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Regular paper

C-C motif chemokine ligand 5 and C-C chemokine receptor type 5: possible diagnostic application in breast cancer patients

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The chemokine CCL5 and its receptor CCR5 play important roles in cancer invasion and metastasis. Based on our knowledge, our results were the first that presented the diagnostic usefulness of CCL5 and CCR5 in breast cancer (BC) patients, based on receiver operating characteristic (ROC) curve analysis. We wished to examine further if CCL5 and CCR5 are appropriate to be applied as BC markers for early screening. Values of tested parameters in patients' plasma were determined by CMIA method (Chemiluminescent Microparticle Immunoassay, CA 15-3) as well as by ELISA method (Enzyme-Linked Immunosorbent Assay, CCL5 and CCR5). Levels of CCL5 in the plasma were markedly increased, while those of CCR5 were remarkably lower in BC patients when compared to the control groups. Moreover, higher levels of CCL5 in BC corresponded to advanced tumor stage, while the levels of CCR5 decreased with increasing the disease stage. CCL5 concentration was characterized by high sensitivity (SE) (68.04%) and high specificity (SP) (100.00%) in the BC patients. Results indicated that area under the curve (AUC) corresponding to CCL5 (0.8116) had a higher value than this corresponding to CA 15-3. The AUC value of CCL5 was significantly increased in the early phase of BC (stage I – 0.7089; stage II – 0.8313). The maximum range in the BC patients was observed for the combined analysis of the tested measurands with CA 15-3 (0.8335). In conclusion, our research indicates that examination of plasma CCL5 and CCR5 may be useful in BC diagnosis at the early stage of the disorder, especially when combined with CA 15-3.

Key words: CCL5; CCR5; Area Under Curve; Receiver Operating Characteristics

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Abbreviations: BC, Breast Cancer; CA 15-3, Cancer Antigen 15-3; ELISA, Enzyme-linked Immunosorbent Assay; CMIA, Chemiluminescent Microparticle Immunoassay; AUC, Area Under Curve; ROC, Receiver Operating Characteristics; SE, Sensitivity; SP, Specificity; PPV, Positive Predictive Value; NPV, Negative Predictive Value

INTRODUCTION

Cancer is a pathology with the highest mortality rate overall. Cancer of the famale breast represents 29% of all new cancer cases and 14% of all cancer deaths in women(Torre *et al.*, 2015; Siegel, Miller & Jemal, 2016). Nevertheless, it is critical to detect the disease at the earliest possible stage. Immunohistochemistry markers including estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) are used for breast cancer diagnosis and prognosis (Hussein *et al.,* 2020). The discovery of new valuable serum biomarkers for the early detection of breast cancer has thus been a high priority.

Furthermore, the good tumor marker should be highly sensitive, easy to measure, and reproducible. A large number of observations suggested that intrinsic properties of the tumor cells, as well as macro-environmental factors, with a direct interaction of the tumor cells with chemokines and their receptors, are involved in breast tumorigenesis and tumor progression (Liu *et al.,* 2020; Suman *et al.,* 2016). Chemokines and chemokine receptors play a crucial role in these processes since they participate in the trafficking of cells into and out of the tumor microenvironment (Ali & Lazennec, 2007). There are at least 50 ligands and 20 G-protein-coupled chemokine receptors, which are named according to their structure (Allen *et al.*, 2007; Zlotnik & Yoshie, 2012).

Chemokines and their receptors are small molecules that play a role in the control of the migration of the immune cells (Raman *et al.*, 2011). The chemokines were divided into four categories according to the spacing of the first two cysteines: C, CC, CXC and CX3C (Bachelerie *et al.*, 2014). CCL5, a classical pro-inflammatory chemokine, belongs to the CC chemokine family. This chemokine also called Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES) is released by activated T lymphocytes, monocytes/macrophages and epithelial cells (Haberstroh *et al.*, 2002). It was shown that pre-metastatic neutrophils conditioned medium contains CCL2 and LTB4. It was found that CCL5 mediated by leukotrienes signal promotes tumorigenicity (Wculek & Malanchi, 2015) and that leukotrienes LTB4, LTC4, LTD4 and LTE4 influence the growth of human mammary carcinoma cells MCF-7 (Przylipiak *et al.*, 1998). The biological activity of CCL5 is mediated through its binding to the G protein-coupled receptors CCR1, CCR3, and mainly CCR5 (Bennett *et al.*, 2011; Nomenclature, 2003; Walens *et al.,* 2019). The CCL5 chemokine and its receptor CCR5 are molecules with important immunological functions, which have further relevant roles in the context of several cancer types, including breast cancer (Soria & Ben-Baruch, 2008). The tumor microenvironment plays an important role in cancer maintenance and progression (Maman & Witz, 2018). CCL5 plays a key role in recruiting several cells to the tumor sites, which facilitates the disease progression in breast cancer (Allavena *et al.*, 2011; Aldinucci & Colom-

batti, 2014). Thus, the significance of these pair of molecules in breast cancer invasion and metastasis has been widely discussed.

We, therefore, hypothesized that plasma concentrations of CCL5, CCR5 may be helpful in both determining the clinical applicability of the analyzed parameters (separately and in conjunction with CA 15-3) in the diagnosis of breast cancer and in the differentiation of its subtypes. To test this hypothesis, we examined the concentration of the mentioned proteins in the plasma of 3 groups of individuals: 1. the breast cancer group; 2. the benign breast disease group; 3. the healthy volunteers and in different stages of BC. Additionally, we defined the criteria for the diagnosis based on investigated marker set. The present study is a continuation of our earlier studies on the importance of chemokines and their receptors in breast cancer patients.

MATERIALS AND METHODS

Patients. The study was performed on specimens from 100 patients suffering from BC. Clinical stage of BC was taken into account when dividing individuals into groups. All patients were diagnosed histopathologically as *ductal adenocarcinoma*. Samples were collected before any therapies were applied. Specimens from 35 women with diagnosed benign breast tumor (BBT) like *adenoma* or *fibroadenoma* and 35 healthy individuals were used as controls (Table 1). Control individuals as well as BBT patients were clinically examined to be BC-free and free of BC anamnesis. Individuals from the control group were also free of inflammations and heart diseases. Local Ethics Committee approved the study. All patients gave their informed consent.

Plasma collection and storage. The blood samples from the patients were obtained before the treatment. The blood samples were collected from each patient and stored at −85°C until assayed. Blood was collected into EDTA tubes (S-Monovette, SARSTEDT, Germany), centrifuged 1000 × *g* for 15 min at 2–8°C to obtain plasma samples.

Examination of CCL5 and CCR5. Chemokine ligand 5 and chemokine receptor 5 concentrations were estimated using ELISA (EIAab Science, Wuhan, China and/or R&D Systems, Abingdon, United Kingdom). CA 15-3 concentration was estimated by CMIA (Abbot Laboratories, Chicago, IL, USA). The intra- and inter-assay coefficients were checked by the procedure of kits to comply with the standards. No interference nor cross-reactivity with any human material was found. Each sample was measured twice for each patient.

Statistical analysis. STATISTICA 12.0 program was used to conduct statistic analysis (Dell Software, Round Rock, TX, USA). The concentration of all the proteins did not follow a normal distribution in the preliminary statistical analysis (using Shapiro-Wilk test), and thus nonparametric statistical analyses were employed. Consequently, the statistical analysis between the groups was performed by using the U-Mann Whitney test, the Kruskal–Wallis test and multivariate analysis by the post-hoc Dwass-Steel–Critchlow–Fligner test. Statistically significant differences were defined as comparisons resulting in $p<0.05$.

We estimated SE and SP and used Youden's index to find the *cut off* values.

RESULTS

Our experiments showed enhanced values of AUC of CCL5 (0.8116) and CCR5 (0.7691) when compared to that measured for CA 15-3 (0.7354). Moreover, the values apparently exceeded the value of 0.5 which is an edge indicator for diagnostic use. Employment of CCL5 together with CA 15-3 resulted in larger AUC (0.8292). Furthermore, the combined use of all three examined parameters reached the highest value (0.8335; $p<0.001$) (Fig. 1) (Table 2).

Higher BC tumor stage was associated with enhanced AUC values of CCL5 and CCR5 as well as CA 15-3. Stage I of BC showed the highest AUC value for CCL5

Table 1. Characteristics of breast cancer patients and the control group.

Study group			Number of patients
Examined groups	**Breast cancer patients**	*Adenocarcinoma ductale*	100
	Median age (range)		57 (21-84)
	Tumor stage	I	34
		II	41
		III and IV	25
	Menopausal status:		
	– premenopausal		22
	– postmenopausal		78
Control groups	**Benign breast tumor group**		35
		Adenoma	12
		Fibroadenoma	23
	Median age (range)		39 (21-63)
	Menopausal status:		
	– premenopausal		12
	– postmenopausal		23
	Healthy women		35
	Median age (range)		37 (21-58)
	Menopausal status:		
	– premenopausal		15
	– postmenopausal		20

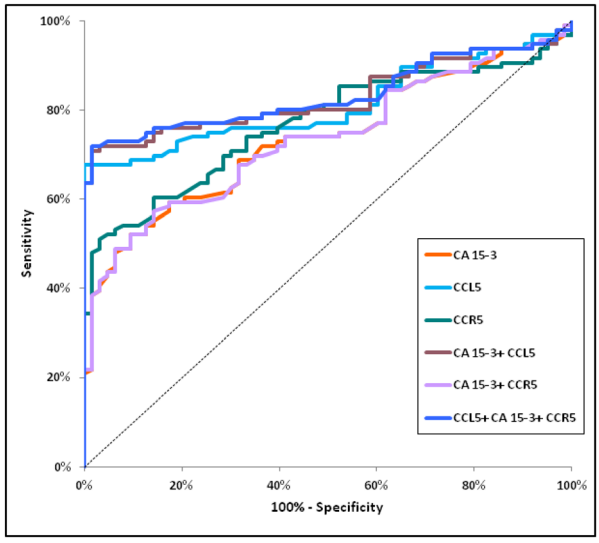

Figure 1. ROC curve analysis for studied measurands and in combination with CA 15-3 in total BC group.

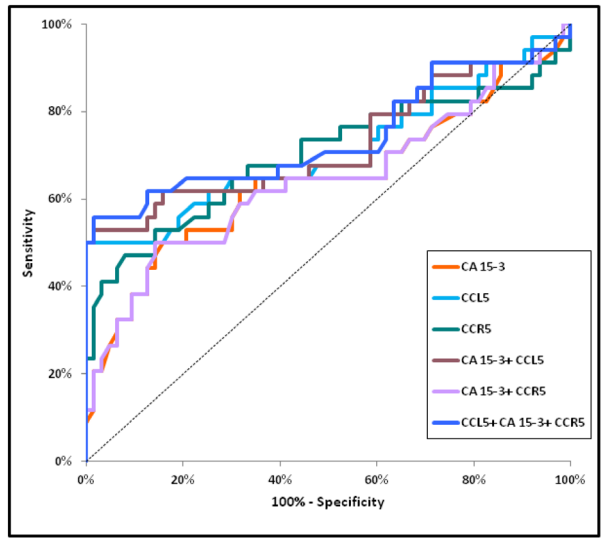

Figure 2. ROC curve analysis for studied measurands and in combination with CA 15-3 in stage I of BC.

Table 2. Diagnostic criteria of ROC curve for the tested parameters and CA 15-3.

Tested parameters	AUC	SE	95% C.I. (AUC)	*p* (AUC=0.5)
	ROC criteria in breast cancer (total group)			
CCL5	0.8116	0.0344	0.744–0.879	<0.001
CCR5	0.7691	0.0370	0.697–0.842	<0.001
CA 15-3	0.7354	0.0389	0.659–0.812	<0.001
CCL5+CA 15-3	0.8292	0.0333	0.764–0.894	<0.001
CCR5+CA 15-3	0.7350	0.0389	0.659–0.811	<0.001
CCL5+CCR5+CA 15-3	0.8335	0.0329	0.769–0.898	<0.001
	ROC criteria in breast cancer (stage I)			
CCL5	0.7089	0.0634	0.585–0.833	0.001
CCR5	0.6998	0.0640	0.574–0.825	0.0018
CA 15-3	0.6452	0.0655	0.517–0.774	0.0266
CCL5+CA 15-3	0.7241	0.0628	0.601–0.847	<0.001
CCR5+CA 15-3	0.6431	0.0651	0.515–0.771	0.028
CCL5+CCR5+CA 15-3	0.7402	0.0620	0.619–0.862	<0.001
	ROC criteria in breast cancer (stage II)			
CCL5	0.8313	0.0507	0.732–0.931	<0.001
CCR5	0.7756	0.0534	0.671–0.880	<0.001
CA 15-3	0.7163	0.0551	0.608–0.824	<0.001
CCL5+CA 15-3	0.8512	0.0489	0.755–0.947	<0.001
CCR5+CA 15-3	0.7163	0.0551	0.608–0.824	<0.001
CCL5+CCR5+CA 15-3	0.8494	0.0491	0.753–0.946	<0.001
	ROC criteria in breast cancer (stage III and IV)			
CCL5	0.9343	0.0414	0.853–1.015	<0.001
CCR5	0.8644	0.0476	0.771–0.958	<0.001
CA 15-3	0.9098	0.0426	0.826–0.993	<0.001
CCL5+CA 15-3	0.9517	0.0373	0.878–1.025	<0.001
CCR5+CA 15-3	0.9113	0.0423	0.828–0.994	<0.001
CCL5+CCR5+CA 15-3	0.9488	0.0382	0.874–1.024	<0.001

p – statistically significantly larger AUC compared to AUC=0.5 AUC, Area Under Curve; ROC, Receiver Operating Characteristics; S.E., Standard Error; C.I., Confidence Interval

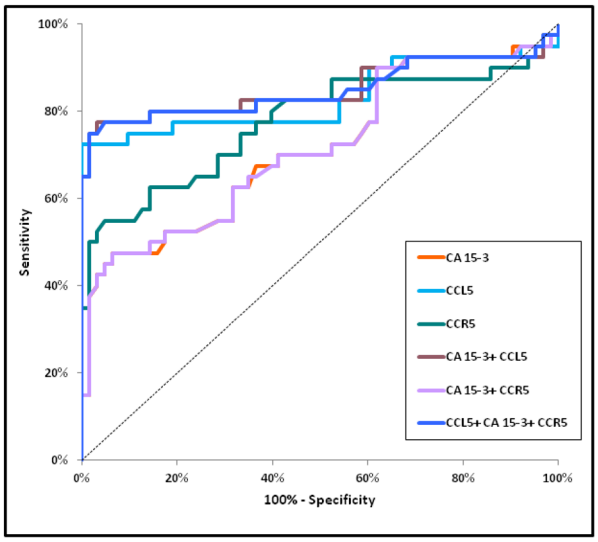

Figure 3. ROC curve analysis for studied measurands and in combination with CA 15-3 in stage II of BC.

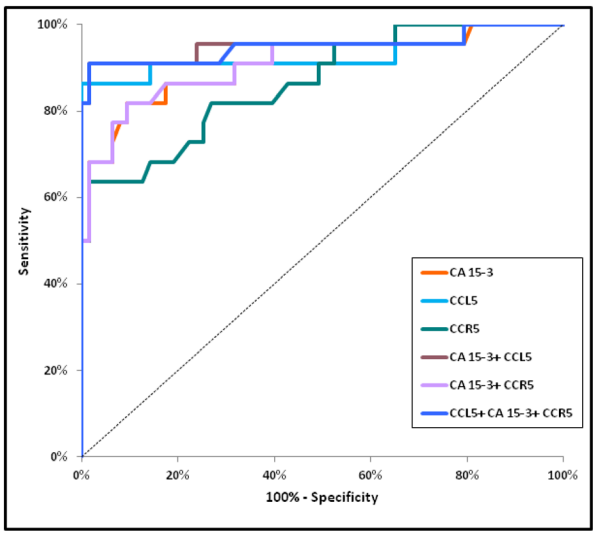

Figure 4. ROC curve analysis for studied measurands and in combination with CA 15-3 in stages III and IV of BC.

(0.7089) (Fig. 2) (Table 2). Stage II showed larger AUC for CCL5 (0.8313; $p<0.001$) when compared to the other parameters: CCR5 (0.7756) and CA 15-3 (0.7163). The combination of CCL5 with CA 15-3 showed an increase in AUC values ($p<0.001$, when compared to AUC=0.5) (Fig. 3) (Table 2).

Using CCL5 together with CA 15-3 resulted in enhancement of AUC value ($p<0.001$). CCL5 had the largest AUC value (0.9343; $p<0.001$) for stage III and IV of BC. It was larger than that of CCR5 (0.8644; $p<0.001$). Also, the results for CCL5 and CCR5, CA 15-3, were significantly higher in comparison to AUC=0.5 ($p<0.001$ in all cases). CCL5, CCR5 and CA 15-3 applied together had a higher AUC value (Fig. 4) (Table 2).

Moreover, we found that median levels of CCL5 and CA 15-3 in BC patients were markedly larger compared to healthy individuals ($p<0.001$). At the same time median concentration of CCR5 was significantly smaller ($p<0.001$, when compared to healthy individuals) (Table 3).

Analyzing the tested proteins' levels versus the tumor stage of BC, we found that the plasma concentrations of CCL5 for stage II, III and IV were significantly higher in patients with BC than in the healthy group ($p<0.001$). Nevertheless, the levels of CCR5 for every tumor stage were lower than in the healthy individuals ($p<0.001$) in opposite to CA 15-3 (Table 3).

We documented that there is a statistically significant difference between the entire BC group and benign breast tumors. Moreover, the plasma levels of CCL5 and CA 15-3 were significantly higher than in BBT patients ($p<0.001$, similarly when compared to every BC stage). Interestingly, the median level of CCR5 for each stage of cancer was significantly lower in comparison to the patients with BBT ($p=0.002$; $p<0.001$; respectively) (Table 3).

Increased plasma levels of CCL5 and CA 15-3 were revealed when we compared tumor stage III and IV versus stage I ($p<0.001$; $p=0.005$; respectively); or versus stage II ($p=0.001$; $p=0.032$; respectively). Reduced values of CCR5 were found when tumor stage III and IV were compared to stage I ($p=0.041$). Interestingly, the statistical differences between patients with benign breast tumors and healthy controls were demonstrated for CCL5 and CCR5 concentrations ($p<0.001$; $p=0.001$; respectively). There was no statistical difference in CA 15-3 level between healthy subjects and BBT. Our present study indicated that CCL5 and CCR5 may be applied to differentiate between BBT and healthy individuals (Table 3).

We indicated that the sensitivity of tested proteins in the total cancer group was the highest for CCL5 (68.04%), and was higher than those of CCR5 (62.89%) and CA 15-3 (59.79%). The maximum diagnostic sensitivity (86.60%) was obtained for the combination of CCL5 and CCR5 with CA 15-3. Moreover, for the early stages of BC the highest SE was observed for CCL5 or CCR5 (for stage I of BC – CCR5 58.82%; for stage II – CCL5 73.17%). The specificity measure for CCL5 levels (100.00%) was higher than for CA 15-3 (85.71%) in the BC group. Also, the predictive value of CCL5 was 100% in BC patients group while those of CA 15-3 was 86.57%. The highest PPV (67.02%) in the BC group was calculated for CCL5, compared to CCR5 (60.44%) and CA 15-3 (58.06%). The NPV in the group of BC was higher for CCL5 (67.02%) than for CCR5 (60.44%) and CA 15-3 (58.06%) (not presented).

DISCUSSION

In the recent years, an intensive search for biomarkers to provide useful information for better laboratory diagnosis of BC has focused on preoperative CA 15-3 levels (Shao *et al.*, 2015; Kazarian *et al.*, 2017; Lee *et al.*, 2013). In the present study, we sought to elucidate the significant diagnostic value of the chemokine CCL5 and its receptor CCR5 alone and in combination with CA 15-3 in BC patients.

In our studies, we observed several unexpected trends. The data presented here revealed that CCR5 is a negative biomarker whose median level was significantly lower in BC patients compared to the control groups. Furthermore, the plasma level of CCR5 in the BC group decreased with the tumor stage (Table 3). To date, there has been no research on CCR5 plasma concentrations in BC patients, especially in comparison to CA 15-3 levels. The previous studies concerned only the tissue expression of CCR5. In the study by Hartmann et al. (Hartmann *et al.*, 2011) real-time quantitative PCR was used

Table 3. Plasma levels of the examined proteins and CA 15-3 in patients with breast cancer and in the control groups.

Groups tested	CCL5 (ng/mL)	CCR5 (ng/mL)	CA 15-3 (U/mL)
	Breast cancer (median, range)		
Stage I	11.78 (27.31–63.30)[b/f]	9.84 (8.22–67.05)[a/f]	17.15 (6.20–50.30)[a/b/f]
Stage II	21.10 (0.88–396.67)[a/b/f]	9.45 (8.27–18.55)[a/b/f]	17.60 (4.40–48.10)[a/b/f]
Stage III and IV	35.52 (6.90–72.84)[a/b/c/d/f]	9.16 (5.12–12.38)[a/b/c/f]	27.75 (8.90–250.00)[a/b/c/d/f]
Total group	21.14 (0.88–396.67)[a/b/f]	9.58 (5.12–67.05)[a/b/f]	19.20 (4.40–250.00)[a/b/f]
	Control groups (median, range)		
Benign breast tumor	6.19 (2.90–11.38)[e]	10.34 (9.11–15.50)[e]	14.00 (5.20–20.70)
Healthy women	8.86 (6.63–11.38)	12.14 (9.77–16.95)	13.40 (6.30–28.40)
Total control group	8.57 (2.90–12.14)	11.02 (9.11–16.95)	13.60 (5.20–28.40)

Notes: [a]Statistically significant for patients with BC compared to healthy women. [b]Statistically significant for patients with BC compared to benign breast tumor group. [c]Statistically significant for patients with BC stage III and IV compared to the patients with BC stage I. [d]Statistically significant for patients with BC stage III and IV compared to the patients with BC stage II. [e]Statistically significant for patients with benign breast tumor compared to the healthy women. [f]Statistically significant for patients with BC compared to the total control group.

to detect CCR5 expression in corresponding tumor tissue, normal tissue, and isolated tumor and normal stromal cells. We would like to note that, in contrast to our study, the authors observed that CCR5 expression was significantly higher in tumor in comparison to the normal tissue. Moreover, a positive correlation was observed between the expression of CCL5 and CCR5, which indicates a high affinity between the ligand and receptor (Hartmann *et al.*, 2011). We suggest that the increased concentrations of CCL5 and low levels of its specific receptor in BC might be the results of the improved ability of CCR5 receptor to bind the higher amount of CCL5 in cancer patients.

In the present study, BC patients had significantly higher levels of CCL5 and CA 15-3 compared to the control groups. Additionally, the level of CCL5 in BC patients increased as the breast cancer became more advanced, similarly as CA 15-3 (Table 3). Concerning the association of CCL5 levels in plasma with breast cancer progression, Niwa and others (Niwa *et al.*, 2001) observed that concentrations of CCL5 were significantly increased in the subjects with active disease, compared to those in remission. In addition, both groups showed increased CCL5 levels compared to healthy subjects. The researchers observed that higher levels of CCL5 correlated with more advanced stage of breast cancer. In the study by Zhang and others (Zhang *et al.*, 2009), expression of CCL5 correlated with the development of the disease, especially at the stages III and IV according to the TNM classification. By contrast, Hartmann and others (Hartmann *et al.*, 2011) demonstrated no significant difference in CCL5 concentrations in the BC group compared to the healthy volunteers.

The significant difference between the entire BC group and benign breast tumors was also demonstrated for CCL5. The median level of CCL5 in the BC group was significantly higher than in the benign breast diseases group. By contrast, the plasma level of CCR5 was statistically significantly decreased. Moreover, the elevation in plasma CCL5 may be an early event during the development of BC because plasma CCL5 concentrations were significantly different between women with stage I compared to those with benign breast tumors (Table 3). Results of the study by Soria et al. (Soria *et al.*, 2011) revealed that expression of CCL5 in the tumor cells in all groups of BC patients was significantly elevated when compared to the normal cells in biopsied material of the benign patients. Our observations highlight the possible role of CCL5 and CCR5 as differentiation markers between benign breast diseases and healthy subjects.

The diagnostic characteristics for tumor markers are sensitivity, specificity and AUC. The CCL5 exhibited high diagnostic SE (68.04%) and diagnostic SP (100.00%) in the entire BC group. The number of plasma samples from patients with early stage of BC was large (n=75), so the findings indicate that CCL5 and CCR5 are potentially useful biomarkers for breast carcinoma. Moreover, our data indicate that CCL5 and CCR5 are better than CA 15-3 in distinguishing patients with BC from healthy women at high specificity. Tsukishiro and others (Tsukishiro *et al.*, 2006) showed that the sensitivity of CCL5 was 81%, and the specificity was 64% in patients with ovarian cancer. Similar values of CCL5 sensitivity and specificity were obtained by Wang and others (Wang *et al.*, 2016) in gastric cancer (80.00%, 69%, respectively).

The area under the ROC curve shows the clinical applicability of a tumor marker. As shown in this study, the ROC area of CCL5 (0.8116) was the largest of all the tested measurands in the group of BC, similarly as for all stages of cancer (Table 2). To date, there have been no reports of combined analysis of CCL5 and CCR5 using area under the ROC curve in breast cancer

patients, particularly in comparison to CA 15-3. In the paper by Gonzalez and others (Gonzalez *et al.*, 2011), the AUC for CCL5 ranged from 0.76 to 0.82, depending on breast cancer subtype. The results obtained from the study conducted with the serum of gastric cancer patients demonstrated that AUC for CCL5 was 0.795, and was lower than in our experiments (Wang *et al.*, 2016). Moreover, ROC curve analysis using the combination CCL5+CCR5+CA 15-3 (AUC=0.8335) showed an improvement in BC diagnosis as compared to the chemokine or its receptor alone. Based on our previous studies, it can be concluded that the proper assessment of diagnostic power should be based on a combined analysis of the tested measurands in BC subjects (Lubowicka *et al.*, 2018) (Zajkowska *et al.*, 2016; Ławicki *et al.*, 2013; Ławicki *et al.*, 2016).

CONCLUSIONS

In conclusion, the present study demonstrated that concentrations of CCL5 and CCR5 are significantly different in BC women in comparison to the control groups. The results of the present study indicate that CCL5 is a more sensitive and more specific marker of breast cancer than CA 15-3. The AUC value was higher for CCL5 and CCR5 than those of CA 15-3, which shows a possible clinical utility of CCL5 and CCR5 measurements in the BC diagnosis, especially at the early stages of the disease. This study also identified a potential association between CCL5 and CCR5 in the plasma and tissue of BC patients which guaranties further examination of tissue expression in the studied groups in the future.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics approval and informed consent

This study was approved by the local Ethics Committee at the Medical University of Bialystok (R-I-002/51/2015). All of the patients gave their informed consent for participation in the study.

Ethical declaration

This work was conducted in accordance with the Declaration of Helsinki (1964).

Contribution Statement

ED conceived the idea for the study. ED, SŁ contributed to the design of the research. All authors were involved in data collection and analyzed the data. ED coordinated funding for the project. All authors edited and approved the final version of the manuscript

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