

# The Relationship Between Cytokine Production, CSF2RA, and IL1R2 Expression in Mammary Adenocarcinoma, Tumor Histopathological Parameters, and Lymph Node Metastasis

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## Abstract

**Objective:** The aim of this study was to evaluate the relationship between cytokine production, GM-CSF receptor (CSF2RA), and IL-1 receptor (IL1R2) expression in mammary adenocarcinoma and their association with its histopathological parameters and lymph node metastasis. **Methods:** We analyzed tumor biopsy samples (cultured *in vitro*) from 50 women (aged 43-75) with invasive ductal mammary adenocarcinomas. Enzyme-linked immunosorbent assay method the concentrations of interleukin 2, interleukin 6, interleukin 8, interleukin 10, interleukin 17, interleukin 18, interleukin 1 $\beta$ , interleukin 1Ra, tumor necrosis factor  $\alpha$ , interferon  $\gamma$ , granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, and vascular endothelial growth factor A were determined in culture supernatants. The expression of CSF2RA and IL1R2 in tumor biopsy was evaluated by immunohistochemical method. **Results:** We showed that the “cytokine profile” of a tumor (the ability of tumor cells and its microenvironment to produce different cytokines) is very individual. It has been shown that the features of the cytokine profile of the mammary adenocarcinoma are important for the formation and realization of the metastatic potential of the mammary adenocarcinoma. We found correlations between some histopathological parameters of mammary adenocarcinoma and coefficients  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$ , which are the ratios of concentrations of granulocyte macrophage colony-stimulating factor and interleukin -1 $\beta$  to expression of CSF2RA and IL1R2, respectively.  $K_{GM-CSF/CSF2RA}$  positively correlated with highly differentiated cells, and  $K_{IL-1\beta/IL1R2}$  positively correlated with the number of mitoses, poorly differentiated cells, and a number of lymph nodes with metastases.  $K_{GM-CSF/CSF2RA}$  positively correlated with the concentrations of interleukin 6, interleukin 8, interleukin 1Ra, and granulocyte colony-stimulating factor.  $K_{IL-1\beta/IL1R2}$  positively correlated with concentrations of interleukin 1 $\beta$  and interferon  $\gamma$  and negative correlated with the concentrations of vascular endothelial growth factor A and tumor necrosis factor  $\alpha$ . It is shown that  $K_{IL-1\beta/IL1R2}$  can be considered as a prognostic indicator predicting the probability of mammary adenocarcinoma metastasis to regional lymph nodes. **Conclusions:** The ratios of granulocyte macrophage colony-stimulating factor and interleukin 1 $\beta$  cytokines, produced in tumor, to the expression of CSF2RA and IL1R2 depend on levels of interleukin 6, interleukin 8, tumor necrosis factor  $\alpha$ , interferon  $\gamma$ , granulocyte colony-stimulating factor, and vascular endothelial growth factor A and are important factors affecting the progression and metastasis of the breast cancer.

## Keywords

mammary adenocarcinoma, CSF2RA, IL1R2, receptors, cytokines, metastasizes

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## Abbreviations

AUC, area under the curve; DMEM, Dulbecco modified Eagle medium; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; IFN- $\gamma$ , interferon  $\gamma$ ; MAC, mammary adenocarcinoma; NST, no special type; ROC, receiver operating characteristic; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; VEGF-A, vascular endothelial growth factor A.

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## Introduction

To date, multiple research articles have been published showing that cytokines should be considered an important factor affecting pathological mechanisms of malignant tumor formation, progression, and metastases.<sup>1,2</sup> During tumor growth, cytokines are produced not only in immune organs and in the immunocompetent cells that infiltrate the tumor but also directly by the tumor cells together with the cells of the connective tissue that form the microenvironment of the tumor.<sup>3,4</sup> Moreover, the cytokine network that develops in the tumor becomes one of the most important regulators of cell–cell interaction within the tumor.<sup>5,6</sup>

Several studies have shown that depending on the concentration and balance of the levels of cytokines and their antagonists, they can enhance or inhibit breast cancer growth and metastases.<sup>7,8</sup> For example, changes in the relative concentration of some cytokines (interleukin [IL]-1, IL-6, IL-8, IL-11, IL-18, and IL-19) and growth factors (granulocyte colony-stimulating factor [G-CSF], granulocyte macrophage colony-stimulating factor [GM-CSF], and vascular endothelial growth factor A [VEGF-A]) in blood can directly or indirectly stimulate breast cancer growth or progression.<sup>9,10</sup> However, the mechanisms of this effect have not been studied enough, since most of the data on the role of cytokines in tumor progression are based, as a rule, only on the comparison of data on the concentration of certain cytokines detected in the blood with tumor growth rates.

Recently, in our laboratory, it was shown that mammary adenocarcinoma (MAC) biopsy samples, obtained from tumors metastasizing to regional lymph nodes, produced in tissue culture *in vitro* significantly more GM-CSF and IL-1 $\beta$  cytokines than biotates of nonmetastatic breast tumors.<sup>11</sup> We have also shown that the high-level production of IL-1 $\beta$  and IL-18 by MAC, overexpression of VEGF-R2 in tumor (at relatively low VEGF-A production), and the high level of interferon  $\gamma$  (IFN- $\gamma$ ) production are attributed factors contributing to the formation of a population of low-grade cells in the tumor.<sup>12</sup> In this regard, from a theoretical and practical point of view, it was interesting to study the possibility of using the same model to evaluate the production of some cytokines produced by the tumor as well as some receptors for cytokines expressed in tumor cells, in relation to histopathological parameters of the tumor, as prognostic biological markers indicating the probability of MAC metastasis to regional lymph nodes.

The aim of our research to study the relationship between cytokine production, CSF2RA, and IL1R2 expression in MAC

and their association with it histopathological parameters and lymph node metastasis.

## Materials and Methods

### Patients

The object of the study was cultured tumor biopsy samples from 50 women aged 43 to 75 with invasive ductal breast cancer (according to modern classification revised World Health Organization in 2012—“Invasive Breast Carcinoma of No Special Type” [NST]), treated at the Novosibirsk Regional Oncological Center, which was classified according to histological type as MAC grade II to III. The exclusion criteria from the study were signs of hematogenous metastasis to distant organs and the presence of concomitant hormonal, chronic, inflammatory, and infectious diseases. The study and all study protocols have been approved by the Ethics Committee of the Institute of Molecular Biology and Biophysics (Approval No. 2016-3) and Subdivision of Federal Research Center of Fundamental and Translational Medicine (Novosibirsk, Russia). All procedures performed in this study were conducted in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Each patient was informed about the study conducted and its objectives and methods. Written informed consent for participation in the study and for the tumor biopsy procedure was signed by each patient and verified by a physician.

### Method of Measurement of Cytokine Production

Tumor biopsy samples (8 mm<sup>3</sup>), obtained using core biopsy,<sup>11</sup> were washed with culture medium Dulbecco modified Eagle medium (DMEM)–F12 3 times to wash off the remaining blood cells on their surface and then were placed into a glass vial with 1 mL of the DMEM–F12 growth medium and incubated for 72 hours in order to accumulate in the supernatant sufficient (for an accurate assessment of each cytokine) concentration of all the cytokines studied by us. Before collection of the supernatant, the tumor biopsy samples were retrieved from the vial and placed in 10% neutral formalin. After culturing the biopsy samples in the supernatant, there was a small number (no more than 50–100 cells for the whole supernatant) of cellular elements (single tumor, lymphoid, and monocytic cells) that were removed from supernatant by precipitating with centrifugation at 900  $\times$  g for 15 minutes. By enzyme-linked immunosorbent assays (the assay kits produced by AO Vector-Best (Novosibirsk Region, Russia)), the concentrations of IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1 $\beta$ , IL-1Ra, tumor necrosis factor  $\alpha$

(TNF- $\alpha$ ), IFN- $\gamma$ , G-CSF, GM-CSF, and VEGF-A were determined in the culture supernatant.

### Immunohistochemical Analysis

The MAC biopsy samples fixed in neutral formalin were dehydrated and embedded in paraffin. Dewaxing and rehydration of the MAC paraffin sections were carried out according to the standard procedure through xylene and alcohol. The expression of CSF2RA and IL1R2 receptors was evaluated in 2 tumor samples of each patient obtained simultaneously. One sample was examined immediately, and the second after 72 hours of cultivation. The expression of CSF2RA and IL1R2 in MAC sections was detected using antibodies of appropriate specificity (anti-CSF2RA, MBS711361; MyBioSource [San Diego, California, USA]; anti-IL1R2, LS-B377, LifeSpan [Seattle, Washington, USA]), and visualization system VECTASTAIN Elite ABC Kit (Vector Laboratories [Burlingame, California, USA], PK-7200) according to the manufacturer's recommendations. The slices were additionally stained with Azure II-eosin, dehydrated, and embedded in balsam.

### Computer Morphometric Analysis

Histological samples stained for CSF2RA and IL1R2 were photographed (at magnification 400 $\times$ ) using an image analysis system based on a Micros MC 300A microscope (Austria) and a digital CMOS camera based on the Aptina MT9J003 sensor (China). Computer morphometric quantitative evaluation of expression of receptors CSF2RA and IL1R2 was performed in the ImageJ 1.50a software (National Institute of Health, Bethesda, Maryland). Immunohistochemical indicators of CSF2RA and IL1R2 expression were as squares of colored zones that were specific for CSF2RA and IL1R2 expression (% , percentage of colored area from a total area of tested image, based on 8 digital photographs).  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$  coefficients were then determined, representing the ratio of the concentration of cytokines GM-CSF and IL-1 $\beta$  to expression value of CSF2RA and IL1R2 receptors, respectively. The coefficients are expressed in arbitrary units.

### Histopathological Analysis

After surgical resection, a pathologist carried out a histopathological examination of the MAC tissue sections stained with hematoxylin and eosin according to the standard procedure. Evaluation of the differentiation degree of the tumor cells and their classification as highly differentiated, moderately differentiated, and poorly differentiated cells were based on the cellular polymorphism degree, nuclear-cytoplasmic ratio, presence of mitoses (including pathological ones), capacity for tissue structure formation (gland development), and preservation of cell functions.<sup>13,14</sup> Highly differentiated tumor cells had a shape similar to that of normal cells and showed the predominance of cytoplasm over nucleus and capacity for gland formation. Poorly differentiated tumor cells had an irregular shape and featured pronounced polymorphism, predominance of the

nucleus over cytoplasm, inability to form tissue structures, diffuse growth, and numerous mitoses (including pathological ones). Moderately differentiated tumor cells were somewhere between and in terms of these features. We calculated the mean number of mitoses and pathological mitoses after analyzing 10 visual fields as well as the percentage of poorly differentiated, moderately differentiated, and highly differentiated tumor cells.

### Statistical Analysis

Histograms was created in the Microsoft Excel, Statistica version 7, and IBM SPSS Statistics version 22.0. Determination of mean values, medians, 25th to 75th percentiles, and Spearman rank correlation coefficient ( $r$ ) was performed by means of software package Statistica version 7. The cluster analysis was performed using the Statistica version 7. Receiver operating characteristic (ROC) curve analysis was performed by means of software package IBM SPSS Statistics version 22.0.

## Results

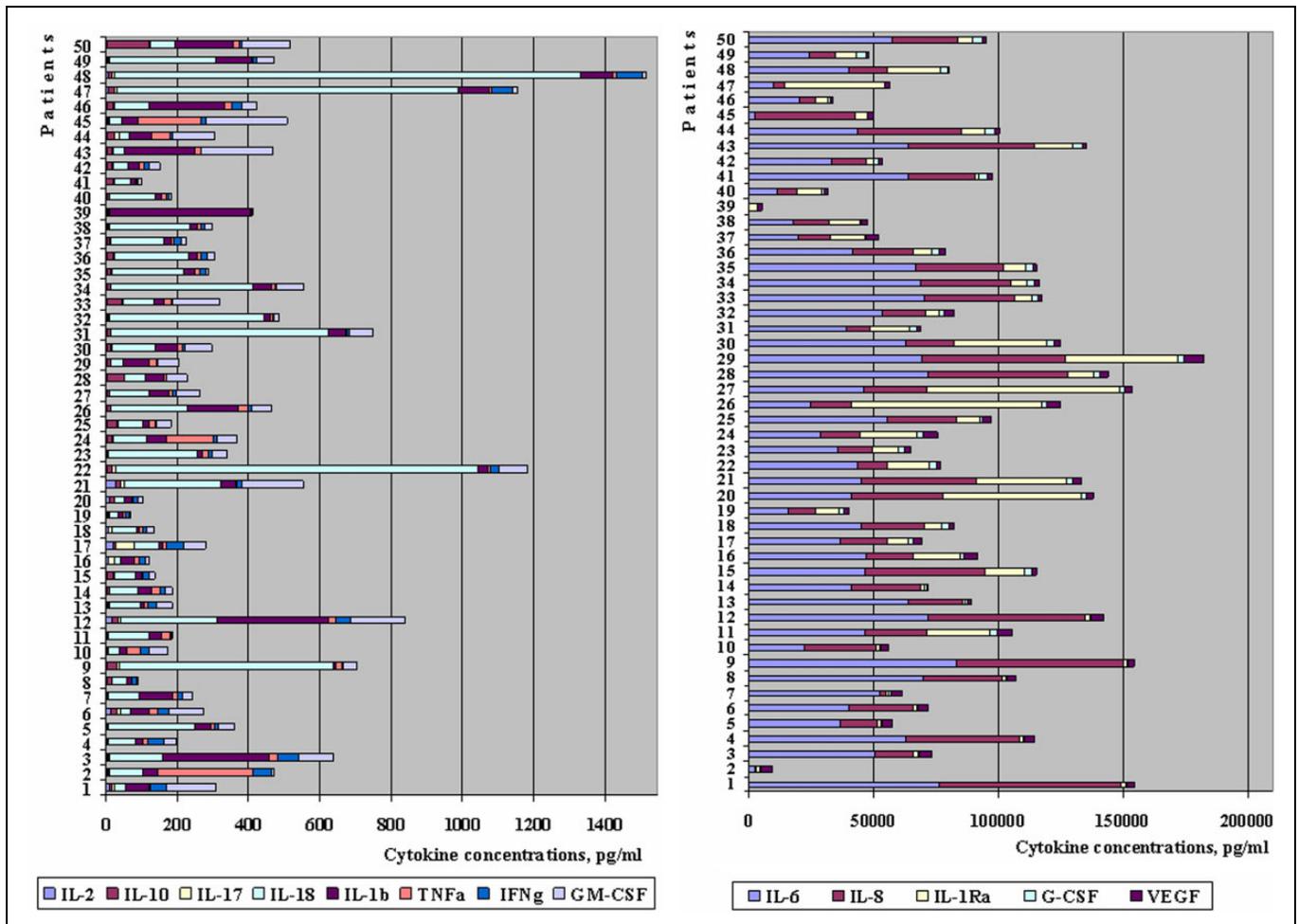
### Cytokine Profiles of the Supernatants of MAC Samples

At the beginning of the study, we examined individual cytokine profiles in MAC samples of different patients. The concentrations of IL-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1 $\beta$ , IL-1Ra, TNF- $\alpha$ , IFN- $\gamma$ , G-CSF, GM-CSF, and VEGF-A were determined in the culture supernatants. The cytokine profiles of MAC samples obtained from different patients differed considerably (Figure 1). This difference manifested itself in considerable variation in concentrations of some cytokines in MAC culture supernatants and, accordingly, different proportions of cytokines in the resulting individual profile of each patient. The generalized estimation of the levels of production of cytokines (medians, 25th and 75th percentiles) is shown in Figure 2. The greatest variability in values was revealed for the following cytokines produced by MAC samples (in ascending order): GM-CSF, IL-1 $\beta$ , IL-8, IL-6, and IL-18.

### Evaluation of the Variability in Cytokine Profiles of MAC Biopsy Samples by Cluster Analysis

To assess the variability in cytokine profiles of MAC biopsy samples, we used hierarchical cluster analysis, which allows us to classify the objects of study by a large number of parameters.

In our study, we used as such parameters the production of 13 cytokines produced by MAC biopsy samples *in vitro* (concentration of cytokines in the supernatant, pg/mL). Using Ward's cluster method, it was found that with Linkage distance within 13 to 18, all cytokine profiles can be divided into 3 clusters and, accordingly, into 3 groups of profiles of cytokine production in MAC (Figure 3). It is important to note that cluster 2 received a large number of cytokine profiles of those MAC samples that were obtained from patients with metastases to the lymph nodes. Cluster 2 contained indicators of cytokine production in 66.7% of patients with metastases in regional lymph nodes, relative to the total number of patients who had metastases.



**Figure 1.** Cytokine profiles in the supernatants of mammary adenocarcinoma (MAC) biopsy samples that were obtained from 50 patients (cumulative histograms): interleukin (IL)-2, IL-10, IL-17, IL-18, IL-1 $\beta$ , tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ), granulocyte macrophage colony-stimulating factor (GM-CSF), IL-6, IL-8, IL-1Ra, granulocyte colony-stimulating factor (G-CSF), and vascular endothelial growth factor A (VEGF-A). Tumor samples (8 mm<sup>3</sup>), obtained using core biopsy, were placed into a sterile vial with 1 mL of the Dulbecco modified Eagle medium (DMEM)–F12 growth medium for 72 hours. By means of a solid-phase immunosorbent assay, the cytokine concentrations were determined in the MAC supernatants (pg/mL).

Cluster 1 and cluster 3 contained indicators of cytokine production in equal numbers—about 16.7% of patients with metastases to regional lymph nodes, relative to the total number of patients who had metastases.

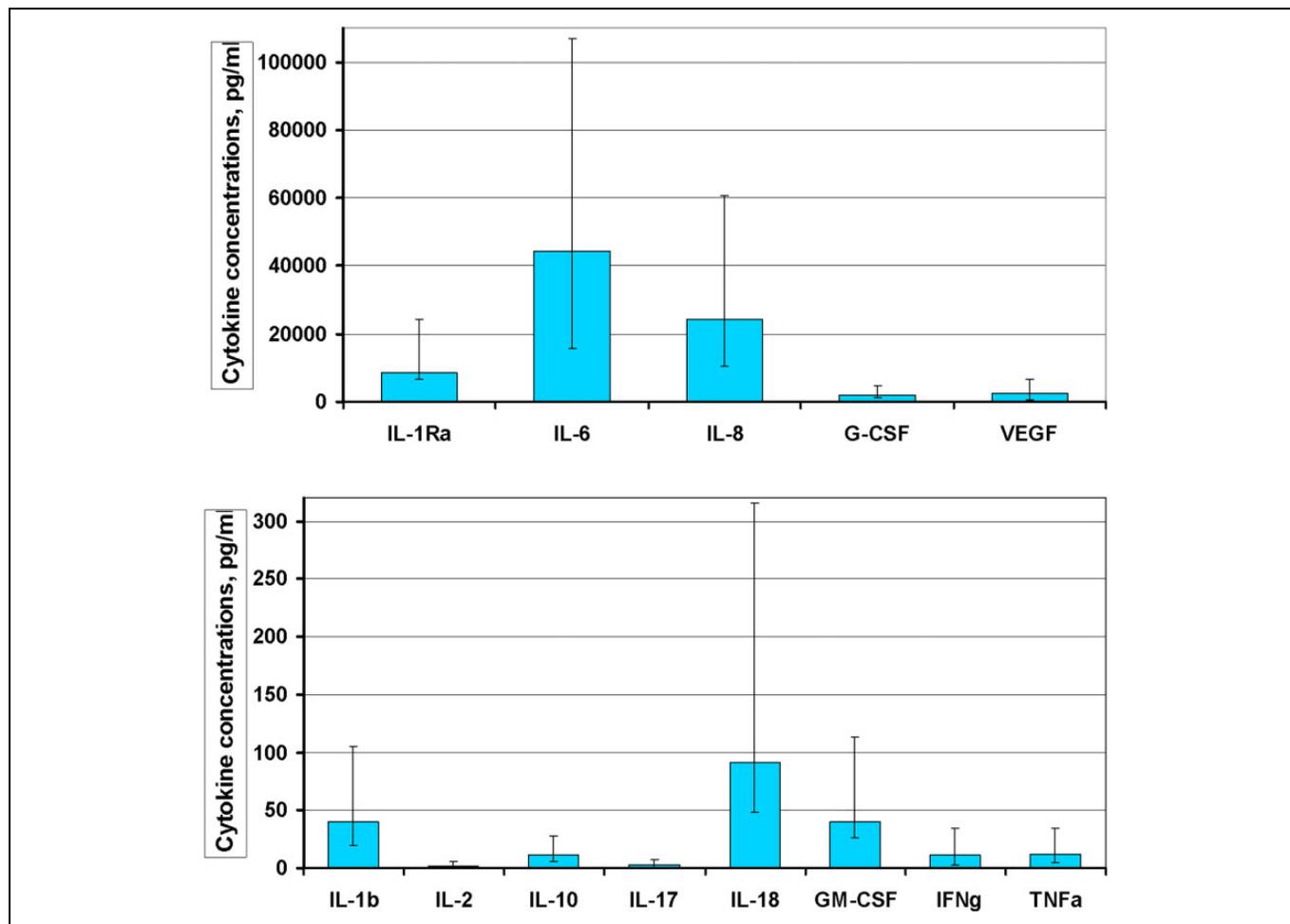
The data obtained indicated that the features of the cytokine profile of the MAC are important for the formation and realization of the metastatic potential of the MAC.

#### *Peculiarities of Expression of CSF2RA and IL1R2 in the Samples of MAC and the Ratios of Concentrations of GM-CSF and IL-1 $\beta$ to Expression of CSF2RA and IL1R2, Respectively*

In the study of tumor samples after cultivation for 72 hours, it was noted that the morphological structure did not differ from the samples of the same tumor before cultivation. They were characterized by a typical structure with the formation of nests, clusters, and trabeculae. No signs of redistribution of cell

elements of lymphocytic and macrophage type due to migration, which could be caused by the process of cultivation in the samples, were identified. The average value of CSF2RA expression (colored area to total area of tested image, %; mean  $\pm$  standard error) in MAC samples without cultivation was  $-2.11 \pm 0.32$ , and in samples after cultivation 72 hours— $1.74 \pm 0.35$  ( $P > .05$ ). The average value of IL 1R2 expression in MAC samples without cultivation was  $4.15 \pm 0.59$ , and in samples after cultivation 72 hours was  $3.79 \pm 0.65$  ( $P > .05$ ). For further study and calculation,  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$  coefficients used the data on the expression of IL1R2 and CSF2RA, expressed in samples of MAC after cultivation for 72 hours, because from these samples were obtained supernatants in which were determined the concentrations of various cytokines.

Figure 4A and B presents data on the distribution of data by the following indicators: percentage of CSF2RA-colored area (to total area of tested image) as an indicator of CSF2RA



**Figure 2.** The value of concentrations (pg/mL) of interleukin (IL)-2, IL-6, IL-8, IL-10, IL-17, IL-18, IL-1 $\beta$ , IL-1Ra, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\gamma$  (IFN- $\gamma$ ), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and vascular endothelial growth factor A (VEGF-A) in the supernatants of mammary adenocarcinoma (MAC) biopsy samples that were obtained from 50 patients. Tumor samples (8 mm<sup>3</sup>), obtained using core biopsy, were placed into a sterile vial with 1 mL of the Dulbecco modified Eagle medium (DMEM)–F12 growth medium for 72 hours. By means of a solid-phase immunosorbent assay, the cytokine concentrations were determined in the MAC supernatants. The data were presented as median  $\pm$  25th to 75th percentiles.

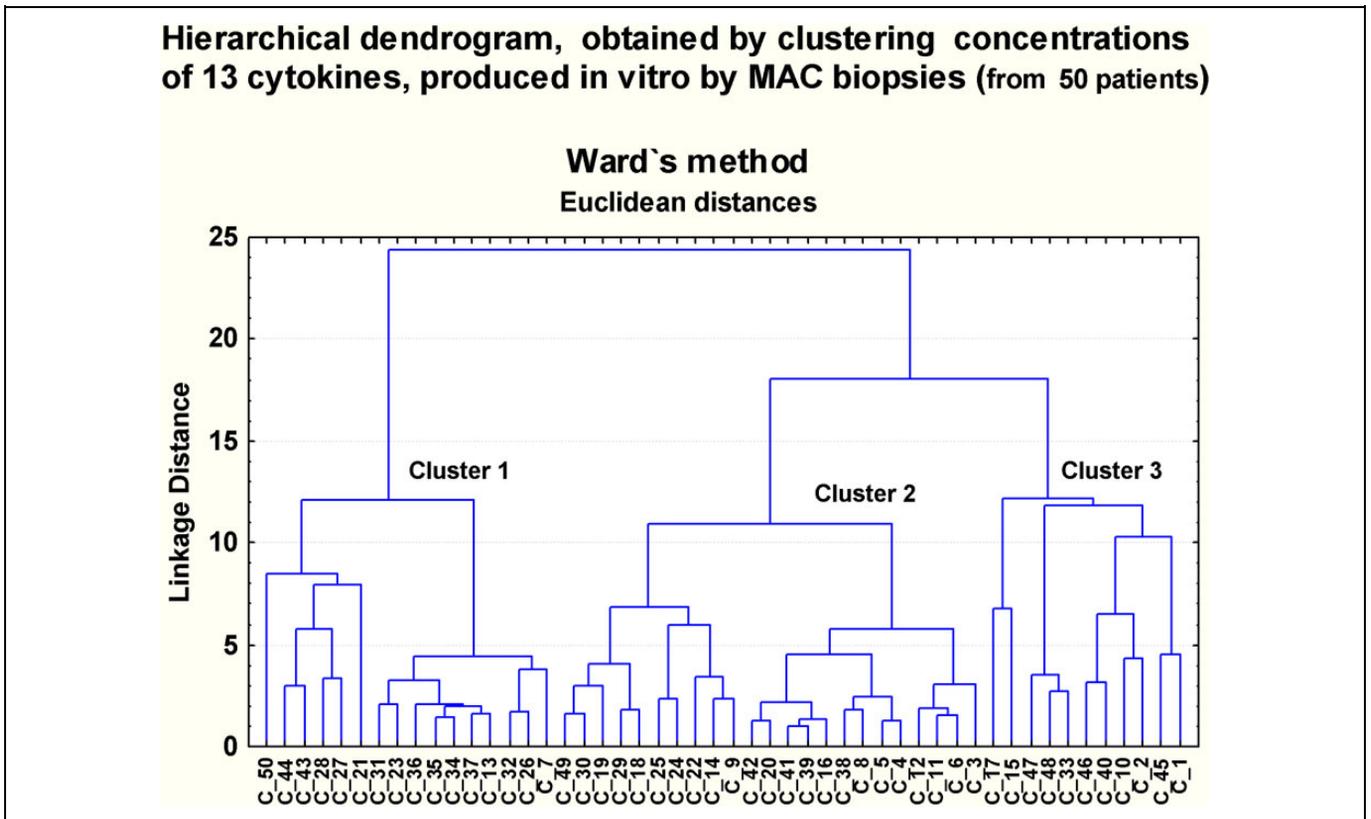
expression and  $K_{GM-CSF/CSF2RA}$  coefficient representing the ratio of the concentration of GM-CSF to expression value of CSF2RA. The least variability was determined for the expression of CSF2RA.

The greatest variability was found for  $K_{GM-CSF/CSF2RA}$  coefficient. The histopathological and immunohistochemical analyses of MAC biopsy samples show that the receptor CSF2RA in the samples was expressed predominantly in monocytes/macrophages of infiltrating the tumor. Outlier and extreme CSF2RA expression values were due to the presence of macrophage infiltrates expressing CSF2RA in some samples of MAC (Figure 4A). Figure 4C and D presents data on the distribution of data on the following indicators: percentage of IL1R2-colored area (to total area of tested image) as expression  $K_{IL-1\beta/IL1R2}$  coefficient representing the ratio of the concentration of IL-1 $\beta$  to expression value of IL1R2. The smallest variability was determined for the expression of IL1R2 (IL1R2 percentage of colored area). The greatest variability was found

for  $K_{IL-1\beta/IL1R2}$  coefficient (Figure 4D). The histopathological and immunohistochemical analyses of MAC biopsy samples show that the receptor IL1R2 was expressed predominantly in monocytes/macrophages. The outlier and extreme values of IL1R2 expression were due to the presence of tumor cells expressing IL1R2 in some of the MAC samples (Figure 4C).

#### *Correlation Between CSF2RA and IL1R2 Expression, $K_{GM-CSF/CSF2RA}$ , and $K_{IL-1\beta/IL1R2}$ Coefficients With Histopathological Indicators and Metastasis Rate in Regional Lymph Nodes*

As for CSF2RA and IL1R2 expression correlations, we found a correlation only between CSF2RA expression and the percentage of highly differentiated cells in MAC samples (a positive correlation) and between IL1R2 expression and the proportion of highly differentiated cells in MAC samples (a negative correlation; Table 1) and number of regional lymph nodes with



**Figure 3.** Hierarchical dendrogram obtained by clustering mammary adenocarcinoma (MAC) biopsies of 50 patients by concentrations of 13 cytokines produced by MAC biopsies. Ward's cluster method. Cluster 1 and cluster 3 contained indicators of cytokine production in equal numbers—about 16.7% of patients with metastases to regional lymph nodes, relative to the total number of patients who had metastases. Cluster 2 contained indicators of cytokine production in 66.7% of patients with metastases in regional lymph nodes, relative to the total number of patients who had metastases.

metastases (a positive correlation). More often, a correlation of coefficients  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$  with histopathological parameters (Table 1) was identified.

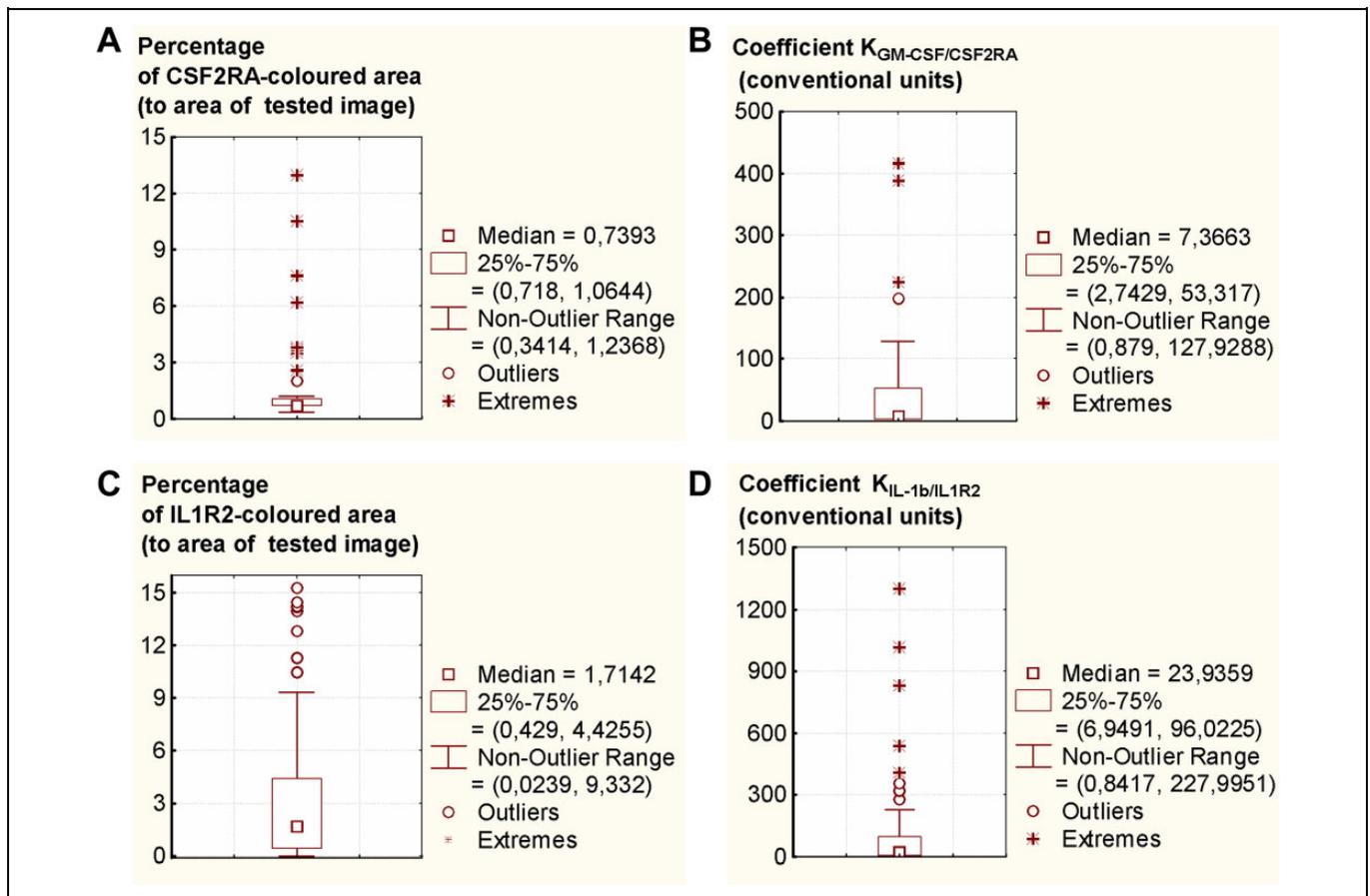
Table 1 indicates that parameter  $K_{GM-CSF/CSF2RA}$  negatively correlated with the percentage of moderately differentiated cells ( $r = -.44$ ) and positively correlated with the percentage of highly differentiated cells ( $r = .59$ ). Parameter  $K_{IL-1\beta/IL1R2}$  showed a negative correlation with the percentage of highly differentiated tumor cells in MAC ( $r = -.51$ ) and number of lymph nodes with metastases and a positive correlation with the histopathological parameters of MAC that determine the grade of tumor malignancy: the numbers of mitoses ( $r = .50$ ) and pathological mitoses ( $r = .48$ ) and the percentage of poorly differentiated cells ( $r = .57$ ).

**Search and Evaluation of the Quality of ROC Models, Including  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$ , to Explore the Possible Influence of the Interaction of CSF2RA and IL1R2 With the Corresponding Cytokines on the Process of Metastasis MAC in Regional Lymph Nodes**

In order to evaluate the quality of models, including  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$ , to explore the possible influence of the

interaction of CSF2RA and IL1R2 with the corresponding cytokines on the process of metastasis MAC in regional lymph nodes, we used the ROC analysis. The ROC curves allowed us to evaluate the quality of the models by dividing all patients into the groups (or classes). In our model, such groups include patients with metastases in regional lymph nodes and patients without metastases in regional lymph nodes. Despite the lack of correlations between number of lymph nodes with metastases and GM-CSF concentrations and  $K_{GM-CSF/CSF2RA}$  (Table 1), using indicators GM-CSF and  $K_{GM-CSF/CSF2RA}$  of all patients, we obtained an average quality model (AUC = 0.625) for the division of patients into 2 groups—with metastases in the lymph nodes and without metastases (Figure 5A).

The AUC values for GM-CSF (pg/mL), CSF2RA (colored area, %), and  $K_{GM-CSF/CSF2RA}$  (conventional units) were, respectively, 0.633, 0.573, and 0.625. The quality of the ROC model for the division of the patient group into a subgroup of patients without lymph node metastases and, accordingly, into a subgroup of patients with metastases, based on the use of indicators GM-CSF and  $K_{GM-CSF/CSF2RA}$ , practically did not change when taking into account the indicators of only those patients ( $n = 31$ ), in which the relative content of highly



**Figure 4.** Histograms showing a distribution of data by the following indicators in mammary adenocarcinoma (MAC) biopsy samples. A, Percentage of CSF2RA-colored area (to total area of tested image) as an indicator of CSF2RA expression; (B)  $K_{GM-CSF/CSF2RA}$  coefficient representing the ratio of the concentration of granulocyte macrophage colony-stimulating factor (GM-CSF; pg/mL) to expression value of CSF2RA (percentage of CSF2RA specific colored area); (C) percentage of IL1R2-colored area (to total area of tested image) as an indicator of IL1R2 expression; (D)  $K_{IL-1\beta/IL1R2}$  coefficient representing the ratio of the concentration of interleukin (IL)-1 $\beta$  (pg/mL) to expression value of IL1R2 (percentage of IL1R2 specifically colored area). The data were presented as median  $\pm$  25th to 75th percentiles.

differentiated tumor cells in MAC biopsy specimens was in the range of 10% to 30% (Figure 5B).

As shown in Figure 5C, the quality of the model showing the dependence of MAC metastasis process in lymph nodes on  $K_{IL-1\beta/IL1R2}$  was very good (AUC = 0.770). The AUC values for IL-1 $\beta$  (pg/mL), IL1R2 (colored area, %), and  $K_{IL-1\beta/IL1R2}$  (conventional units) were, respectively, 0.616, 0.226, and 0.770. It was found that at  $K_{IL-1\beta/IL1R2} \leq 100$  the percentage of patients with metastases was equal to 24.0%, at  $K_{IL-1\beta/IL1R2} \leq 25\%$  to 36.7% and at  $K_{IL-1\beta/IL1R2} \leq 5\%$  to 50.0%. The quality of the ROC model for the division of the patient group into a subgroup of patients without lymph node metastases and, accordingly, into a subgroup of patients with metastases, based on the use of indicators IL-1 $\beta$  and  $K_{IL-1\beta/IL1R2}$ , markedly increased when taking into account the indicators of only those patients (n = 31), in which the relative content of highly differentiated tumor cells in MAC biopsy specimens was in the range of 10% to 30%. The value of AUC has increased by almost 30% (AUC = 0.910), and its quality has acquired the status of “excellent model” (Figure 5D).

### Correlation Between CSF2RA and IL1R2 Expression, $K_{GM-CSF/CSF2RA}$ , and $K_{IL-1\beta/IL1R2}$ Coefficients and Cytokine Concentrations in the MAC Culture Supernatants

To determine the specific features of cytokine regulation of CSF2RA and GM-CSF and IL1R2- and IL-1 $\beta$ -dependent pathways of tumor cell activation, an analysis of correlation was performed on not only CSF2RA and IL1R2 expression in the samples but also on the secretion levels of a set of cytokines in the culture supernatants of the corresponding MAC samples (Table 2). When we studied the association between receptor CSF2RA expression and cytokine concentration in the tumor culture supernatant, we found only 1 correlation between CSF2RA expression and the expression of IL-1Ra (Table 2). Parameter  $K_{GM-CSF/CSF2RA}$  showed a positive correlation with the concentrations of IL-6, IL-8, IL-1Ra, and G-CSF in MAC culture supernatants. When we studied the association between receptor IL1R2 expression in MAC and cytokine concentrations in the tumor culture supernatant, we identified only 1 negative correlation between IL1R2 expression and IFN- $\gamma$  concentration (Table 2).

**Table 1.** Coefficients (*r*) of Correlation Between of CSF2RA and IL1R2 Expression,  $K_{GM-CSF/CSF2RA}$ , and  $K_{IL-1\beta/IL1R2}$  With Histopathological Parameters of MAC.

Histopathological Parameters of MAC	CSF2RA (Colored Area, %) <i>r</i> ( <i>P</i> Value)	IL1R2 (Colored Area, %) <i>r</i> ( <i>P</i> Value)	$K_{GM-CSF/CSF2RA}$ (Conventional Units) <i>r</i> ( <i>P</i> Value)	$K_{IL-1\beta/IL1R2}$ (Conventional Units) <i>r</i> ( <i>P</i> Value)
Number of mitoses	.11 (.6576)	.35 (.1421)	-.18 (.4623)	.50 (.0327)
Number of pathological mitoses	.01 (.9552)	.23 (.3371)	-.03 (.9042)	.48 (.0445)
Percentage of poorly differentiated cells	-.16 (.5215)	.45 (.0522)	-.16 (.5168)	.57 (.0133)
Percentage of moderately differentiated cells	-.44 (.1944)	.18 (.4642)	-.44 (.0500)	.18 (.6680)
Percentage of highly differentiated cells	.53 (.0197)	-.50 (.0310)	.59 (.0069)	-.51 (.0352)
Number of regional lymph nodes with metastases	-.12 (.4140)	.38 (.0051)	-.16 (.2716)	-.36 (.0085)

Abbreviations: GM-CSF, granulocyte macrophage colony-stimulating factor; MAC, mammary adenocarcinoma.

In contrast, when we studied the association between coefficient  $K_{IL-1\beta/IL1R2}$  and cytokine concentrations in the tumor culture supernatant, 2 positive correlations—with IL-1 $\beta$  and IFN- $\gamma$  concentrations—and 2 negative correlations were found: with VEGF-A and TNF- $\alpha$  concentrations in MAC culture supernatants.

## Discussion

It is known that any cytokines can act directly only on those target cells that express their corresponding receptors.<sup>15,16</sup> This mechanism also works in the interaction of tumor cell receptors with complementary cytokines. According to the published data, the IL1R2 receptor for IL-1 $\beta$  and CSF2RA for GM-CSF can be expressed on neutrophils, monocytes, polarized M2 macrophages, some regulatory T cells, and B cells.<sup>17-22</sup> Moreover, according to the information in The Human Protein Atlas (an international scientific database), some tumor cells of breast cancer can express IL1R2 (<https://www.proteinatlas.org/ENSG00000115590-IL1R2/pathology/tissue/breast+cancer#img>). At the same time, it was found that IL-1 $\beta$  and GM-CSF are produced by different types of cells included in the microenvironment of breast tumors (monocytes, macrophages, B-lymphocytes, endothelial cells) as well as fibroblasts (GM-CSF) and directly by some breast cancer cells (IL-1 $\beta$ ).<sup>21-25</sup>

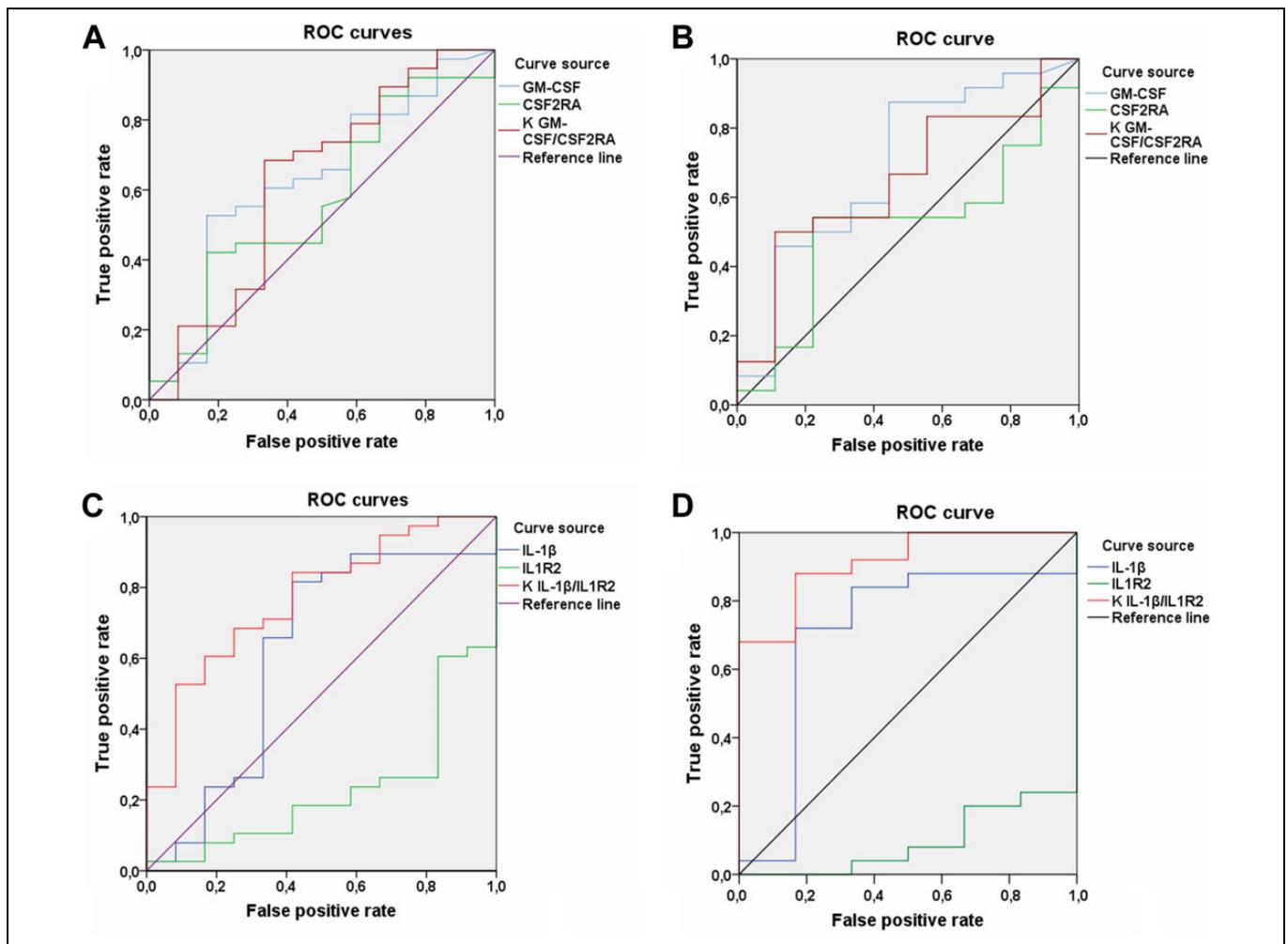
The data on the role of different cytokines in tumor progression and metastasis are inconsistent and ambiguous, making it difficult to accurately predict the effects of cytokine therapy in cancer, including breast cancer.<sup>25-29</sup> The ambiguousness of the effect of multiple cytokines on the tumor growth and metastasis in different patients can be caused by various reasons: by different proportions of tumor cells that express receptors interacting with the mediators that regulate tumor growth and by different production levels of the corresponding cytokines. Based on this, it follows that the search for biological predictive markers of metastasis is, first of all, the search for key biological factors that are directly or indirectly related to many

other factors that affect the process of metastasis. In our work, data were obtained showing that, as of such key factors may include variables such as  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$ , the value of that is correlated with several histopathological parameters and level of production of many cytokines that can affect cytophysiological characteristics of tumor cells (mitotic, migration, and hydrolytic activity), determining of metastatic potential.

In our study, the positive correlations found between  $K_{IL-1\beta/IL1R2}$  and the concentrations of IL-1 $\beta$  in the culture supernatant suggest that an increase in the production of IL-1 $\beta$  by the tumor is not associated with an increase in the expression of IL1R2. A negative correlation is found between  $K_{IL-1\beta/IL1R2}$  and the concentrations of VEGF-A. As known, VEGF-R2-mediated intracellular signals increase tumor cell survival during hypoxia because of secretion of VEGF via an autocrine pathway, which can contribute to the development of a more aggressive phenotype of tumor cells.<sup>7</sup> When VEGF-R2 is expressed by tumor cells, secreted VEGF can act as an autocrine factor that triggers proliferation and increases their survival by blocking apoptosis.<sup>7</sup>

It was shown that the increased production of GM-CSF in MAC (which is accompanied by a decreased in CSF2RA expression) is associated with a reduction in the proportion (%) of highly differentiated tumor cells. On the contrary, decreased production of IL-1 $\beta$  in MAC (which is accompanied by an increase in IL1R2 expression) is associated with an increase in the proportion of highly differentiated tumor cells and a decrease in the proportion of poorly differentiated tumor cells. The observed positive correlation between the number of mitoses in a tumor tissue sample and the  $K_{IL-1\beta/IL1R2}$  shows that proliferation of tumor cells depends on the IL-1 $\beta$ -IL1R2 ratio in MAC. The data on the correlations between CSF2RA and IL1R2 expression levels and  $K_{GM-CSF/CSF2RA}$  and  $K_{IL-1\beta/IL1R2}$  on the one hand and cytokine concentrations in the culture supernatant of MAC samples on the other hand can be interpreted as follows.

A positive correlation between CSF2RA and IL-1Ra may indicate a stimulating effect of antagonist IL-1Ra on CSF2RA



**Figure 5.** Receiver operating characteristic (ROC) curves, characterizing the quality of the ROC models for dividing all patients with mammary adenocarcinoma (MAC) into 2 groups—patients with metastases to regional lymph nodes and patients without metastases, by values granulocyte macrophage colony-stimulating factor (GM-CSF; pg/mL), CSF2RA (colored area, %), and  $K_{GM-CSF/CSF2RA}$  (conventional units; A) for all patients: for GM-CSF area under the curve (AUC) = 0.633, for CSF2RA AUC = 0.573, for  $K_{GM-CSF/CSF2RA}$  AUC = 0.625; (B) provided that highly differentiated tumor cells in MAC biopsy specimens were in the range of 10% to 30% for GM-CSF AUC = 0.692, for CSF2RA AUC = 0.514, for  $K_{GM-CSF/CSF2RA}$  AUC = 0.653. ROC curves, characterizing the quality of the ROC models by dividing the 2 groups of patients—with metastases to regional lymph nodes and without metastases, by values (B) IL-1 $\beta$  (pg/mL), IL1R2 (colored area, %), and  $K_{IL-1\beta/IL1R2}$  (conventional units); (C) for all patients: for IL-1 $\beta$  AUC = 0.616, for IL1R2 AUC = 0.226,  $K_{IL-1\beta/IL1R2}$  AUC = 0.770; (D) provided that highly differentiated tumor cells in MAC biopsy specimens were in the range of 10% to 30%: for IL-1 $\beta$  AUC = 0.707, for IL1R2 AUC = 0.093, for  $K_{IL-1\beta/IL1R2}$  AUC = 0.913.

expression in tumor cells (this effect is likely macrophage mediated). A positive correlation of cytokines IL-6 and IL-8 with coefficient  $K_{GM-CSF/CSF2RA}$  also confirms the influence of the said cytokines on this stimulating effect. The positive correlation between IL1R2 and IFN- $\gamma$  found in our study indicates a stimulating effect of IFN- $\gamma$  on IL1R2 expression in tumor cells. The modifying or regulatory effect of IFN- $\gamma$  on the IL1R2- and IL-1 $\beta$ -dependent pathway of cell activation in MAC is supported by the positive correlation between  $K_{IL-1\beta/IL1R2}$  and the IFN- $\gamma$  concentration in MAC culture supernatant. The correlation between  $K_{IL-1\beta/IL1R2}$  and the VEGF-A concentration probably means the importance of the IL1R2/IL-1 $\beta$  ratio for the regulation of production of angiogenic factor

VEGF-A, which is required for vascular growth in the tumor, which in turn determines tumor growth and metastasis.

From a practical and theoretical point of view, the results obtained by us with the help of ROC analysis are important, which showed that  $K_{IL-1\beta/IL1R2}$  index is a fairly significant complex biological marker that allows to identify a group of patients who have no metastases to regional lymph nodes. With the help of ROC analysis, we also showed that  $K_{IL-1\beta/IL1R2}$  is a biological marker, allowing additional consideration of the number of differentiated forms of tumor cells in MAC with even greater probability to identify a group of patients who have no metastases in the regional lymph nodes. This is very important from a practical point of view. Oncologists often

**Table 2.** Coefficients (*r*) of Correlation of CSF2RA Expression, IL1R2 Expression,  $K_{GM-CSF/CSF2RA}$ , and  $K_{IL-1\beta/IL1R2}$  With Cytokine Concentrations in MAC Culture Supernatants.

Cytokines, pg/mL	CSF2RA (Colored Area, %) <i>r</i> ( <i>P</i> Value)	IL1R2 (Colored Area, %) <i>r</i> ( <i>P</i> Value)	$K_{GM-CSF/CSF2RA}$ (Conventional Units) <i>r</i> ( <i>P</i> Value)	$K_{IL-1\beta/IL1R2}$ (Conventional Units) <i>r</i> ( <i>P</i> Value)
IL-2	-.01 (.9713)	.10 (.6649)	-.16 (0.5027)	.04 (.8571)
IL-6	-.09 (.7005)	-.17 (.4858)	.63 (0.0032)	.13 (.5912)
IL-8	-.32 (.1686)	-.11 (.6359)	.50 (0.0240)	.04 (.8700)
IL-10	-.12 (.6045)	-.05 (.8305)	.44 (0.0528)	.12 (.6179)
IL-17	.11 (.6352)	-.29 (.2178)	-.14 (0.5454)	.27 (.2584)
IL-18	.05 (.8256)	-.30 (.1952)	-.03 (0.8949)	.04 (.8700)
IL-1 $\beta$	.01 (.9749)	-.04 (.8650)	.11 (0.6541)	.59 (.0058)
IL-1Ra	-.51 (.0217)	-.08 (.7431)	.39 (0.0356)	.04 (.8601)
TNF- $\alpha$	-.16 (.4958)	-.19 (.4160)	.17 (0.4777)	-.39 (.0491)
IFN- $\gamma$	.13 (.5949)	-.56 (.0108)	-.15 (0.5239)	.41 (.0163)
G-CSF	-.29 (.2156)	.11 (.6586)	.83 (0.0001)	.02 (.9273)
GM-CSF	-.10 (.6771)	-.12 (.6267)	.26 (0.6267)	.27 (.2579)
VEGF-A	-.01 (.9749)	.35 (.1317)	-.17 (0.4738)	-.61 (.0044)

Abbreviations: G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN- $\gamma$ , interferon  $\gamma$ ; IL, interleukin; MAC, mammary adenocarcinoma; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; VEGF-A, vascular endothelial growth factor A.

recommend the removal of lymph nodes after the diagnosis of cancer. However, the feasibility of excision of all regional lymph nodes in any form of malignant tumors in the mammary glands has recently been questioned.

In conclusion, this study showed the feasibility of detecting  $K_{IL-1\beta/IL1R2}$  index as fairly significant complex biological marker that allows to identify a group of patients who have no metastases to regional lymph nodes. We found a number of correlations of various histopathological parameters (that characterize the grade of MAC) with  $K_{IL-1\beta/IL1R2}$ ,  $K_{GM-CSF/CSF2RA}$ , CSF2RA, and IL1R2 expression, and a number of correlations of  $K_{IL-1\beta/IL1R2}$ ,  $K_{GM-CSF/CSF2RA}$ , CSF2RA, and IL1R2 expression. The revealed correlations can allow to understand some mechanisms of immunological influence on the process of tumor growth and metastasis, to outline further ways of studying these mechanisms.

### Authors' Note

The study and all study protocols have been approved by the Ethics Committee of the Institute of Molecular Biology and Biophysics (Approval No. 2016-3), Subdivision of Federal Research Center of Fundamental and Translational Medicine (Novosibirsk, Russia). All procedures performed in this study were conducted in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Each patient was informed about the study conducted and its objectives and methods. Written informed consent for participation in the study and the tumor biopsy procedure was signed by each patient and verified by a physician.

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