, it became clear that adaptive and intrinsic immune system take part in their regulation (Park et al., 2015). Lymphocytes that penetrate the damaged tissue produce lymphokines which in turn induce other inflammatory cells, such as macrophages (Eddy, 2014). Studies with the use of mice models revealed that among these subpopulations of lymphocytes, the most relevant for the tissue fibrosis are T helper lymphocytes 1 and 2. At the same time, T helper lymphocytes 2 are profibrotic, their manifestation in the region of inflammation leads to massive deposition of collagen and increase in the development of fibrosis. Several mechanisms are considered responsible for the effect of B-cells on the liver fibrosis. On one hand, the role of B-lymphocytes in pathogenesis of fibrosis is determined in the decrease in the deposition of collagen, observed during CCl<sub>4</sub>-induced fibrosis in mice with deficiency of B-cells. On the other hand, production of B-lymphocytes of profibrinolytic cytokine interleukin 6 contributes to the liver fibrosis by induction of differentiation of perisinusoidal cells into myofibroblasts, proliferation of fibroblasts and increase in the synthesis of tissue inhibitors of metalloproteinases and collagen. Thus, today, lymphocytes are no longer considered "observers" in pathogenesis of liver fibrosis, they take part directly in this process (Pellicoro et al., 2014).

The objective of the study was determining the level of expression of fibronectin 1 in lymphocytes, the level of fibronectin within and on the surface of blood leukocytes, division of lymphocytes, monocytes and granulocytes from the topical and cell-associated fibronectin in blood of patients with chronic diffuse liver diseases.

## Materials and methods

The object of the study was leukocytes of the blood of patients suffering from chronic diffuse diseases of the liver (n = 15) at the age of 28–46 years. The control group comprised practically healthy donors (PHD) aged 25–52 years (n = 15). The studies were performed in accordance with the bioethical norms corresponding to the regulations of the WHO, the World Medical Association's Declaration of Helsinki (1989), the Convention on Human Rights and Biomedicine developed by the Council of Europe (1977), the Council for International Organizations of Medical Sciences, International Code of Medical Ethics (1983), which fact was confirmed by the Committee of Bioethics of the Dnipropetrovsk Medical Academy of the Ministry of Healthcare of Ukraine.

First, for the lysis of erythrocytes, the blood was subjected to the influence of Optilyse C lysis solution (Beckman Coulter, USA, 2018) for 30 minutes, subsequent rinsing with normal saline buffer (centrifuged for 2 minutes at 2,400 rpm) and fixation procedure with 8% paraformaldehyde over 7 min. As a result, we isolated fraction of leukocytes, in which lymphocytes, monocytes and granulocytes were studied. After rinsing, the cells were re-suspended in normal saline buffer, their number was counted in hemocytometer. Vitality of the cells (over 90%) was determined using trypan blue and working concentration of lymphocytes was prepared (300 thou/mL in each sample) (Novikov & Novikova, 1996). Exposure of fibronectin was determined using monoclonal antibodies to matrix FN (AbDSerotec, UK) and corresponding secondary antibodies to mouse's immunoglobulins conjugated with fluorescein

isothiocyanate (FITC, Millipore, USA). For permeabilization of cells, we used digitonin (Fluka, Sweden) with the concentration of 250  $\mu$ g/mL. Control of dead cells was conducted using binding with propidium iodide. The data were recorded on the Beckman Coulter EPICS flow cytometer (Beckman Coulter, USA, 2001). The density of the exposure was calculated according to the program of FCS Express 3 (De Novo Software, USA, 2001).

We performed analysis of the expression of FN1 using quantitative real-time polymerase chain reaction (PCR). RNA from lymphocytes was isolated using Trizol reagent (Invitrogen, USA). For this purpose, 80 mL of Trizol reagent were added to the suspension of lymphocytes, mixed, 20 mL of chloroform was added, mixed again and centrifuged at 16,000 g and +4 °C during 10 min. We selected 50 mL of supernatant and RNA was precipitated using equal volume of isopropanol during two hours in –20 °C. Sediments of RNA obtained as a result of centrifugation were rinsed with 100 mL of 75% ethanol and dissolved in ribonuclease-free sterile water (QIAGEN, Germany).

Amplification of FN1 gene was carried out using a pair of primers: forward 5'-ACCAACCTACGGATGACTCG-3' and reverse primer for mRNA of fibronectin-1: 3'-GCTCATCATCTGGCCATTTT-5'. These oligonucleotides correspond to nucleotide sequences 6,769–6,788 and 6,998–6,979 of published kDNA of fibronectin of humans (GenBank number NM\_002026). The size of amplified fragment of 230 bp.

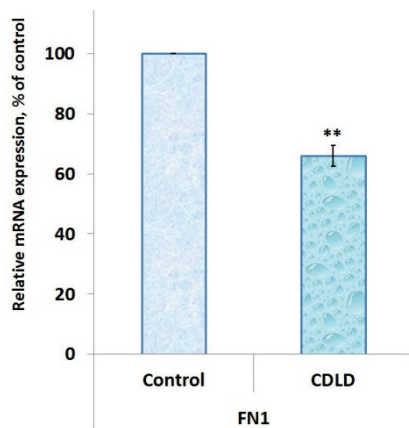
The quantity of RNA taken for the analysis was evaluated according to the level of mRNA expression of beta-actin (ACTB). Amplification of kDNA of beta-actin was performed using forward – 5'-GGACTTCGAGCAAGAGATGG-3' and reverse – 5'-AGCACTGTGTTGGCGTACAG-3' primers. Nucleotide sequences of these primers correspond to the sequences 747–766 and 980–961 of kDNA of human ACTB (GenBank number NM\_0011101). Size of the amplified fragment was 234 bp. Primers were obtained from Sigma-Aldrich Company (USA). For the amplification of kDNA of ribosomal protein S16, we used primers: forward – 5'-GGCAATGGTCTCATCAAGGT-3' and reverse – 5'-TCTCCTTCTTGAAGCCTCA-3'. These oligonucleotides correspond to nucleotide sequences 133–152 and 373–354 of published kDNA of ribosomal protein S16 of human (GenBank number X00351). Reverse transcription of RNA was performed using Quantitect Reverse Transcription Kit toolkit (QIAGEN) according to the manufacturer's protocol. For the reaction of reverse transcription, we took 1 mL of RNA and mixture of nucleases, 1  $\mu$ L of kDNA, 1 mL of 10  $\mu$ mol/L of mixture of primers and 10  $\mu$ L of two-fold mixture for polymerase chain reaction (QIAGEN). Products of amplification were analyzed using electrophoresis in 2% agarous gel, staining the DNA 5xSightDNAstain (EUROMEDEA). Gels were analyzed in the Quantity One Bio-Rad System (USA).

Statistical analysis was performed using R and EasyROC 1.3.1 software. The test of correspondence of the distribution of the quantitative features of the normal law was conducted with the Shapiro–Wilk test. In the conditions of normal distribution of the data for the description of the level of the central tendency of quantitative features, we used mean arithmetic value ( $\bar{x}$ ) and standard error of average value (SE). For the calculation of the difference between the values of the groups, the Tukey test with Bonferroni correction were employed. To analyze the interaction between the signs, we used correlation analysis with calculation of coefficients of Spearman's rank-order correlation ( $\rho$ ). Correlation coefficient in the range of  $0.7 \leq |\rho| < 1$  indicated strong correlation relation;  $0.3 \leq |\rho| < 0.7$  – relation of average strength;  $0 < |\rho| < 0.3$  – low correlation relation. The parameter of the level of statistical significance for all types of analysis equaled 5%. The differences were considered statistically significant if the probability of random occurrence of differences did not exceed 0.05 ( $P < 0.05$ ). To evaluate the efficiency of use of diagnostic markers, ROC-analysis was used (Mandrekar, 2010). ROC-analysis and visualization of ROC-curves were performed in EasyROC 1.3.1 software. The analysis revealed the values of the statistical parameters of the efficiency of the diagnostic tests of sensitivity (Se) and specificity (Sp); to characterize the informativeness, we determined the values of the area under the ROC-curve (AUC – Area Under Curve), Cut-off points for the condition of norm-pathology. The value of the area under the ROC-curve was interpreted in the parameters of

diagnostic accuracy: 0.9–1.0 – excellent, 0.8–0.9 – very well, 0.7–0.8 – good, 0.6–0.7 – average, 0.5–0.6 – unsatisfactory; value of 0.5 corresponds to impracticability of the model. Cut-off point was calculated using the Youden index. To develop the graphs of box-plot, program pack of statistical analysis of “R” data was used (Lang & Sesik, 2011). To predict the possibility of pathology and search of optimum combination of tests, as classifiers we used Support Vector Machine. To implement the method, we used Kenlab R package. SVM allows developing the decision surface using selections of small volume and gives high diagnostic accuracy of predicting in the case of low classification efficiency of ROC-analysis.

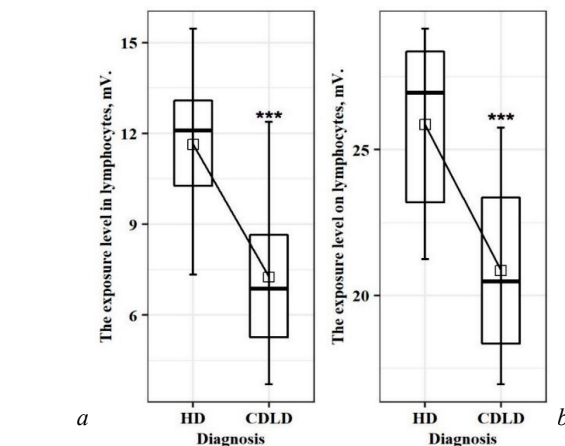
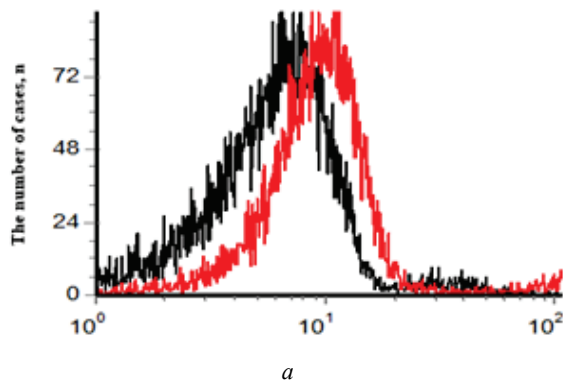
## Results

In the patients with chronic diffusive diseases of the liver, compared with healthy donors, we observed decrease in the level of FN1 mRNA expression by 34.0% ( $P < 0.01$ ). Expression of FN1 (fibronectin-1) in blood lymphocytes was determined in relation to the beta-actin mRNA expression as the control gene.



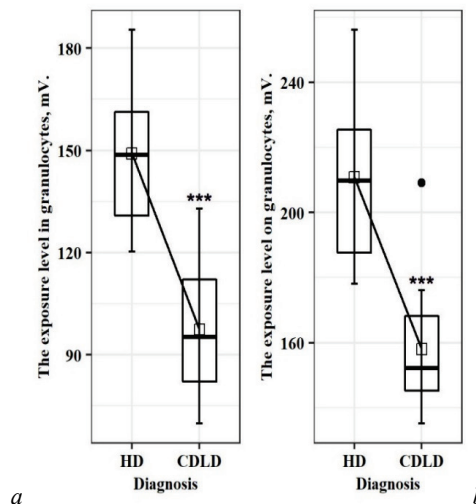
**Fig. 1.** FN1 mRNA expression in lymphocytes of practically healthy donors (control) and patients with chronic diffuse liver diseases (CDLD) using real-time polymerase chain reaction ( $n = 15$ ,  $x \pm SE$ ): \*\* –  $P < 0.01$ ; as the control, values of beta-actin mRNA expression, were considered 100%

The assays of the intensity of fluorescence of antibodies to fibronectin of blood lymphocytes of patients with CDLD compared to the group of practically healthy donors demonstrated decrease during chronic liver diseases both inside and on the surface of the cells by 45.3% ( $P < 0.05$ ) and 16.2%, respectively (Fig. 2).

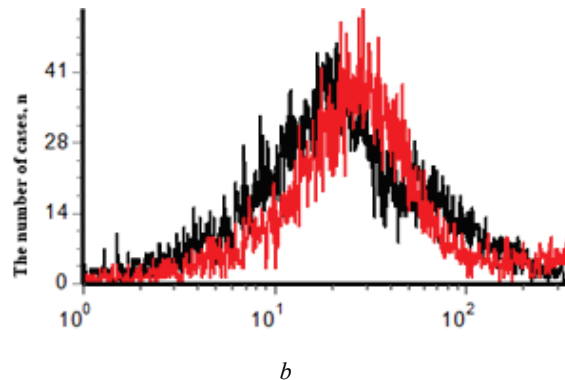


**Fig. 2.** Average level of exposure of fibronectin within (a) and on the surface (b) of lymphocytes of the group of practically healthy donors (PHD) and patients with chronic diffuse liver diseases (CDLD): \*\*\* –  $P < 0.001$

On the surface of and within granuloocytes, during chronic diffuse liver diseases, decrease in the density of the exposure of fibronectin was  $25.0 \pm 2.0\%$  ( $P < 0.05$ ) and  $36.5 \pm 3.2\%$  ( $P < 0.05$ ), respectively (Fig. 3, 4).



**Fig. 3.** Average level of the exposure of fibronectin within (a) and on the surface (b) of granuloocytes of the groups of practically healthy donors (PHD) and patients with chronic diffuse liver diseases (CDLD); \*\*\* –  $P < 0.001$

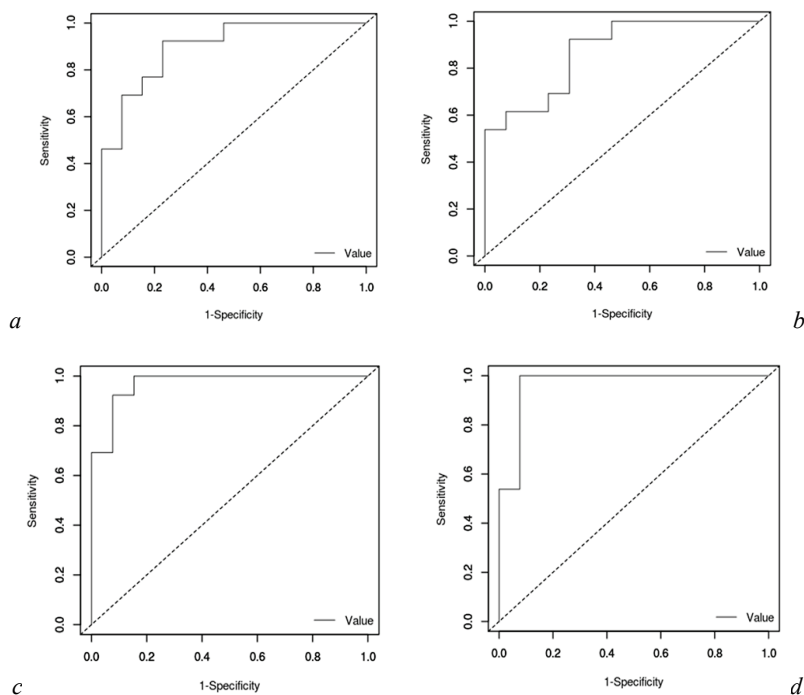


**Fig. 4.** Intensity of fluorescence (mV) of antibodies to fibronectin on the surface of granuloocytes (a) and lymphocytes (b) of practically healthy donor (black line) and patient with chronic diffuse liver diseases (red line) according to the data of flow cytometry on Beckman Coulter EPICS: the density of the exposure was calculated in accordance with FCS Express 3 program

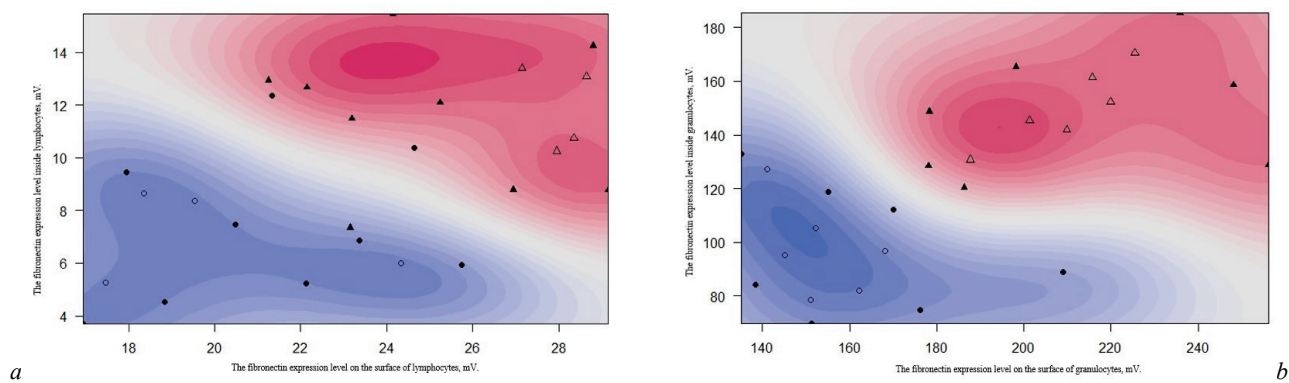
It should be noted that the level of fibronectin exposure within and on the surface of monocytes in the group of patients with chronic liver diseases and healthy donors practically did not differ (results are not given).

The analysis of ROC-curves of the level of fibronectin exposure in lymphocytes and granulocytes in blood plasma (Fig. 5) revealed statistical values of the informativeness of the tests. Diagnostic efficiency of the test of the level of fibronectin in lymphocytes equaled  $Se = 0.923$ ,  $Sp = 0.769$ . Cut-off point corresponds to the value of 8.76 mV (Youden index of 0.692), pathology is indicated by the values of the exposure level lower than this parameter.  $AUC = 0.899$  ( $P = 2.01 \times 10^{-11}$ ), excellent diagnostic informativeness of the method. The test of the diagnostic of CDLD according to the level of fibronectin exposure on the surface of lymphocytes equaled  $Se = 0.923$ ;  $Sp = 0.692$ . The Cut-off point

corresponds to the value of 11.15 mV (Youden index of 0.615), pathology is diagnosed by values below this parameter.  $AUC = 0.869$  ( $P = 6.57 \times 10^{-8}$ ), good diagnostic informativeness of the method. Diagnostic possibilities of the test of the level of fibronectin exposure in granulocytes:  $Se = 1.0$ ;  $Sp = 0.846$ . The Cut-off point corresponds to the value of 128.35 mV (Youden index of 0.846), pathology is indicated by the value of the level of exposure lower than this parameter.  $AUC = 0.970$  ( $P = 6.46 \times 10^{-65}$ ), excellent diagnostic informativeness. Diagnostic efficiency of the test of fibronectin exposure on granulocytes  $Se = 1.0$ ,  $Sp = 0.923$ . Cut-off point corresponds to the value of 178.15 mV (Youden index of 0.923), pathology is indicated by values lower than this parameter.  $AUC = 0.964$  ( $P = 8.44 \times 10^{-36}$ ), excellent diagnostic informativeness of the method.



**Fig. 5.** ROC-curves of use of parameters of the level of fibronectin exposure within lymphocytes (a), on the surface of lymphocytes (b), within granulocytes (c), on the surface of granulocytes (d)



**Fig. 6.** Results of the classification of groups of practically healthy patients and patients with CDLD using SVM according to the parameters: a – average level of FN exposure within lymphocytes and on the surface of lymphocytes, b – average of the level of FN exposure within granulocytes and on the surface of granulocytes; optimum surfaces which separate the groups of PHP and patients with CDLD are indicated in the figures with lighter tone

Correlation analysis using Spearman coefficient indicates direct relation of average strength between all the analyzed tests. Highest correlation dependence was found between the exposure of intracellular and topical fibronectin in granulocytes; Spearman coefficient ( $\rho$ ) and the level of statistical significance ( $P$ ) for this fraction respectively equaled:  $\rho = 0.672$ ,  $P = 0.046$ . The relationship between the intracellular fibronectin fraction of granulocytes and surface-associated fraction of lymphocytes was  $\rho = 0.477$ ,  $P = 0.015$ . Practically equal quantitative correlation was found bet-

ween the tests: the level of exposure of FN within lymphocytes and granulocytes,  $\rho = 0.415$ ,  $P = 0.036$ ; level of exposure of FN within lymphocytes and associated on the surface of granulocytes,  $\rho = 0.414$ ,  $P = 0.036$ .

The analysis of the surveyed parameters using the SVM (Fig. 6) revealed predicted possibilities of the tests. The location of the data of the test in relation to the separating surface: higher – pathology is absent, lower – contrary. In case of the development of classifier according to the parameters of the level of FN exposure within and on the surface of

lymphocytes, overlapping of the studied classes was seen. In the case of presence of linear indivisibility of groups, the expected level of the classification error increases, accuracy of the developed classifier in this case equals 88.4%. During the use of data of FN exposure within and on the surface of granulocytes, observation clouds of the studied groups do not become overlapped; the accuracy of classifier equals 100%. We determined the percentage of the share of lymphocytes, granulocytes and monocytes which contain fibronectin from the general amount of these cells

in blood (Table 1). Compared with the control group, in the blood of patients with chronic diffuse diseases, we determined reliable ( $P < 0.001$ ) increase by 32.3% and in 2.78 times in the percentage of the share of lymphocytes and granulocytes which contain the topical fibronectin, respectively. The level of monocytes (in percents) with cell-associated FN and FN localized in the cells, by contrast, reliably ( $P < 0.001$ ) decreased by 2.07 and 4.50 times respectively compared with the values observed in the blood of practically healthy donors (Table 1).

**Table 1**

Quantity of leukocytes of blood which contain topical and intracellular fibronectin of the group of practically healthy donors ( $n = 15$ ) and patients with chronic diffuse diseases of the liver ( $n = 15$ )

Characteristic of the group of the examined donors	$\bar{x} \pm SE$	Median	Quartile 1 – Quartile 3	Min–Max
Lymphocytes (%) with fibronectin within the cells of the group of PHP	26.8 ± 0.4	26.8	25.9–28.3	23.1–29.3
Lymphocytes (%) with fibronectin within cell in the group of CDLD	32.8 ± 3.0	30.8	23.4–39.0	18.2–58.1
Lymphocytes (%) with surface-bound fibronectin in the group of PHP	53.3 ± 1.3	54.2	49.8–57.2	41.9–59.3
Lymphocytes (%) with surface-bound fibronectin in the group of CDLD	70.4 ± 1.9***	70.8	65.9–76.1	57.7–82.2
Granulocytes (%) with intracellular fibronectin in the group of PHP	0.21 ± 0.04	0.18	0.09–0.29	0.07–0.62
Granulocytes (%) with intracellular fibronectin inside cells in the group of CDLD	0.23 ± 0.03	0.18	0.14–0.27	0.07–0.59
Granulocytes (%) with surface-bound fibronectin in the group of PHP	3.0 ± 0.2	2.9	2.4–3.4	1.6–5.1
Granulocytes (%) with surface-bound fibronectin in the group of CDLD	8.4 ± 1.0***	7.4	5.4–9.3	3.8–18.4
Monocytes (%) with intracellular fibronectin in the group of PHP	1.44 ± 0.18	1.34	1.09–1.79	0.47–2.79
Monocytes (%) with intracellular fibronectin in the group of CDLD	0.32 ± 0.03***	0.33	0.20–0.39	0.13–0.56
Monocytes (%) with surface-bound fibronectin in the group of PHP	20.1 ± 2.2	19.9	13.9–25.7	7.1–34.3
Monocytes (%) with surface-bound fibronectin in the group of CDLD	9.7 ± 0.8***	8.6	8.0–10.2	5.7–17.0

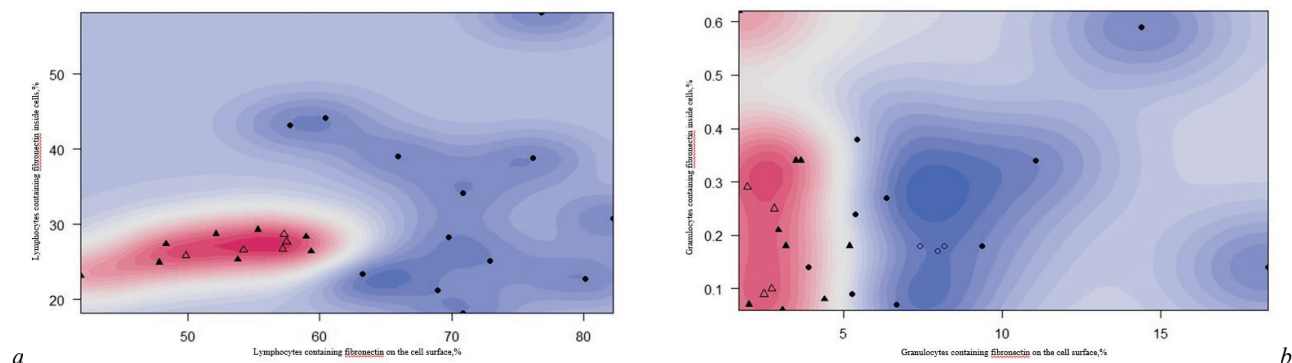
Note: \*\*\* –  $P < 0.001$ , compared with the control group (PHP) according to the Tukey test with Bonferroni correction.

The method of traditional ROC-analysis revealed the efficiency of the studied tests for diagnostics of chronic diffuse liver diseases. Unsatisfactory diagnostic informativeness was seen for the tests for diagnostics of CDLD according to the quantity of lymphocytes having FN inside,  $AUC = 0.609$ , ( $P = 0.408$ ) and quantity of granulocytes with FN inside,  $AUC = 0.532$ , ( $P = 0.785$ ). Excellent diagnostic informativeness was shown by the tests: according to the quantity of lymphocytes bearing FN on the surface,  $Se = 0.923$ ,  $Sp = 1.0$ ,  $AUC = 0.987$  ( $P = 1.01 \times 10^{-255}$ ), Cut-off point of 60.4% (values higher than the parameter indicate pathology); according to quantity of monocytes with intracellular FN,  $Se = 1.0$ ,  $Sp = 0.846$ ,  $AUC = 0.982$  ( $P = 8.67 \times 10^{-157}$ ), Cut-off point of 5.2% (values below this parameter indicate pathology). Diagnostic efficiency of the test according to the number of monocytes with surface-bound FN  $Se = 0.923$ ,  $Sp = 0.769$ . Cut-off point equals 12.4%, pathology is indicated by values below this parameter.  $AUC = 0.887$  ( $P = 8.89 \times 10^{-8}$ ), indicating very good diagnostic informativeness of the method.

Correlation analysis of share of lymphocytes, granulocytes and monocytes (%) in the total quantity of the type of cells which contain fibronectin indicate strong relationship between some tests. Positively correlating were the fractions of lymphocytes with cell-associated fibro-

nectin and granulocytes with surface-bound fibronectin. A similar tendency was exhibited by two fractions of monocytes: with cell-associated and endogenous fibronectin. These tests exhibited the following values of the Spearman coefficient ( $\rho$ ) and the level of statistical significance ( $P$ ) respectively:  $\rho = 0.797$ ,  $P = 3.07 \times 10^{-6}$  and  $\rho = 0.742$ ,  $P = 1.43 \times 10^{-5}$ . Negative correlation of average strength was seen between the following fractions of leukocytes: lymphocytes with surface-bound FN and monocytes with cell-associated fibronectin,  $\rho = -0.580$ ,  $P = 0.0023$ ; granulocytes with topical fibronectin and monocytes with endogenous FN,  $\rho = -0.626$ ,  $P = 0.0006$ ; granulocytes and monocytes with surface-associated fibronectin,  $\rho = -0.552$ ,  $P = 0.0039$ . Fraction of lymphocytes with fibronectin associated on the surface of the cells and monocytes with intracellular fibronectin demonstrated strong reverse correlation dependency,  $\rho = -0.747$ ,  $P = 1.14 \times 10^{-11}$ .

Analysis of the tests according to the SVM revealed the separating surfaces which divided the groups of PHP and CDLD, demonstrating 100% accuracy according to the number of lymphocytes with surface-bound and intracellular fibronectin; 92.3% accuracy according to the number of granulocytes with surface-bound and intracellular fibronectin; and 96.2% accuracy according to the quantity of monocytes with surface-bound and intracellular fibronectin (Fig. 7).



**Fig. 7.** The results of classification of the groups of PDP and CDLD by the SVM according to the quantity of the cells (%) which contain fibronectin: *a* – on the surface of and within lymphocytes, *b* – on the surface of and within the granulocytes

## Discussion

Fibronectin on the surface of lymphocytes can be both of endogenous and exogenous origin, because it is synthesized in lymphocytes and is affinitive to the receptors and on their surface (Hauzenberger, 1996). This

glycoprotein is involved in the reaction of inter-cellular interaction, migration of lymphocytes in the fibrous tissue, processes of their activation and proliferation and directly affects on the course of inflammatory process (Schreiber et al., 2013). The role of fibronectin on the surface of lymphocytes during fibroses is studied insufficiently. This protein is considered to

have immunomodulating action: T-helper lymphocytes 1 express high levels of fibronectin which stimulates the appearance of anti-inflammatory phenotype of monocytes which can activate the macrophages and expression of cytokines. Particularly these cells are in the areas of chronic inflammatory diseases, as indicated during rheumatoid arthritis and multiple sclerosis (Sandig et al., 2009). There is confirmation of the effect of fibronectin on proliferation of CD-4- and CD-8-positive cells and its binding with lymphokines for the stimulation of interaction between antigen-presenting cells and T-cells (Forget et al., 2014). The studies on the levels of plasmatic and cellular-binding fibronectin were undertaken (Maslak et al., 2013) during pathological conditions of different etiology: inflammatory and viral diseases (Netronina et al., 2018), revealing changes in the structure of N- and O-glycated regions of glycoprotein, cell-associated with leukocytes of blood (Maslak, 2013).

During chronic diffuse liver diseases, the level of fibronectin within lymphocytes reliably decreases, which was confirmed by the results obtained using the method of quantitative real-time polymerase chain reaction: the level of FN1 mRNA expression in the blood lymphocytes was also lower than the control values. In the physiological conditions, the level of FN1 expression was insignificant in practically healthy donors and unstudied in cases of fibrotic liver disorders. Milner et al. (2008) confirmed the effect of the level of the FN1 expression on the growth completion and growth of the vessels during angiogenesis, particularly: first, the increase during the development of cerebral capillaries was determined, then rapid decrease after their maturation. Particularly the low parameter of the level of fibronectin expression is considered to be responsible for reduced survivability and proliferation of endothelial cells (Milner et al., 2008). This could explain the results we obtained, because there is a direct pathogenic relationship between the processes of angiogenesis and the intensity of the further fibrosis (Radenska-Lopovok et al., 2015). In addition, the authors proved the effect of hypoxia on the expression of many genes, including mRNA COL6A1 – collagen 6A1 (Minchenko et al., 2017).

No differences between the levels of fibronectin exposure in monocytes of blood of patients with CDLD and practically healthy donors were observed. According to the results of our studies, the density of fibronectin exposure on the surface of and within the granulocytes during chronic diffuse liver diseases was reliably lower than the values of the control. These studies were conducted because the activated granulocytes are cells involved in the inflammatory process and their content significantly increases during fibrosis (Downey et al., 2009). The role of fibrosis inside or on the surface of activated granulocytes still remains undetermined. It is included in specific granulocytes of neutrophils (Lee et al., 2010), and in the conditions of their activation, dehydration occurs and FN can be released from cells (Abbonante et al., 2015). It should be noted that the migration and adhesion of activated neutrophils to fibroblasts during inflammation take place due to interaction of integrins (CD18, CD11) and fibronectin. Downey et al. (2009) report that the processes of transendothelial migration of neutrophils across the fibrous tissue are significantly affected by proteins of extracellular matrix, fibonectin, etc., and while moving, the neutrophils release large amount of proteinases and choose the route with the lowest amount of the ECM proteins, because the second ones can induce their apoptosis. Therefore, granulocytes are active participants of fibrinolytic processes, and decrease in the levels of intracellular and cell-associated fibronectin could be associated with its release and active involvement of fibronectin in the processes of migration of granulocytes across the fibrous tissue.

Surveys of the distribution of leukocytes with intracellular and cell-associated fibronectin in the blood of patients with CDLD revealed increased percentage share of lymphocytes and granulocytes with surface-associated fibronectin and decrease in the level of monocytes according to this parameter. Therefore, re-distribution of leukocytes with topical fibronectin was determined in the studied group of patients compared with the control values. Search for the hematologic analysis-based cellular markers of pathologic conditions is ongoing. The suggested indicator of the ratio of the level of neutrophils to the number of lymphocytes in blood (NLR) is a simple and available marker for the evaluation of inflammation, two variables of which could be easily obtained with blood analysis. NLR includes two components of the immune system-neutrophils which reflect the process of the inflammation which illustrate its regulation (Peng et al.,

2018). We have for the first time demonstrated the re-distribution of leukocytes and localization of topical fibronectin, which could have higher specificity in use, because NLR is also broadly used as a prognostic marker for the evaluation of the condition of the patients with various oncologic diseases (Allan et al., 2017).

Fibrosis is an extremely complex and multi-stage process, when leukocytes act as pro- and anti-fibrosis agents (Kryczka, 2015), and fibronectin, similarly to glycoprotein of the extracellular matrix, is an important participant of these processes and a target for the cells (Liu et al., 2016). Search for prognostic, diagnostic and therapeutic agents (Duval et al., 2015) continues, but always concerns the liver tissue itself, requiring the biopsy, which carries the risk of complications, such as infection and bleeding (Germani et al., 2011). Recently, scientists have agreed on the idea that non-invasive methods could be sufficiently informative (Verloh et al., 2019).

## Conclusions

We presumed that in the blood of patients with CDLD, changes could occur in the distribution and the level of fibronectin exposure within and on the surface of the circulating cells. The results of flow cytometry were confirmed using quantitative real-time polymerase chain reaction and were statistically analyzed, revealing the practicability of using the indicators selected in the study as diagnostic and prognostic criteria of CDLD. Excellent diagnostic informativeness was exhibited by the tests of the level of the fibronectin exposition within and on the surface of granulocytes, and the method of support vectors SVM demonstrated that the accuracy of the classifier in the case of using the data of the tests equaled 100%. High levels of diagnostic informativeness were seen also for the tests of all types of the analyzed leukocytes with cell-associated fibronectin, and the classifiers based on the pair combinations of the tests with cell-associated fibronectin and fibronectin localized within the cells provide high diagnostic accuracy of the prognosis. The data we obtained, in our opinion, indicate the practicability of performing further studies in order to find reliable strong markers for the diagnostics and evaluation of complexity of chronic diffuse liver diseases, which would allow decreasing the use of paracentetic trepanobiopsy, a painful and risky procedure which still remains the main type of diagnostics.

The article is performed according to the plan of scientific research of the work of State Institution Dnipropetrovsk Medical Academy of the Ministry of Health of Ukraine and is a fragment of complex scientific research study “Structural-functional changes in proteins during oxidative- carbonyl stress and their correction under the effect of therapeutic measures” (state registration 0118U006025).

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