BLOOD EXPRESSION OF CD39 AND CD73 ECTONUCLEOTIDASES IN PATIENTS WITH VARIOUS FORMS OF METABOLIC-ASSOCIATED FATTY LIVER DISEASE

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Experimental studies have demonstrated the protective role of ectonucleotidases — particularly CD39 and CD73 — in limiting inflammation connected to a liver pathology. However, their expression in metabolic-associated fatty liver disease (MAFLD) has not been thoroughly investigated. This study aimed to evaluate the mRNA levels of the *ENTPD1* and *NT5E* genes, which encode CD39 and CD73, respectively, in patients with different forms of MAFLD (liver steatosis (LS) and metabolic-associated steatohepatitis (MASH)), and to assess the expression of CD39- and CD73-positive cells following immune cell activation *in vitro*. The sample included 29 healthy donors and 56 MAFLD patients. We measured the mRNA levels of the *ENTPD1* and *NT5E* genes, pro-inflammatory cytokines (IL-6, TNFα), fragmented cytokeratin-18, and the blood content of $CD39^+$ cells. Another parameter measured in vitro was the effect of immune cell activation on the proportion of $CD39^+$ and $CD73^+$ cells in patients with MASH and healthy donors. The expression of the *ENTPD1* gene ($p = 0.007$ vs. control group; $p = 0.010$ vs. LS group) and the proportion of $CD39^+$ cells among monocytes ($p = 0.004$ vs. control group; $p = 0.003$ vs. LS group) and lymphocytes ($p = 0.034$ vs. control group) were lower in the MASH group compared with both the control and LS groups. Activation of cells from MASH patients increased the proportion of $CD39^+$ lymphocytes, but not that of $CD14^+$ monocytes. It also increased the proportion of $CD73^+$ cells among both lymphocytes and $CD14^+$ monocytes. Thus, further investigation into the roles of CD39 and CD73 in the context of MAFLD progression represents a promising avenue for future research.

Keywords: metabolic-associated fatty liver disease, metabolic-associated steatohepatitis, CD39, CD73, $CD14^+$ monocytes, $CD4^+$ T cells

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ЭКСПРЕССИЯ ЭКТОНУКЛЕОТИДАЗ CD39 И CD73 В КРОВИ БОЛЬНЫХ РАЗНЫМИ ФОРМАМИ МЕТАБОЛИЧЕСКИ АССОЦИИРОВАННОЙ ЖИРОВОЙ БОЛЕЗНИ ПЕЧЕНИ

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Экспериментальные исследования показали защитную роль экспрессии эктонуклеотидаз, в частности CD39 и CD73, в ограничении воспаления при патологии печени, но их экспрессия у больных метаболически ассоциированной жировой болезнью печени (МАЖБП) мало изучена. Целью работы было оценить уровни мРНК генов *ENTPD1* и *NT5E*, кодирующих CD39 и CD73 соответственно, у пациентов с разными формами МАЖБП: стеатозом печени (СП) и метаболически ассоциированным стеатогепатитом (МАСГ), а также оценить содержание CD39- и CD73-экспрессирующих клеток при активации иммунных клеток *in vitro*. Обследовали 29 здоровых доноров и 56 пациентов с МАЖБП. Проводили оценку уровня мРНК генов *ENTPD1* и *NT5E*, уровня провоспалительных цитокинов (ИЛ-6, ФНОα), уровня фрагментированного цитокератина-18 и содержания $CD39^+$ клеток в крови обследованных людей. Кроме того, оценивали влияние активации иммунных клеток на содержание $CD39^+$ и $CD73^+$-клеток у пациентов с МАСГ и здоровых доноров *in vitro*. Установлено, что экспрессия гена ENTPD1 ($p = 0{,}007$ при сравнении с контролем; $p = 0{,}010$ — с СП) и содержание $CD39^+$-клеток в популяции моноцитов ($p = 0{,}004$ — с контролем; $p = 0{,}003$ — с СП) и лимфоцитов ($p = 0{,}034$ — с контролем) снижены при МАСГ относительно групп контроля и СП. Показано, что активация клеток пациентов с МАСГ увеличивает уровень $CD39^+$-лимфоцитов, но не $CD14^+$-моноцитов, а $CD73^+$-клеток — среди лимфоцитов и $CD14^+$-моноцитов. Таким образом, представляется перспективным дальнейшее изучение CD39 и CD73 в механизмах прогрессирования МАЖБП.

Ключевые слова: метаболически ассоциированная жировая болезнь печени, метаболически ассоциированный стеатогепатит, CD39, CD73, $CD14^+$-моноциты, $CD4^+$ Т-клетки

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Metabolic-associated fatty liver disease (MAFLD) is a chronic, non-communicable, slowly progressing multifactorial disease that is extremely common in the world and in Russia in particular. In the recent reports, the prevalence of MAFLD among the adult population in Russia and worldwide is put at 29–35% [1, 2].

According to current concepts, the complex pathogenesis of MAFLD involves insulin resistance, impaired autophagy, lipotoxicity, inflammation, imbalances in cytokines and adipokines, activation of innate immunity and the microbiota, as well as environmental and genetic factors [3, 4]. Several clinical and morphological forms of MAFLD are distinguished according to the disease stage: steatosis, metabolic-associated steatohepatitis (MASH) with or without fibrosis, and liver cirrhosis (LC) [3]. Liver steatosis (LS) typically follows a benign clinical course, whereas MASH may have a progressive course and develop into LC and hepatocellular carcinoma. Early MASH is often asymptomatic for a long time, but ultimately, even with only minimal abnormalities in routine liver function tests, it can lead to several comorbidities — such as type 2 diabetes mellitus, hypertension, and chronic kidney disease — that adversely affect both the employability and quality of life of patients. At the same time, the mechanisms of progression of MAFLD remain poorly understood [4]. One of the key problems of modern gastroenterology is that of finding alternatives to liver biopsy to differentiate MASH from LS [5].

MAFLD involves a complex interaction among inflammation, apoptosis, and fibrosis, in which immune cells and cytokines are crucial mediators of these interconnected processes [6-8]. Many authors have convincingly demonstrated the relationship of fragmented cytokeratin-18 (FCK-18) with laboratory and histological signs of inflammation in MASH [9, 10].

Currently, one promising avenue for controlling inflammation is the study and therapeutic targeting of the ATP–adenosine balance. Extracellular ATP is released from the cell during necrosis, apoptosis, or hypoxia; it directs phagocytes to the inflammation loci, thus activating the inflammasome, which is followed by the release of pro-inflammatory cytokines. Extracellular adenosine then acts as a limiter of inflammation and tissue damage, exerting an anti-inflammatory effect [11]. The key enzymes in extracellular adenosine formation are ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD1, also known as CD39), which hydrolyzes ATP and ADP to AMP, and ecto-5'-nucleotidase (NT5E, also known as CD73), which converts AMP to adenosine.

Accumulating evidence suggests that CD39 and CD73 play an important role in liver diseases [12–14]. In experimental models, the deletion of CD39 was shown to lead to insulin resistance and exacerbation of inflammation in the liver [15]. In another study, CD39 deficiency contributed to bile duct damage and fibrosis by attracting cytotoxic T cells [16]. Deficiency of the adenosine A2A receptor in both macrophages and hepatocytes was associated with increased severity of MAFLD, likely due to enhanced inflammation and lipogenesis [17]. By dephosphorylating ATP to adenosine, CD39 and CD73 ectonucleotidases play a protective role and prevent local and systemic inflammation. The significance of this mechanism in MAFLD has not been investigated. This study aimed to evaluate the mRNA levels of the *ENTPD1* and *NT5E* genes, which encode CD39 and CD73, respectively, in patients with different forms of MAFLD (LS, MASH), and to assess the expression of CD39- and CD73-positive cells following immune cell activation *in vitro*.

METHODS

We examined 56 patients with MAFLD: 31 with simple liver steatosis (LS group) and 25 with moderate to high activity MASH (MASH group). The control group consisted of 29 healthy people.

The inclusion criteria for the control group were a normal BMI, normal liver function test results, normal liver sonographic structure, and normal liver stiffness.

The exclusion criteria for the control group were the presence of cardiometabolic risk factors, a diagnosis of NAFLD, or the use of medications.

The exclusion criteria common to all study groups included infectious or inflammatory diseases within the past month, pregnancy or lactation, smoking, and alcohol consumption.

The inclusion criteria for the treatment group were an established diagnosis of NAFLD (LS or NASH), and presence of at least one cardiometabolic risk factor.

The criteria for exclusion of NAFLD patients were as follows: viral genesis of liver damage as proven by the absence of serological markers of HBV, HCV infection; autoimmune genesis, as proven by the absence of autoantibodies to hepatocyte structures; alcoholic genesis, as proven by anamnestic, clinical data, data from the CAGE, AUDIT scales; drug genesis, as proven by anamnestic data; diabetes mellitus types 1 and 2; liver cirrhosis; active course of hepatotropic or other therapy at the time of sampling the material for the study.

Healthy donors and MAFLD patients were examined by general practitioners at *RZD-medicine* clinical hospital (Petrozavodsk). The diagnosis of MAFLD, including determination of its form (LS or MASH), was established based on traditional clinical, laboratory, instrumental, and histological data in accordance with clinical guidelines [3]. All non-healthy participants (patients) had signs of metabolic syndrome and at least one cardiometabolic risk factor, most often — dyslipidemia (in 64% of LS and 93% of MASH patients) and visceral obesity (all patients, BMI > 25 kg/m^2). The patients underwent an ultrasound examination of the abdominal organs using a Vivid Pro-7 system (General Electric, USA), which revealed increased echogenicity of the liver tissue exceeding that of the right kidney. Liver stiffness was assessed by ARFI shear wave elastometry using a Mindray DC-80 device (Mindray, China). None of the patients showed signs of portal hypertension: there was no ascites, and esophagogastroscopy did not reveal esophageal or gastric varices. The patients underwent a blind percutaneous liver biopsy, and histological activity and fibrosis were assessed according to the method of Brunt et al. [18]. Based on these findings, the form of MAFLD was verified as either steatosis (≥5% of hepatocytes) without inflammation and hepatocellular ballooning, or as steatohepatitis characterized by active injury, including hepatocellular balloon degeneration and lobular inflammation (predominantly lymphocytic with some neutrophils), along with varying degrees of steatosis. According to the results of histological examination, fibrosis in all patients did not exceed stage F2.

For the study, fasting venous blood was drawn from the ulnar vein into K_2EDTA tubes. Clinical evaluation of liver function tests, isolation of peripheral blood leukocytes (PBLs) and plasma, as well as analysis of CD39-expressing cells, were performed within one hour after blood sampling. Blood plasma, total RNA isolated from PBLs, and peripheral blood mononuclear cells (PBMCs) obtained by Ficoll density gradient centrifugation (density = 1.077 g/cm^3; Biolot) were placed in a biobank for storage at –80 °C for no more than 3 months. The collection of samples took place after the patients had been admitted to the hospital but before therapy was prescribed, approximately 2–3 days prior to liver biopsy and elastography.

Assessment of liver function parameters — alanine aminotransferase (ALT), aspartate aminotransferase (AST), total

bilirubin, alkaline phosphatase (ALP), glucose, total cholesterol (TCH), high-density lipoprotein (HDL), triglycerides, and C-reactive protein (CRP) — was carried out using a Random Access F-15 analyzer (BioSystems, Spain) with reagents from Vector-Best (Russia). LDL levels were calculated using the Friedewald formula. The erythrocyte sedimentation rate (ESR) was determined by the Westergren method.

The ELISA method was used to determine the concentration of tissue polypeptide-specific antigen (hepatocyte apoptosis index FCK-18), tumor necrosis factor alpha (TNF) and interleukin-6 (IL-6) in blood plasma on a SuPerMax 3100 tablet multimodal reader (Flash Spectrum, China). The test systems used were TPS ELISA (Biotech, Sweden), Human TNFa Platinum ELISA (eBioscience, Austria), Interleukin-6 — IFA – Best (Vector-Best, Russia).

Total RNA from PBLs was isolated using ExtractRNA reagent (Eurogen, Russia). The relative expression level of the *ENTPD1* and *NT5E* genes in PBLs was assessed by real-time PCR using a LightCycler instrument (Roche, Germany) and the qPCRmix-HS SYBR kit (Eurogen, Russia) Primers for amplification of the *ENTPD1* gene are forward 5'-AGCAGCTGAAATATGCTGGC-3', reverse 5'-GAGACAGTATCTGCCGAAGTCC-3'; for the *NT5E* gene, forward 5'- ATTGCAAAGTGGTTCAAAGTCA-3', reverse 5'-ACTTGGCCAGTAAAATAGGG-3'. The *18S rRNA* gene was used as a reference, with forward primer 5'-AGAAACGGCTACCACATCCA-3' and reverse primer 5'-CACCAGACTTGCCCTCCA-3'. The PCR conditions were as follows: cDNA denaturation for 5 min 95 °C; 40 cycles: denaturation at 95 °C for 30 seconds, annealing at 64 °C for 30 seconds, elongation at 72 °C for 30 seconds. The specificity of the amplification products was verified by melting curve analysis. Each PCR analysis was performed in triplicate to assess repeatability. The relative level of transcripts was assessed by Livak and Schmittgen [19].

The relative cell content of the studied populations was determined using a Cytomics FC500 flow cytometer (Beckman Coulter, USA). Blood samples or cell suspensions were stained with FITC-, PE-, PerCP, and PE-Cy7–labeled monoclonal antibodies against CD39, CD4, CD25, CD161, and CD14 antigens, as well as with appropriate isotypic controls (Sony Biotechnology, USA; eBioscience, USA). The erythrocytes were lysed with the FACS Lysing Solution reagent (BD Biosciences, USA). FMO (fluorochrome minus one) control was used to identify CD39- and CD73-expressing cells. The absolute number of cells was determined based on the data of a clinical blood test.

To assess the effect of activation on the proportions of $CD39^+$ and $CD73^+$ cells, PBMCs isolated from MASH patients ($n = 6$) and healthy donors ($n = 6$) were seeded in 48-well plates (3.5×10^5 cells per well) in RPMI-1640 medium (Servicebio) supplemented with 10% heat-inactivated ETS (Biolot), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Servicebio). The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. The cells were activated with classical stimulants: lymphocytes with PHA at a final concentration of 10 µg/ml (Biolot), and monocytes with LPS (Pyrogenal, MEDGAMAL of N. F. Gamaleya National Center of Epidemiology and Microbiology) at a concentration of 1 µg/ml.

Statistical analysis was performed using GraphPad Prism v.8 (GraphPad Software Inc.; San Diego, CA, USA). The data on the graphs are given as Me (Q_1–Q_3). The differences were considered significant at $p < 0.05$. Due to non-normal distributions of indicators in the groups (confirmed by the Shapiro-Wilk test), nonparametric tests were used for analysis. To compare the studied indicators across groups, we used the Kruskal–Wallis test, and for pairwise comparisons, the Mann–Whitney *U* test. A paired comparison of related indicators was performed using the Wilcoxon signed-rank test. The relationship of the indicators was assessed by calculating the Spearman's rank correlation coefficient (*r*). The strength of the correlation between the two variables was evaluated using the Chaddock scale.

RESULTS

Table 1 shows the clinical and laboratory characteristics of patients with LS, MASH, and healthy participants. In MASH patients, compared with LS patients, blood aminotransferase activity, clinical markers of inflammation (CRP, ESR), LDL levels, and FCK-18 concentrations were increased (Tables 1 and 2). The blood plasma levels of the main pro-inflammatory cytokines (TNF-α and IL-6) in patients with MAFLD were higher than in healthy controls and similar between subgroups with different forms of the disease (Table 2).

We performed a comparative analysis of the mRNA expression levels of the *ENTPD1* gene (which encodes CD39) and the *NT5E* gene (which encodes CD73) in PBLs obtained from patients with MAFLD and healthy donors. As shown by the Kruskal–Wallis ANOVA, the study groups differ significantly in the mRNA levels of *ENTPD1* ($H = 8.830$, $p = 0.012$), but not in the mRNA levels of *NT5E* ($H = 0.618$, $p = 0.734$) (Fig. 1). It has been established that the expression level of the *ENTPD1* gene in MASH patients is lower than in healthy people and LS patients. At the same time, the mRNA level of the *ENTPD1* gene does not differ from the control in LS (Fig. 1A).

Since we found that *ENTPD1* expression differed significantly between the study groups, the next step was to perform a correlation analysis of *ENTPD1* mRNA levels in MAFLD patients and healthy donors with blood biochemical parameters, including routine clinical indicators, FCK-18 concentrations, and the pro-inflammatory cytokines TNFα and IL-6 (Tables 1 and 2). This analysis revealed a moderate positive correlation between the relative mRNA expression level of *ENTPD1* and the blood LDL level in LS patients ($r = 0.379$, $p = 0.039$).

As shown in Table 1, the blood levels of lymphocytes and monocytes did not differ between the MAFLD groups and the control group. However, the data obtained earlier in this study (Fig. 1) necessitated the evaluation of CD39 ectonucleotidase expression on PBLs in MAFLD patients. This enzyme is known to be expressed on the surface of various immune cells [20]. We therefore assessed the frequency of $CD39^+$ granulocytes, monocytes, and lymphocytes. Among lymphocytes, the focus was on the proportion of $CD39^+$ T cells within the $CD4^+$ T helper population as a whole, as well as within its subpopulations — immunosuppressive Treg cells ($CD4^+CD25^{hi}$) and pro-inflammatory Th17 cells ($CD4^+CD161^+$). The data is presented in Table 3 and Fig. 2.

We found that the relative content of $CD39^+$ monocytes in the MASH group was significantly reduced compared with the control and with the LS group (Table 3, Fig. 2). The granulocytes from both MAFLD patients and healthy donors were almost all CD39- positive (Table 3). In MASH patients, we also observed a reduced relative and absolute count of $CD39^+$ lymphocytes compared with controls (Table 3). The content of $CD39^+$ Treg cells was higher in the MASH group compared to healthy donors (Fig. 2). A noteworthy fact: the count of Treg cells themselves in LS and MASH patients ($p = 0.007$ and $p = 0.013$, respectively) was lower than in the control group.

To determine whether the expression of the ectonucleotidases CD39 and CD73 on monocytes and lymphocytes from MASH

Table 1. Clinical and laboratory characteristics of the study groups

Indicator	Control (*n* = 29)	LS (*n* = 31)	MASH (*n* = 25)
Age, years	43.72 ± 1.12 (44.81)	48.43 ± 1.63 (48.10)	46.29 ± 2.27 (47.50)
Male/Female (*n*)	14/15	15/16	12/13
ALT, units/l	16.04 ± 1.25 (14.76)	23.16 ± 1.74 (21.90)	116.80 ± 27.67 (84.85)*#
AST, units/l	20.60 ± 0.86 (20.35)	23.92 ± 0.90 (23.90)	75.01 ± 8.48 (65.95)*#
Total bilirubin, mmol/l	15.14 ± 2.20 (12.20)	15.40 ± 1.38 (13.30)	16.85 ± 2.23 (15.92)*
ALP, units/l	96.37 ± 11.12 (83.20)	141.24 ± 16.17 (143.21)*	173.21 ± 16.54 (165.43)*
CRP, mg/l	1.06 ± 0.17 (0.95)	1.18 ± 0.40 (1.25)	4.87 ± 0.11 (4.87)*#
ESR, mm/h	8.65 ± 1.94 (6.00)	10.87 ± 2.06 (7.00)	17.38 ± 4.77 (12.00)*#
Glucose, mmol/l	5.12 ± 0.07 (5.02)	5.20 ± 0.10 (5.20)	5.31 ± 0.11 (5.28)
TC, mmol/l	5.04 ± 0.16 (4.96)	5.09 ± 0.19 (5.03)	5.56 ± 0.22 (5.60)*
HDL, mmol/l	1.40 ± 0.06 (1.30)	1.36 ± 0.13 (1.11)	1.22 ± 0.12 (1.03)
LDL, mmol/l	3.01 ± 0.18 (2.56)	2.74 ± 0.15 (2.68)	3.81 ± 0.24 (3.80)*#
Triglycerides, mmol/l	1.22 ± 0.12 (1.09)	1.81 ± 0.19 (1.55)	1.91 ± 0.14 (1.94)*
BMI, kg/m^2	23.88 ± 1.10 (23.77)	33.81 ± 0.78 (33.10)*	34.40 ± 1.13 (34.25)*
Lymphocytes, %	33.33 ± 0.99 (33.00)	31.36 ± 1.34 (31.00)	32.09 ± 1.1 (31.07)
Lymphocytes, ×10^9/l	2.11 ± 0.10 (2.23)	2.09 ± 0.12 (1.92)	1.91 ± 2.17 (0.14)
Monocytes, %	7.06 ± 0.35 (6.80)	7.63 ± 0.44 (7.35)	7.68 ± 0.46 (7.55)
Monocytes, ×10^9/l	0.44 ± 0.03 (0.41)	0.51 ± 0.03 (0.46)	0.50 ± 0.01 (0.45)

Note: the data are presented as M ± *m*, median in parentheses; * — significant difference from the control group, # — significant difference from the LS group (Mann–Whitney *U*–test with Bonferroni correction).

patients was associated with cellular activation, their cells and those from healthy donors (controls) were stimulated and analyzed by flow cytometry after 24 hours. We assessed the expression levels of CD39- and CD73-positive cells within lymphocyte gates, CD4$^+$ T cells, and CD14$^+$ monocytes. In addition, the median fluorescence intensity (MFI) for CD39 and CD73 was determined, which reflects the density of antigen expression on cells. Fig. 3 shows the results.

It is known that the activation of lymphocytes, particularly T cells, is usually accompanied by an increase in CD39 enzymatic activity and in the number of CD39$^+$ cells [21]. In our study, we observed an increase in the number of CD39$^+$ cells in lymphocyte cultures — particularly among CD4$^+$ T cells — following the addition of a mitogen in the control and MASH groups. (Fig. 3A). Changes in CD39 MFI were noted only in the lymphocyte gate, in both of these groups (Fig. 3B). The presence of LPS had no effect on the content of CD14$^+$CD39$^+$ monocytes (Fig. 3A).

The analysis of CD73$^+$ cell numbers after activation of PBMCs from patients with MASH and healthy donors (Fig. 3B) revealed significant differences. Upon stimulation, the proportion of CD73$^+$ lymphocytes and CD14$^+$ monocytes increased in MASH patients, whereas in healthy donors, significant changes in CD73$^+$ cell numbers were observed only among CD4$^+$ T cells. Both MASH patients and healthy donors showed an increase in CD73 MFI within the lymphocyte gate after activation (Fig. 3G).

DISCUSSION

The progression of hepatocellular dysfunction in MAFLD results from immuno-inflammatory processes, in which leukocytes play a central role [6, 8]. It is known that CD39 and CD73 ectonucleotidases are expressed on the surface of various immune cells in peripheral blood. In addition, enzyme expression can start from exposure to oxidative stress and hypoxia, as well as pro-inflammatory cytokines [22]. The levels of ectonucleotidases, determined mainly using experimental models, have been described in certain acute and chronic liver pathologies [22]. However, the expression of CD39 and CD73 in the blood of MAFLD patients and on individual populations of immune cells has not been investigated. Therefore, we conducted a comparative assessment of ectonucleotidase expression at both the mRNA level and on the surface of PBLs in patients with different forms of MAFLD (LS and MASH).

We discovered that in a simple LS case, the mRNA ENTPD1 levels of and the count of the enzyme on the surface of the PBLs are similar to those seen in the control group. In MASH cases, CD39 expression — both at the mRNA level in PBLs and at the protein level on the surface of monocytes — is significantly lower than in LS cases and in samples from healthy donors (Fig. 1, Fig. 2, Table 3). In addition, it was found that the number of CD39$^+$ cells changes among monocytes as well as Treg cells in MASH patients. We observed a higher proportion of CD39$^+$ cells among Treg cells in patients with MASH compared with the control group, although the overall number of Treg cells

Table 2. Cytokine content and FCK-18 levels in the blood plasma of patients with MAFLD and healthy donors

Indicator	Control (*n* = 29)	LS (*n* = 31)	MASH (*n* = 25)
FCK-18, units/l	80.36 ± 10.06 (83.80)	130.60 ± 13.83 (129.00)*	423.90 ± 84.60 (269.70)*#
TNFα, pg/ml	4.01 ± 0.35 (3.58)	5.86 ± 0.33 (5.89)*	6.50 ± 0.35 (6.67)*
IL-6, pg/ml	1.79 ± 0.18 (1.75)	5.31 ± 0.88 (4.25)*	6.89 ± 1.28 (6.71)*

Note: the data are presented as M ± *m*, median in parentheses; * — significant difference from the control group, # — significant difference from the LS group (Mann–Whitney *U* test with Bonferroni correction).

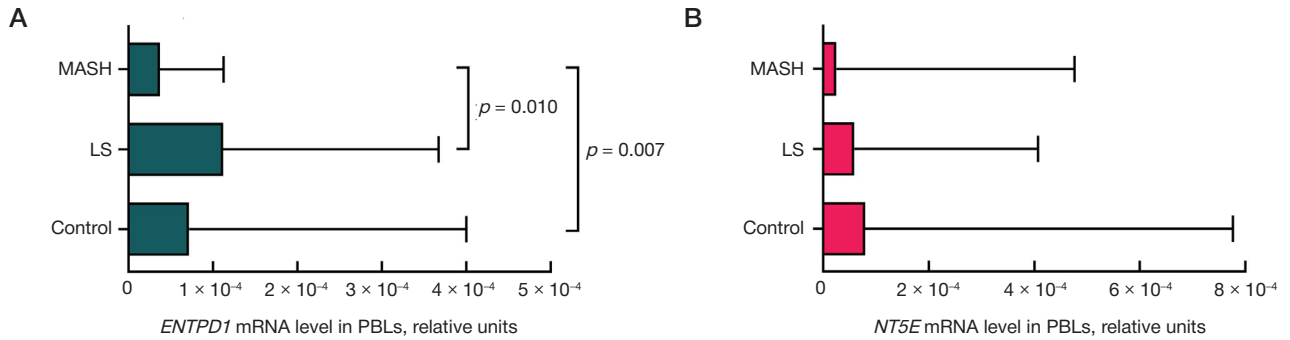

Fig. 1. The mRNA levels of *ENTPD1* (**A**) and *NT5E* (**B**) in the PBLs of MAFLD patients and healthy donors, Me and interquartile range; the differences are significant according to the Mann-Whitney *U* test with Bonferroni correction.

Table 3. Relative and absolute levels of CD39-expressing cells in blood cell populations of patients with MAFLD and healthy donors

	Control	LS	MASH
Lymphocytes CD39⁺ cells, % CD39⁺ cells, x10⁹/l	 12.40 (11.05–14.78) 0.33 (0.26–0.42)	 11.25 (9.84–12.59) 0.24 (0.18–0.30)	 10.65 (9.85–12.10)* 0.20 (0.19–0.30)*
Monocytes CD39⁺ cells, % CD39⁺ cells, x10⁹/l	 80.17 (70.98–88.41) 0.37 (0.31–0.49)	 76.95 (68.88–81.54) 0.35 (0.28–0.40)	 61.46 (53.64–71.94)*# 0.33 (0.24–0.41)
Granulocytes CD39⁺ cells, %	 94.65 (90.68–97.14)	 95.65 (92.49–97.90)	 94.65 (90.68–94.65)

Note: the data are given as Me (Q_1–Q_3). * — significant difference from the control group, # — significant difference from the LS group (Mann–Whitney *U* test with Bonferroni correction). Cell populations were isolated using forward scatter (FSC/FS) and side scatter (SSC/SS).

in these patients was reduced. It is likely that the increased CD39 expression on the surface of these cells is associated with compensation for their low number, since the generation of extracellular adenosine through CD39 production is one of the main mechanisms of Treg-associated immunosuppression [20]. Moreover, their concentrations in the blood of patients with MASH are insufficient to modulate pro-inflammatory cell function.

In addition, we have shown that, upon in vitro activation, the level of CD14⁺CD39⁺ monocytes in MASH patients remains lower than that observed in healthy donors (Fig. 3). It is noted in the literature that low CD39 expression on monocytes may be a poor prognostic sign. For example, a reduced CD39⁺ monocyte count is described as a potential diagnostic biomarker and predictor of an unfavorable prognosis in patients with sepsis [23].

A decrease in CD39 expression in MASH cases can lead to reduced extracellular degradation of ATP and ADP, resulting in lower levels of anti-inflammatory adenosine, which may contribute to the progression of inflammation and insulin resistance. This assumption is supported by data on the pathological effect of ENTPD1/CD39 deficiency on these processes in liver damage [15, 16].

A study of CD39 activity in the platelets of patients with hypercholesterolemia [21] showed that hypercholesterolemia, including elevated LDL levels, is associated with an increased inflammatory response, oxidative stress, and enhanced hydrolysis of ATP and ADP. The authors attributed the increase in CD39 activity to a compensatory response to the inflammatory and pro-oxidant conditions associated with hypercholesterolemia [24]. Interestingly, in cases of LS — the early stage of MAFLD characterized by lipid accumulation in hepatocytes and low-level subclinical inflammation — we did not observe a decrease in CD39 expression in peripheral blood compared with the control group. However, we found a moderate positive correlation between *ENTPD1* gene mRNA

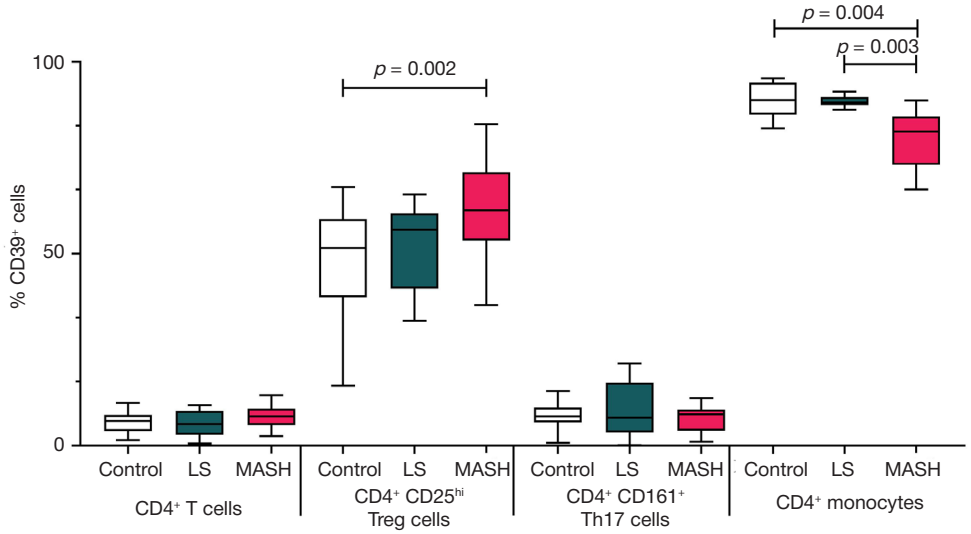

Fig. 2. Relative levels of CD39-expressing cells in blood cell populations of patients with MAFLD and healthy donors. Me, interquartile range, minimum and maximum values

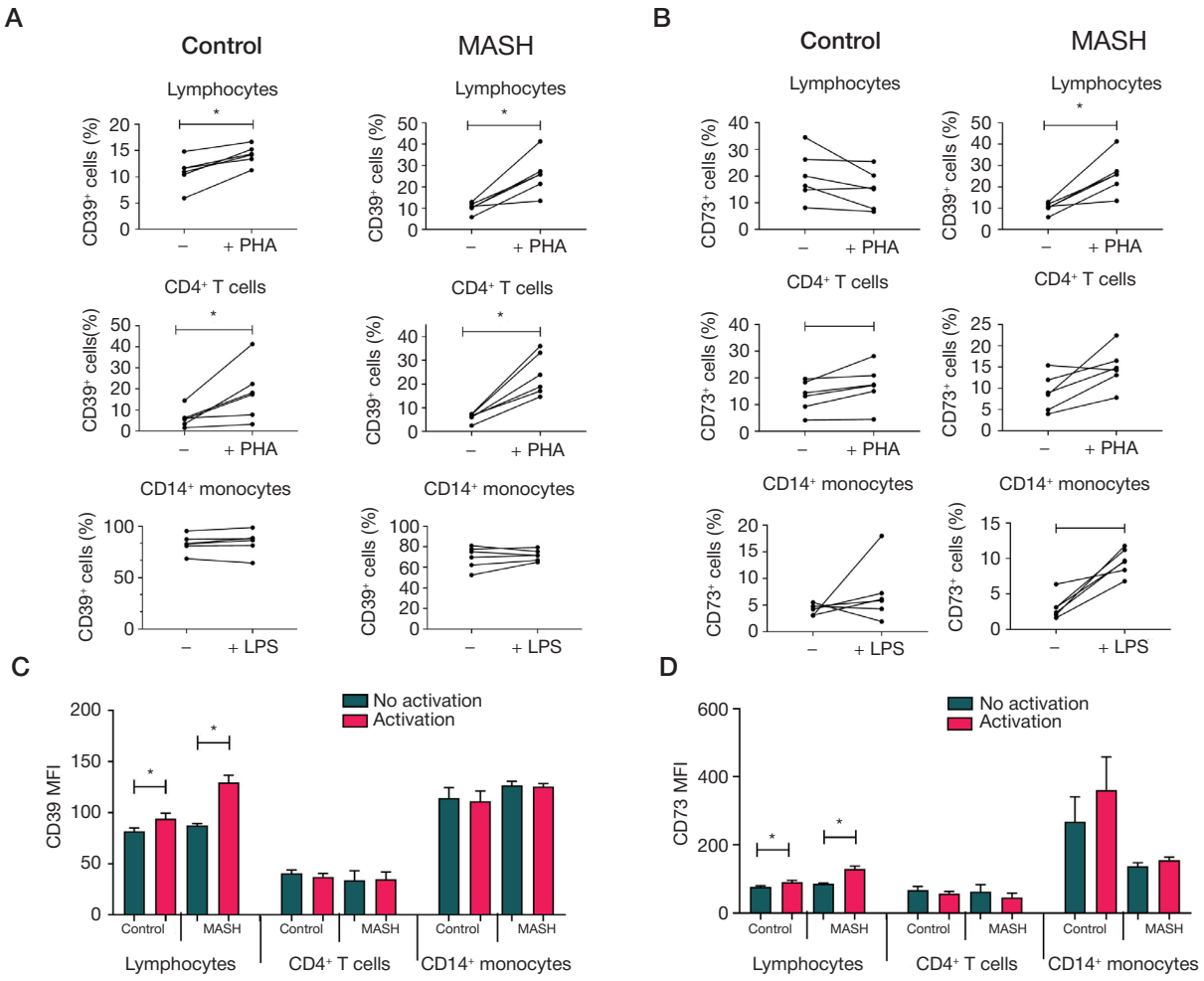

Fig. 3. Changes in the content of cells expressing CD39 and CD73 ectonucleotidases after activation in healthy donors (n = 6) and patients with MASH (n = 6). **A**. The content of CD39⁺ cells, % of the studied population. **B**. The content of CD73⁺ cells, % of the studied population. **C**. MFI of CD39 expression on activated vs. unactivated cells. **D**. MFI of CD73 expression on activated vs. unactivated cells. The data are presented as M ± SE, * — significant differences from inactive cells (Wilcoxon T test)

levels and LDL levels. Previously, evidence of a link between cholesterol metabolism and CD39 activity was identified. *In vitro*, it has been shown that a decrease in the level of cholesterol in the cell membrane leads to inhibition of the enzymatic and antiplatelet activity of CD39, and treatment of membranes with cholesterol completely restores the function of the enzyme [25]. However, unlike in LS patients, no correlation was detected between *ENTPD1* gene mRNA levels and blood lipid concentration in patients with MASH, likely due to severe inflammation and altered lipid status. Nevertheless, a decrease in CD39 expression in peripheral blood was observed relative to both the control group and LS patients. Probably, in MASH, progressive inflammation disrupts compensatory mechanisms that support the dephosphorylation of ATP to adenosine in liver damage. Changes in CD39 ectonucleotidase expression can also be detected for other immune cell populations; this work did not consider B cells that predominantly express CD39 among lymphocytes, as well as NK cells and CD8⁺ T cells.

CONCLUSIONS

Thus, in patients with MASH, the levels of inflammatory indicators (ESR, CRP, IL-6, and TNF-α) are higher than in healthy donors. Along with this, this study as shown that in MASH, the expression of CD39 ectonucleotidase is reduced both at the mRNA level in PBLs and at the enzyme level on the surface of monocytes and lymphocytes. The lack of the enzyme on the surface of immune cells can lead to the accumulation of extracellular ATP and thus support the process of inflammation. Based on the data obtained, it seems promising to further investigate CD39 ectonucleotidase as a diagnostic and prognostic marker in MAFLD.

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