UDC 618.19-006

DOI: 10.34662/2588-0489.2019.2.1.39-48

Plasma mRNA level of certain cytokines in the blood plasma of patients with breast cancer

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Purpose: to evaluate the level of gene expression (TNFα, IL6, IL8, IL18, VEGF121, VEGF165 and VEGF189) associated with tumor formation in the blood plasma of breast cancer patients and the control group of women.

Material and methods. To determine the level of messenger ribonucleic acid (mRNA) representation of the studied genes in blood plasma, the methods of reverse transcription and quantitative polymerase chain reaction were used (reagent kits manufactured by DNA-Technology, Russia). The amplification reaction was carried out in a DTprime 4 detection amplifier (DNA-Technology, Russia).

Results. In the blood plasma of breast cancer patients and women in the control group, the relative amounts of mRNA of the TNFα, IL8, IL18, and VEGF189 genes were significantly increased in patients with breast cancer compared with the healthy control ($p < 0.01$). When comparing the control group with a group of breast cancer patients at various stages, a high level of IL8 and IL18 mRNA expression is observed mainly in the group of breast cancer patients with stage 0 disease. The relative amount of mRNA of IL8 gene in the plasma of patients with breast cancer of all stages (0, I and II) significantly exceeded the relative amount of mRNA of this cytokine in the blood plasma of women in the healthy group ($p < 0.01$), while the differences between the control group and the group of patients with breast cancer III stages were identified at $p < 0.05$. Significant differences in the level of IL18 mRNA expression were detected between the control group and breast cancer groups of stages 0, I and II ($p < 0.01$), while differences in the level of IL18 mRNA expression between the control group and stage III breast cancer patients the diseases were not significant ($p < 0.05$). Significant differences in the level of TNFα mRNA expression were found between the control group and the group of patients with breast cancer stage I disease ($p < 0.01$) and between the control group and the groups of patients with breast cancer with stage II and III disease ($p < 0.05$). Statistically significant differences in the mRNA level of the VEGF189 gene are observed in groups of breast cancer at stages 0 and I compared with the control group, where the level of VEGF189 mRNA is significantly increased in these groups ($p < 0.01$). Significant differences are also observed between the control group and the groups of patients with breast cancer of the II and III stages of the disease ($p < 0.05$).

Conclusion The obtained results on the level of mRNA representation of the studied genes indicate that mRNA of cytokines (TNFα, IL8, IL18) in blood plasma have a statistically significant increased level of expression in breast cancer compared with the control group. To prove that the mRNA of these genes can be used as markers for early diagnosis of breast cancer, the sample of the group of breast cancer patients with stage 0 disease should be increased. To obtain statistically valid conclusions about the increased ex-

Keywords: arterial hypertension, beta-blocker, city, coast, metoprolol

pression of the VEGF189 mRNA gene in the blood plasma of breast cancer patients, additional studies with an increase in the sample of the group of breast cancer patients with stage 0 disease are also required.

For citation: Lolomadze E.A., Savilova A.M., Kometova B.B., Rodionov B.B., Rebrikov D.V. Plasma mRNA levels of certain cytokines in the blood plasma of patients with breast cancer. Ecological medicine. 2019; 2(1):39-48. DOI: 10.34662/2588-0489.2019.2.1.39-48.

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Translated from Ecologicheskaya medicina 2019. Vol. 2, N 1, pp. 42-52.

Translated by Kakvaeva S.B.

Breast cancer (BC) is one of the most common diseases in women. In 2018, 627,000 women died from BC in the world, which is approximately 15% of all cancer deaths among women [35]. Early detection and adequate therapy are an integral part in the fight against high mortality from this disease worldwide. Currently, many scientific papers have obtained data demonstrating an increase in the concentration of extracellular molecules of ribonucleic acids (eRNA) due to the death of a significant number of cells in the tumor process [30, 32, 33].

Messenger ribonucleic acid (mRNA) circulate in the blood plasma (extracellular fraction, eRNA) of both healthy and sick people, thus providing an opportunity to detect differences in gene expression levels in different groups of patients [1-3].

Currently, more studies have been conducted regarding the level of eRNA in serum and tumor tissues than in plasma. At the same time, a qualitative and quantitative analysis of eRNA in blood plasma can now be considered the most promising diagnostic method, since it is relatively inexpensive and minimally invasive, and the correct choice of the studied genes will allow the use of these genes as markers of breast cancer in the near future at different stages of this disease. mRNA circulate in the blood plasma (extracellular fraction, eRNA) of both healthy and sick people, thus providing an opportunity to detect differences in gene expression levels in different groups of patients [1-3].

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Pathological angiogenesis is a distinctive feature of oncological and various ischemic and inflammatory diseases; its development establishes the vasculature for tumor growth and hematogenous metastasis [6]. Consequently, the genes involved in this process are actively studied to develop diagnostic systems and therapeutic approaches to the treatment of cancer and other diseases [37]. Cytokines and growth factors produced by many types of cells present in the microenvironment of solid tumors form a complex dynamic network in which they induce other cytokines, alter the expression of cytokines receptors soluble and bound to the cell surface, and stimulate the proliferation of endothelial cells [22]. Vascular endothelial growth factor (VEGF) is a key molecular factor in angiogenesis.

A number of researchers point to an increase in serum VEGF concentration in cancer patients, noting that the measurement of circulating VEGF is a marker of angiogenesis and / or metastasis [4, 7, 14, 31].

The Hyodo group reported the stability of plasma VEGF levels, in contrast to its instability in serum in cancer of the gastrointestinal tract [16]. A study by Jacqueline Adams [18] also demonstrates that plasma VEGF shows better discrimination than serum VEGF in patients with BC. In addition,

the plasma concentrations of VEGF in patients with localized, metastatic and benign diseases were significantly increased compared with normal control. However, the highest VEGF concentrations were observed in patients with remission compared with normal control, which emphasizes the connection with the applied treatment, which is observed in some patients. The results of the study [17] confirm that in primary endocrine-positive BC, serum VEGF levels are significantly higher in patients with BC than in healthy ones, and show a positive relationship with tumor VEGF and p53 overexpression. In addition, the study of Slawomir Lawicki and his colleagues [21] confirms the significance of determining the level of VEGF in the plasma of BC patients, since the VEGF levels in the general group of patients with BC were statistically significantly higher compared to the control group.

Alternative splicing of VEGF mRNA is known to produce 3 major isoforms of 121, 165 and 189 amino acids in humans [36]. In [39], it is indicated that the VEGF121 isoform exhibits more pronounced angiogenic and oncogenic effects than the isoforms 165 and 189.

Purpose: to assess the level of gene expression (TNFα, IL6, IL8, IL18, VEGF121, VEGF165 and VEGF 189) associated with tumor formation in the blood plasma of BC patients and the control group of women.

Material and methods

The group diagnosed with BC was 126 women with histologically confirmed BC. The age of patients ranged from 31 to 82 years, the average age was 56 years.

The degree of spread of BC: 5 patients - early stage 0 BC (T1N0M0); 58 patients - stage I (T1N0M0), 52 patients - stage II: IIA in 34 (T1N1M0 - 10 and T2N0M0 - 24), stage IIB (T2N1M0) - in 18; 13 patients - stage III: IIIA - in 9 (T2N2M0 - 5 and T3N1M0 - 2, T1N2M0 - 2) and IIIC stage (T2N3M0) in 4 patients. There were 2 patients with cancer of both mammary glands at different stages**.** The histological variant of the tumor structure is predominantly represented by a non-specific ductal histological variant (66 cases), the lobular variant was diagnosed in 17 patients, apocrine cancer in 8, medial cancer in 8, mucinous cancer in 5, and intraductal cancer - in 3 patients, special forms of cancer - in 21 patients.

The control group consisted of 60 clinically healthy women (age varied from 26 to 67 years, the average age was 48 years).

To determine the level of mRNA representation of the studied genes in blood plasma, the methods of reverse transcription and quantitative polymerase chain reaction were used (reagent kits manufactured by DNA-Technology, Russia). The amplification reaction was carried out in a DTprime 4 detection amplifier (DNA-Technology, Russia).

Blood samples (4 ml) were taken in single BD Vacutainer tubes with EDTA (Becton, Dickinson and Company, USA), then centrifuged at 3000 rpm for 20 min to obtain plasma. After centrifugation, the upper fraction was transferred to new tubes. RNA was isolated using the Proba-NK kit (DNA-Technology, Russia) according to the attached instructions, then the isolated RNA in a volume of 16.5 μl was immediately used for reverse transcription. The reaction was carried out at a temperature of 40 °C for 30 minutes, followed by inactivation of reverse transcriptase at 95 °C for 5 minutes. The resulting complementary DNA solution was either immediately used for quantitative Polymerase chain reaction or stored at 20 °C. Amplification with recording the results in the “real time” mode was carried out in a volume of 35 μl according to the following program: 50 cycles 94 °C - 10 s, 64 °C - 20 s, 72 °C - 10 s. The measurement of the fluorescence level was carried out on each cycle at a temperature of 64 °C. The studied genes included: tumor necrosis factor-α (TNFα), interleukins IL6, IL8, IL18, vascular endothelial growth factors VEGF121, VEGF165, VEGF189.

Normalization genes included: β2-microglobulin (B2m), β-glucuronidase (GUSB). Normalization values for the mRNA of each gene were calculated using the ΔΔCt method [28].

The level of gene expression was expressed in arbitrary units relative to normal-

ization genes (B2m, GUSB), which have a relatively stable level of expression.

To assess the statistical significance of the differences, the method of nonparametric statistics (Mann-Whitney test) was used. The difference in the groups was considered significant at $p < 0.01$ and $p < 0.05$.

Research results

Figure 1 shows the profile of the studied genes (TNFα, IL6, IL8, IL18, VEGF121, VEGF165, VEGF189) and 2 normalization genes (B2m, GUSB) in the blood plasma of BC patients and the control group. Based on the results obtained, all of the figures in Figure 1 genes can be divided into two groups: genes with a low level of representation (IL6, VEGF121, VEGF165, VEGF189) and genes with a relatively high level of representation (B2m, GUSB, TNFα, IL8, IL18).

Figure 1 shows data on the mRNA expression level of the studied genes (TNFα, IL6, IL8, IL18, VEGF121, VEGF165, VEGF189) in the blood plasma of BC patients and women in the control group. Our results do not contradict the data of [1], where the relative amounts of mRNA of the IL8 and IL18 genes were significantly increased in patients with BC compared with the healthy control ($p < 0.05$).

Significant differences were also found in the mRNA of the TNFα and VEGF189 genes between the group of BC patients in comparison with the healthy control. However, for three of the seven studied mRNA genes (IL6, VEGF121, VEGF165), there were no significant differences between the BC patients and the control.

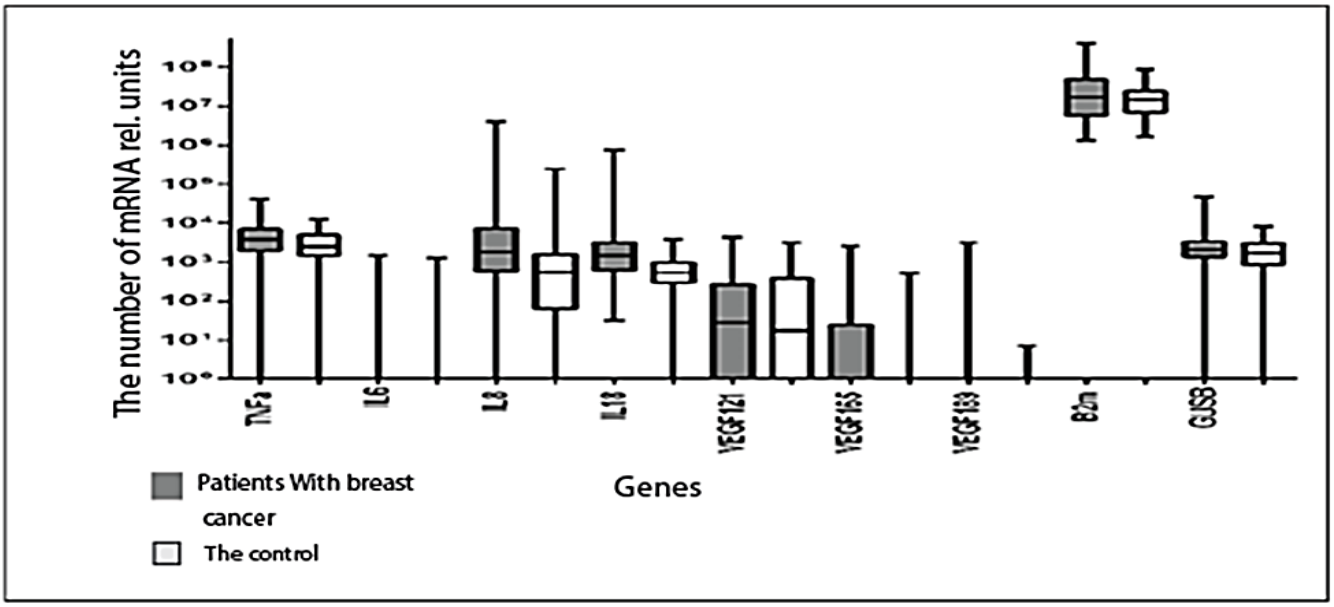


Figure 1. Profile of mRNA gene representation in the blood plasma of breast cancer patients and women in the control group: along the abscissa axis, the mRNA of the studied and normalizing genes; along the ordinate axis, the number of mRNA in relative units.

In addition, the level of IL6 gene mRNA representation was rather low both in the group of BC patients and in the control group.

Figure 2 shows the experimental groups along the abscissa axis (IL8-K - control group, IL8-0 - stage 0, IL8-I - stage I, IL8-II - stage II, IL8-III - stage III), along the axis ordinate - the amount of mRNA in relative units. As can be seen from Figure 3(A) and 3(B), a high level of mRNA expression of the IL8 and IL18 genes is observed mainly in the group of breast cancer patients with stage 0 disease. Moreover, high values are also found in groups of patients with stages I, II and III of the disease compared with the control group. The relative amount of mRNA of IL8 gene in the plasma of patients with breast cancer of all stages (0, I and II) significantly exceeded the relative amount of mRNA of this cytokine in the blood plasma of women in the healthy group ($p < 0.01$), with differences between the group controls and a group of patients with breast cancer with stage III were detected at $p < 0.05$.

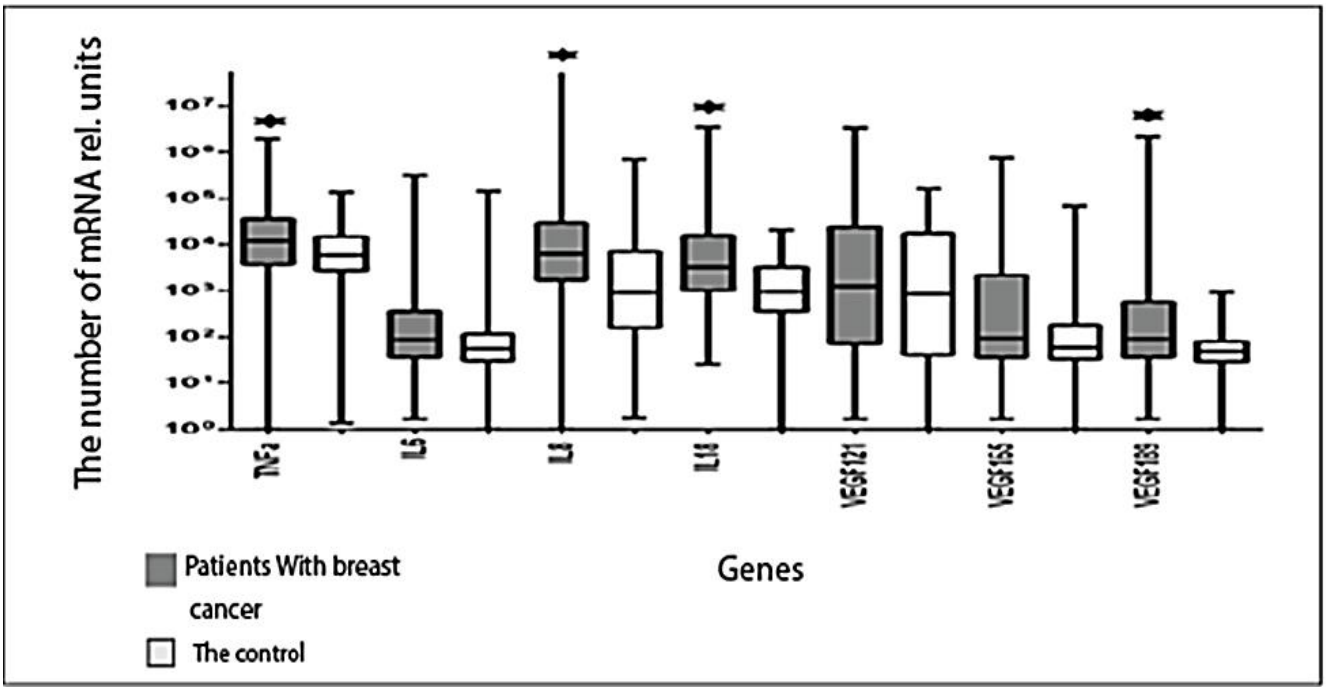
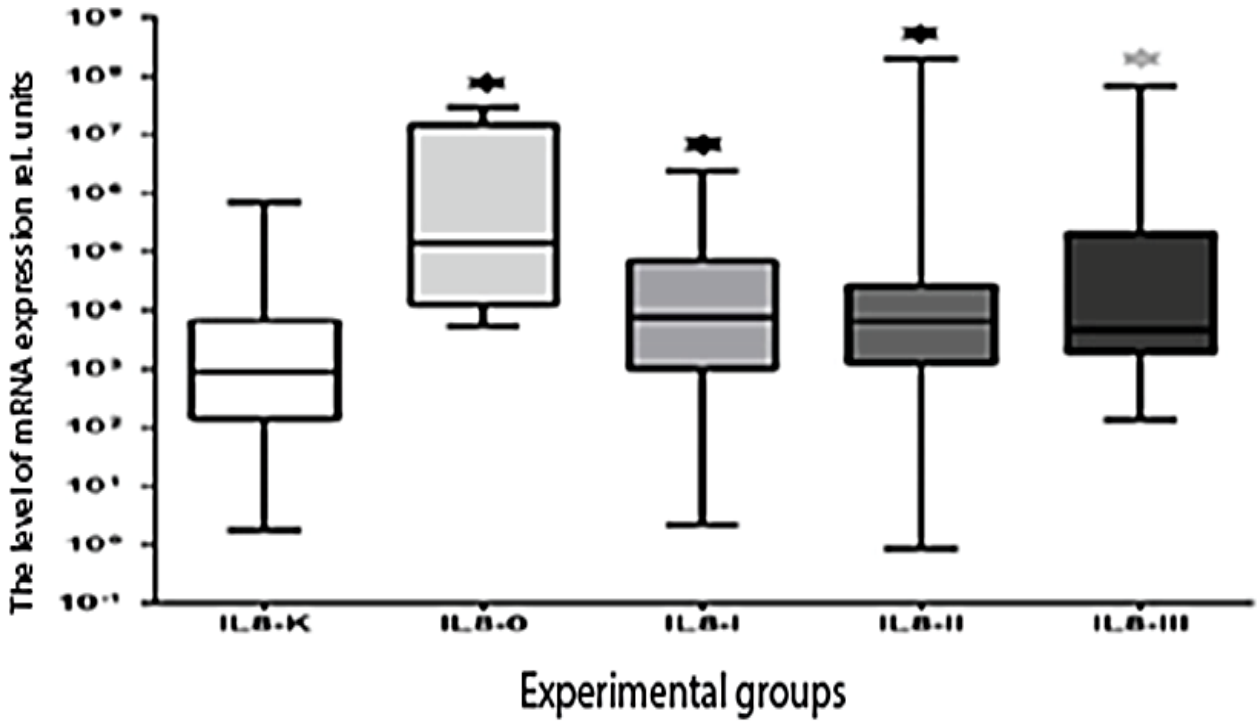
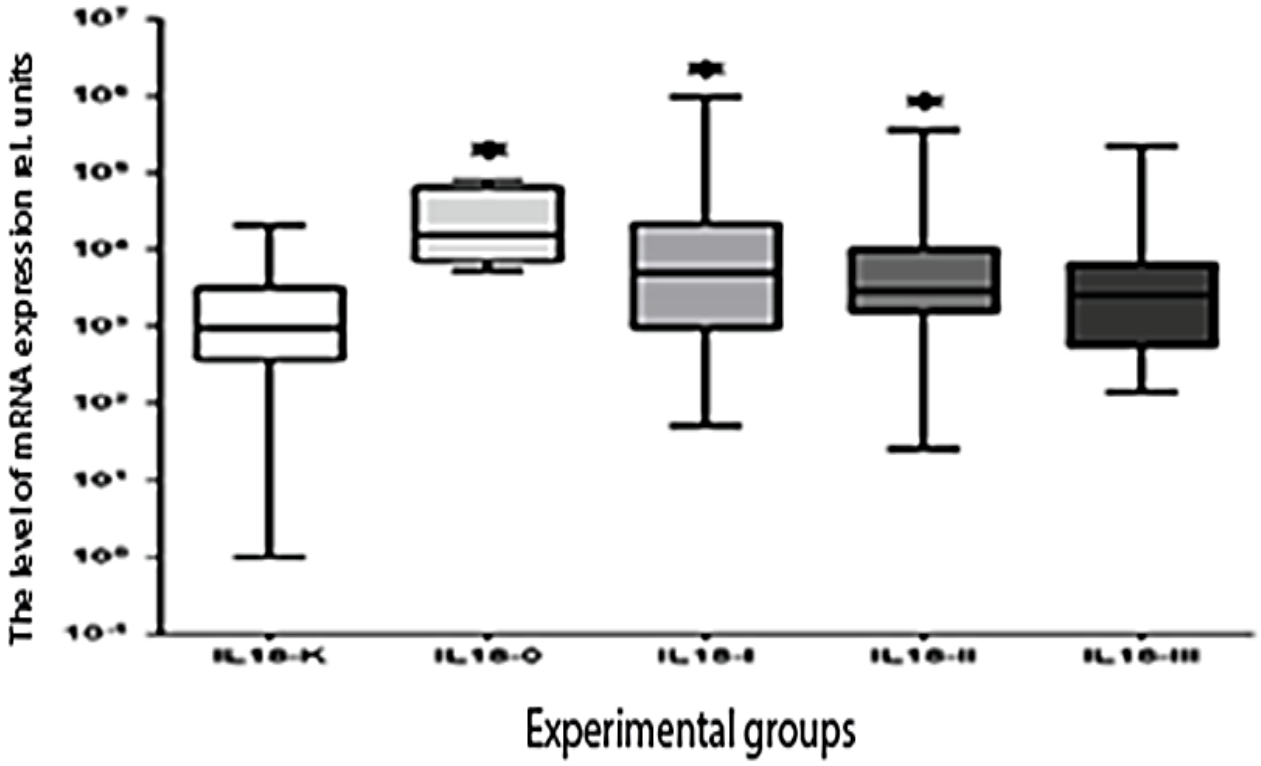


Figure 2. The level of mRNA expression of the studied genes relative to normalization genes (B2m, GUSB) in the blood plasma of breast cancer patients and women in the control group ($p < 0.01$).

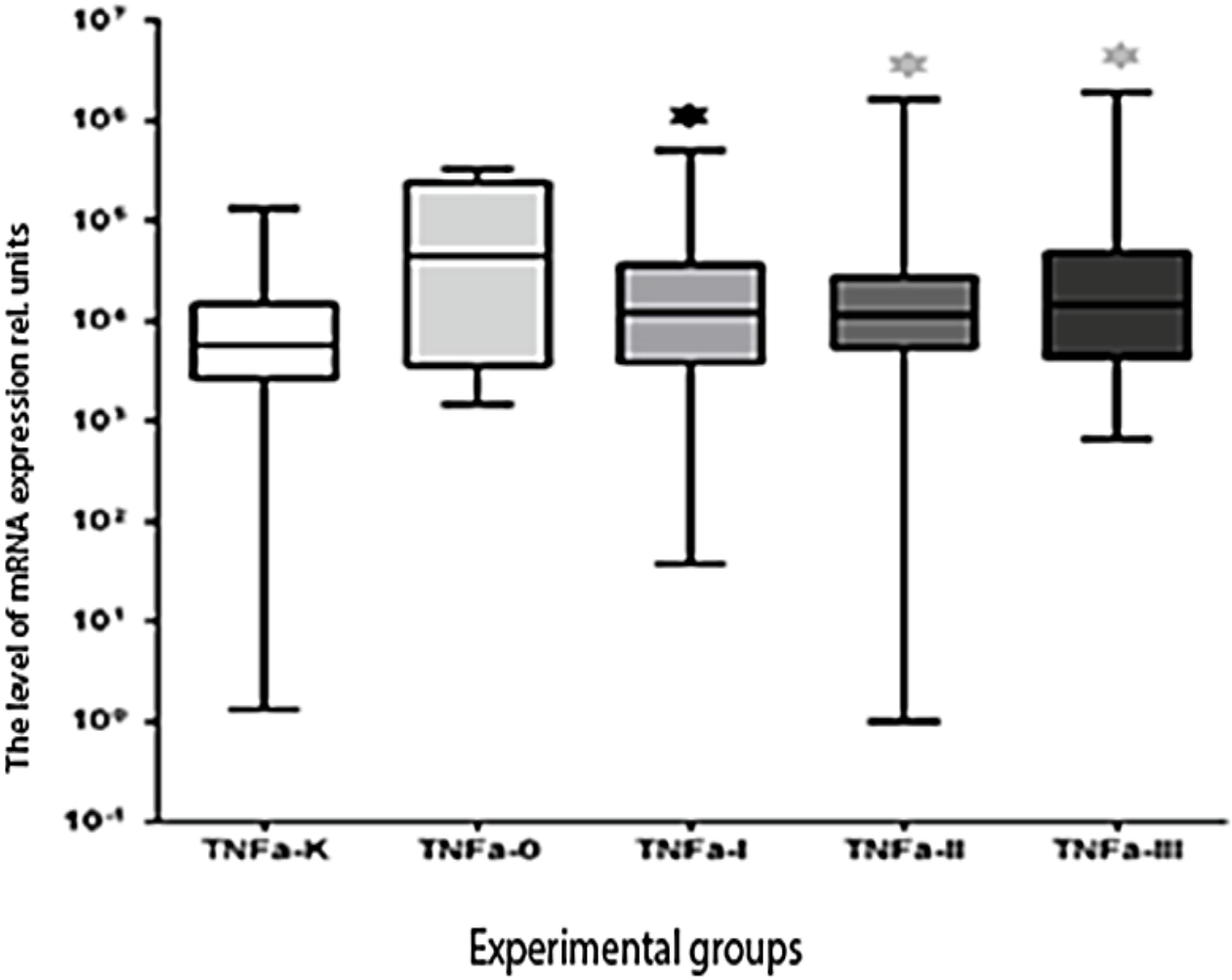
As for the IL18 gene mRNA, significant differences in the expression level were revealed between the control group and the groups of patients with breast cancer 0, I and II stages ($p < 0.01$), with differences in the level of IL18 gene mRNA expression between the control group and the group of breast cancer patients with stage III disease were not significant ($p < 0.05$). It should be noted that our data are consistent with previous studies, where the mRNA levels of the IL8, IL18 genes were significantly increased in patients with breast cancer compared with the control [1-3, 23].



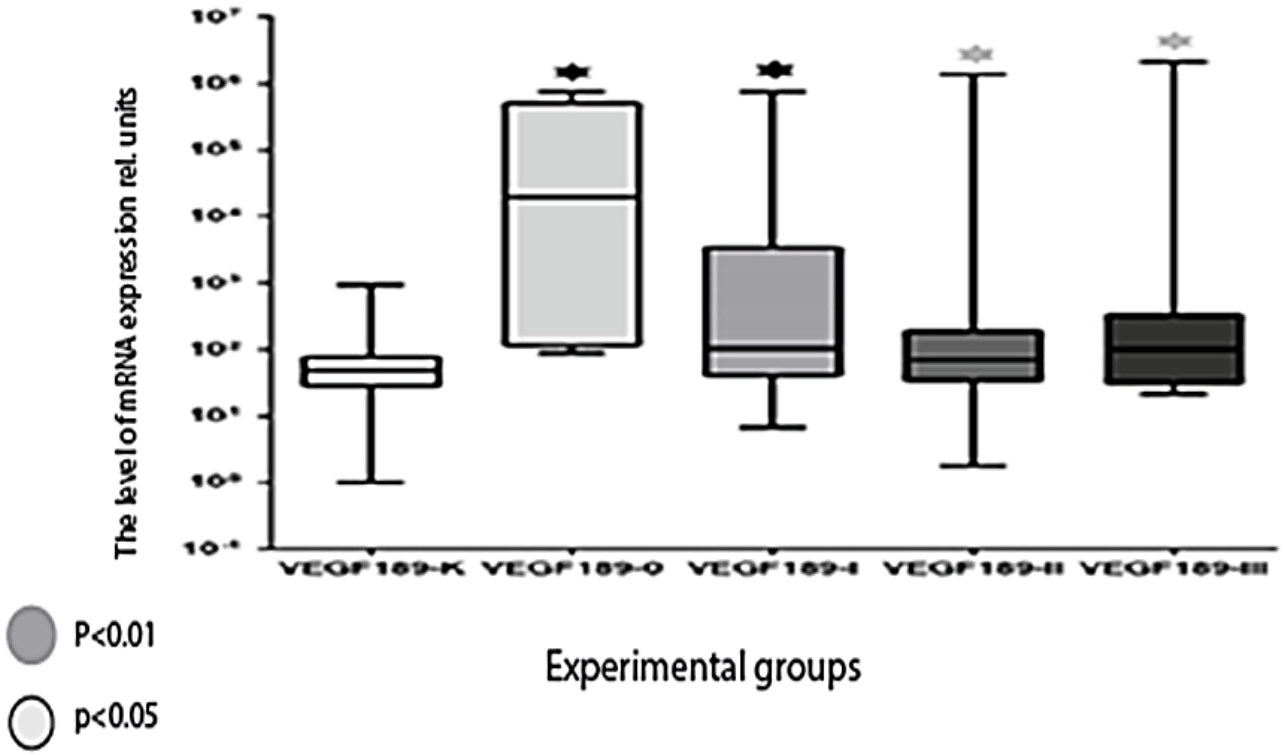
A)


The level of mRNA expression rel. units
IL10-K
IL10-0
IL10-I
IL10-II
IL10-III
Experimental groups

B)


The level of mRNA expression rel. units
TNFa-K
TNFa-0
TNFa-I
TNFa-II
TNFa-III
Experimental groups

C)



D)

Figure 3. The level of mRNA expression of the IL8 (A) and IL18 (B), TNFα (C), VEGF189 (D) genes in the blood plasma of breast cancer patients by stages and in women of the control group.

However, when comparing the expression level of IL8 in the control groups and BC at different stages, we did not find differences between the groups of BC patients at different stages (I, II, and III), as, for example, it was described in the study by Enas A. Hamed [11]. At the same time, a group of researchers [11] also claims that plasma IL8 levels were significantly increased in benign and malignant breast formations compared with healthy ones, as well as in patients with malignant formations compared with blood plasma in patients with benign breast formations. In addition, plasma IL8 levels were significantly increased in patients with stage III and IV BC compared with blood plasma in patients with stages I, II, and IV. IL18 can be considered as an important marker that may indicate metastasis [26]. These articles by E.A.L. Soheir [8] indicate that the protein product of the IL18 gene can also be a marker of metastatic BC.

However, our results demonstrate that IL18 gene mRNA is expressed at a higher level at the initial stage of the disease development, indicating its potential as an early diagnostic marker and confirming the previous study [26]. It is worth noting that insufficient sampling in the group of BC patients with stage 0 disease does not allow us to unambiguously decide whether mRNAs of the IL8 and IL18 genes can be a marker for early diagnosis of BC, and therefore it seems reasonable to study a greater number of samples from BC patients at stage 0 to obtain reliable statistical conclusions.

TNFα is involved in the stimulation of tumor invasion and metastasis [34]. The expression and localization of mRNA and TNFα protein was studied in various benign and malignant tissues of the mammary gland. A group of researchers [13] observed a high level of expression of various genes encoding TNFα and IL8, which are regulated by NF-κB and are associated with the progression of BC after knockdown of the suppressor gene of the cylindromatosis tumor suppressor (CYLD). In addition, there is evidence that TNFα increases estrogen-induced proliferation of estrogen-

dependent breast tumor cells through a complex containing nuclear factor NF-κB [29]. It is also known that TNFα is an important anti-cancer target [12, 25]. Some authors also reported that in patients with BC in plasma the levels of TNFα were higher than in the control group [10, 35]. Our data also demonstrate an increased mRNA level of this cytokine in the group of BC patients (Figure 2) compared with the control group, and when comparing the control group with the group of BC patients at various stages (Figure 3B), we found Significant differences were found between the control group and the group of patients with BC of the first stage of the disease ($p < 0.01$) and between the control group and the groups of patients with BC with the II and III stages of the disease ($p < 0.05$).

VEGF189 is generally considered to be present in low amounts. A group of researchers [15] found that VEGF189, like VEGF165, contributes to the progression of BC and angiogenesis. In addition, the results of other researchers show [20] that in case of human BC, overexpression of cyclooxygenase-2 is associated with overexpression of VEGF189 and, therefore, with tumor angiogenesis. However, data on the level of VEGF189 in the blood plasma of BC patients and healthy people could not be found in the literature at the moment. Moreover, despite the low plasma mRNA representation of this gene, our data demonstrate an increase in the VEGF189 gene mRNA level in the group of BC patients compared with the control group at a statistically significant level ($p < 0.01$) (see Figure 2). Statistically significant differences are also shown in Figure 3D, where the VEGF189 gene mRNA level in the group of BC patients at stages 0 and I is shown, and it can be seen that it is significantly increased in these groups compared with the control group ($p < 0.01$). Significant differences were also observed between the control group and the groups of BC patients with stage II and III disease ($p < 0.05$).

Conclusion

The obtained results on the level of mRNA representation of the studied genes indicate that mRNA of cytokines (TNFα, IL8, IL18) in the blood plasma have a statistically significant increased level of expression in BC compared with the control group. To prove that the mRNA of these genes can be used as markers for early diagnosis of BC, the sample of the group of BC patients with stage 0 disease should be increased. To obtain statistically reliable conclusions about the increased expression of VEGF189 mRNA gene in the blood plasma of BC patients, additional studies with an increase in the sample of the group of BC patients with stage 0 disease are also required.

Funding and Conflict of Interest Information

Financing. The study was carried out as part of the State Assignment No. AAAA-A18-118053190012-9 "Development of a test system for the early diagnosis of breast cancer and ovarian cancer based on the analysis of freely circulating (extracellular) peripheral RNA blood."

The authors declare no apparent and potential conflicts of interest related to the publication of this article.

Search and analytical work on the preparation of the manuscript was carried out at the personal expense of members of the team of authors.

The participation of the authors: the concept and design of the research - Lolomadze E.A.; collection and processing of materials - Savilova A.M.; analysis of the data obtained, writing and editing the text - Kometova V.V., Rodionov V.V., Rebrikov D.V.

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