Possible Diagnostic Application of CXCL12 and CXCR4 as Tumor Markers in Breast Cancer Patients

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Abstract. *Background/Aim: Chemokines are cytokines involved not only in inflammatory but also in inappropriate response of the immune system in breast cancer (BC) progression. We examined the diagnostic usefulness of CXCL12, CXCR4 and CA 15-3 in BC patients, based on ROC curve analysis. Materials and Methods: The study group consisted of 100 patients with BC; the control group consisted of 35 women with benign breast disease and 35 healthy patients. The median concentration of chemokines was measured by ELISA and that of CA 15-3 by chemiluminescent microparticle immunoassay. Results: The concentrations of CXCL12 and CXCR4 in the BC group were significantly higher than those in the control groups. The AUC value of CXCL12 (0.7502) was the highest of all the chemokines measured in the BC patients. Conclusion: There may be a link between CXCL12, CXCR4 and BC that can assist in the diagnosis, markedly when combined with CA 15-3.*

Breast cancer is the most common cancer in women and one of the major causes of death globally (1, 2). The exact causes of breast cancer remain largely unknown. Known risk factors for breast cancer include gender, old age, menopause, positive family history, genetic mutations (*BRCA1*, *BRCA2*), nulliparity, smoking and overweight and obesity (3, 4). Due to the growing number of cases, diagnosis at an early (often asymptomatic) stage seems to be crucial. Significant progress in this area has been achieved thanks to imaging

methods, *e.g.* computed tomography. However, these techniques are not effective in assessing small lesions (5). Thus, significant research is directed to identify new imaging methods and diagnostic markers, including biochemical or molecular markers, which would allow for early diagnosis of cancer processes. Tumor markers have particular value not only in monitoring treatment, but also in detecting, localizing metastases, determining the severity of cancer and detecting early recurrence (6). In cancer patients, pathological angiogenesis and significant deregulation of extracellular matrix proteins is observed. Increasing evidence insinuates that a group of inflammatory cytokines such as chemokines are crucial regulators of angiogenesis (7). Among more than 50 human chemokines, CXCL12 also known as alpha-chemokine stromal cell-derived-factor-1 (SDF-1) is of high importance. CXCL12 binds to the chemokine receptor CXCR4 and plays a crucial role in different physio-pathological processes: it maintains tissue homeostasis, plays a role in the survival and guidance of many migrating cells, acting as an attractant and may also play a role in the development and metastatic progression of cancer (8, 9). CXCL12 also stimulates the formation of capillary blood vessels *in vitro* and induces VEGF secretion (10). It has been shown that CXCR4 is highly expressed in many types of cancers, including prostate, ovarian and breast cancer and its levels have been correlated with poor prognosis (11-14). The molecular mechanisms by which CXCR4 promotes primary breast cancer growth include angiogenesis, increased cell proliferation and recruitment of immune cells (15). The CXCR4/CXCL12 axis has been associated with tumor grade, invasion, lymphatic and distant metastasis (16). The significance of CXCR4/CXCL12 axis in breast cancer has been widely discussed.

The aim of the study was to assess the plasma concentrations of CXCL12 and its receptor CXCR4 and the levels of CA 15-3. Patients were divided into 3 groups: the

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first consisted the BC patients, the second of patients with benign tumor lesions in breast tissue, and the last of healthy women, which constituted the control group. Furthermore, the significance of the measured markers for diagnosis was also assayed

Materials and Methods

Human subjects. Study was performed on specimens of 100 BC patients. The clinical stage of BC was considered in dividing individuals into groups. All patients were diagnosed histopathologically as having ductal adenocarcinoma. Samples were collected before any therapies. Specimens of 35 cases diagnosed with benign breast tumor (BBT) like *adenoma* or *fibroadenoma* and 35 healthy individuals were used as control (Table I). Healthy individuals as well as BBT patients were clinically examined to be BC-free and free of BC-anamnesis Individuals of control group were also free of inflammations and heart diseases.

The Local Ethics Committee approved the study. All patients gave their informed consent.

Plasma samples. Samples were stored under –85°C before assayed.

Examination of CXCL12, CXCR4 and CA 15-3. C-X-C motif chemokine ligand 12 and C-X-C motif chemokine receptor type 4 values were estimated using ELISA (EIAab Science, Wuhan, PR China) and/or R&D systems (Abingdon, UK). CA 15-3 was estimated by CMIA (Abbot Laboratories, Chicago, IL, USA). The within-run and between-run coefficients were examined according to the manufacturer's instructions. No interference or cross-reactivity with any human material were found. Each sample was measured twice for each patient.

Statistical analysis. The STATISTICA 12.0 program was used to conduct statistical analysis (Dell Software, Round Rock, TX, USA). Dwass Steele–Crichlow–Flinger test, Kruskal–Wallis test as well as *U*-Mann Whitney test, were used for analysis. $p<0.05$ was considered to indicate significant differences.

We have estimated sensitivity (SE) and specificity (SP) of the analysis. Youden's index was used to determine the cut off values for each parameter as follows: CXCL12, 3160.55 pg/ml; CXCR4, 2.39 ng/ml; CA 15-3, 16.85 U/ml. Healthy volunteers and the BBT group were used as controls. Moreover, the AUC values were determined to assess the diagnostic accuracy and to compare AUC for all parameters individually and in combination with CA 15-3.

Results

As shown in Table II, BC patients had significantly higher levels of CXCL12, CXCR4 and CA 15-3 compared to the healthy group ($p<0.001$; $p=0.005$; $p<0.001$; respectively). Analyses of the relationship between plasma concentrations of the tested parameters and stage of BC, indicated that those of CXCL12 in all stages of cancer were significantly higher in patients with BC than in healthy individuals (I stage of BC – $p=0.02$; II stage – $p<0.001$; III and IV stage – $p<0.001$), similarly to CA 15-3. Moreover, the concentrations of CXCR4

Table I. *Characteristics of breast cancer patients and control group.*

Study group	Number of patients
Examined groups	
Breast cancer patients	
Ductal adenocarcinoma	100
Median age (range)	57 (21-84)
Tumor stage	
I	34
II	41
III and IV	25
Control groups	
Benign breast tumor group	35
Adenoma	12
Fibroadenoma	23
Median age (range)	39 (21-63)
Healthy women	35
Median age (range)	37 (21-58)

in stage I and II of BC were significantly higher than in the healthy subjects ($p=0.003$; $p=0.02$; respectively).

The significant differences between the entire BC group and BBT group were also demonstrated for CXCL12 and CA 15-3. The concentrations of CXCL12 and CA 15-3 in BC patients were notably higher than those in the BBT group ($p<0.001$). Plasma CXCL12 levels were significantly increased in all stages of BC in comparison to BBT, similarly to CA 15-3 glycoprotein.

In patients with BC, the median levels of CXCL12 and CXCR4 were significantly higher, compared with the BBT and healthy control group ($p<0.001$; $p=0.008$; respectively), similarly to CA 15-3 ($p<0.001$). A similar trend was noticed regarding the different stages of cancer ($p<0.05$ in all cases, except for CXCR4 in stages III and IV).

Increased plasma values of CXCL12 and CA 15-3 were observed in individuals with grade III and IV *versus* grade I ($p=0.03$; $p=0.005$; respectively).

In the present study, the statistical differences between women with benign tumors in the breast and healthy controls were not demonstrated for CXCL12 and CXCR4. Moreover, there was no statistical difference in CA 15-3 level among healthy subjects and BBT.

As shown in Table III, the sensitivity of CXCL12 (74.2%) was higher than those of CXCR4 (59.8%) and CA 15-3 (59.8%). Moreover, the frequency of increased concentrations was the highest for CXCL12 and CXCR4 in combination with CA 15-3 levels (92.8%). Interestingly, the higher sensitivity in I stage of BC was noted for CXCR4. The highest SE at stages II, III and IV of BC was observed for CXCL12. The combination of the CXCL12 or CXCR4 and CA 15-3 resulted in an increase of sensitivity value in each stage of cancer. The combination of CXCL12 or CXCR4 together with CA 15-3 demonstrated increased values of SE in each grade of BC. The

Table II. *Plasma levels of examined CXCL12, CXCR4 and CA 15-3 in patients with breast cancer and in control groups.*

Groups tested	CXCL12 (pg/ml)	CXCR4 (ng/ml)	CA 15-3 (U/ml)
Breast cancer (median, range)			
Stage I	3312.65 (1200.80-4556.2)[a/b/e]	3.00 (0.61-7.51)[a/e]	17.15 (6.20-50.30)[a/b/e]
Stage II	3602.10 (696.95-4995.70)[a/b/e]	2.54 (0.61-7.05)[a/e]	17.60 (4.40-48.10)[a/b/e]
Stage III and IV	3703.95 (2759.8-5124.3)[a/b/c/e]	2.39 (0.16-7.72)	27.75 (8.90-250)[a/b/c/d/e]
Total group	3559.20 (696.95-5124.30)[a/b/e]	2.39 (0.16-7.72)[a/e]	19.20 (4.40-250.00)[a/b/e]
Control groups (median, range)			
Benign breast tumor	3000.60 (1823.20-3870.30)	1.95 (0.07-6.52)	14.00 (5.20-20.70)
Healthy women	2893.60 (2411.90-3897.10)	1.89 (0.18-8.21)	13.40 (6.30-28.40)
Total control group	2947.10 (1823.20-3897.10)	1.93 (0.07-8.21)	13.60 (5.20-28.40)

[a]Statistically significant when patients with BC compared with healthy women; [b]Statistically significant when patients with BC compared with benign breast tumor group; [c]Statistically significant when patients with BC stage III and IV compared with patients with BC stage I; [d]Statistically significant when patients with BC stage III and IV compared with patients with BC stage II; [e]Statistically significant when patients with BC compared with total control group.

Table III. *Diagnostic characteristics of CXCL12, CXCR4 and CA 15-3 and CXCL12 and CXCR4 in combination with CA 15-3 in breast cancer patients.*

Measurands	Diagnostic criteria (%)	Breast cancer			
		Stage I	Stage II	Stage III/IV	Total group
CXCL12	SE	58.82	78.05	90.91	74.23
	SP	71.43	71.43	71.43	71.43
	PPV	52.63	64.00	52.63	80.00
	NPV	76.27	83.33	95.74	64.29
CXCR4	SE	64.71	60.98	50.00	59.80
	SP	65.08	65.08	65.08	65.08
	PPV	50.00	53.19	33.33	72.50
	NPV	77.36	71.93	78.85	51.25
CA 15-3	SE	50.00	53.66	86.36	59.79
	SP	85.71	85.71	85.71	85.71
	PPV	65.38	70.97	67.86	86.57
	NPV	76.06	73.97	94.74	58.06
CXCL12+ CA 15-3	SE	73.53	85.37	100.00	84.54
	SP	60.32	60.32	60.32	60.32
	PPV	50.00	58.33	46.81	76.64
	NPV	80.85	86.36	100.00	71.70
CXCR4+ CA 15-3	SE	82.35	82.93	95.45	85.57
	SP	57.14	57.14	57.14	57.14
	PPV	50.91	55.74	43.75	75.45
	NPV	85.71	83.72	97.30	72.00
CXCL12+CXCR4+CA 15-3	SE	88.24	92.68	100.00	92.78
	SP	34.92	34.92	34.92	34.92
	PPV	42.25	48.10	34.92	68.70
	NPV	84.62	88.00	100.00	75.86

NPV: Predictive value of a negative test result; PPV: predictive value of a positive test result; SE: sensitivity; SP: specificity.

specificity of the various parameters was the highest for CA 15-3 (85.7%) in the BC group.

A maximum PPV value (86.6%) in the BC group was obtained for CXCL12, in comparison to CXCR4 (72.5%).

Moreover, a maximum PPV was estimated for CA 15-3 in all stages of cancer. The predictive value of a negative test result was higher for CXCL12 (64.3%) than for CXCR4 (51.3%) and CA 15-3 (58.1%). Additionally, the highest

Table IV. *ROC curve analysis for for CXCL12, CXCR4 and CA 15-3 and CXCL12 and CXCR4 in combination with CA 15-3.*

Measurands	AUC	SE	95%CI (AUC)	*p*-Value (AUC=0.5)
Total BC group				
CXCL12	0.7502	0.0393	0.673-0.827	<0.001
CXCR4	0.6239	0.0462	0.533-0.714	0.007
CA 15-3	0.7354	0.0389	0.659-0.812	<0.001
CXCL12+CA 15-3	0.7774	0.0371	0.705-0.850	<0.001
CXCR4+CA 15-3	0.7593	0.0374	0.686-0.833	<0.001
CXCL12+CXCR4+CA 15-3	0.8062	0.0348	0.738-0.875	<0.001
I stage of BC				
CXCL12	0.6650	0.0636	0.540-0.790	0.01
CXCR4	0.6629	0.0561	0.553-0.773	0.004
CA 15-3	0.6452	0.0655	0.517-0.774	0.03
CXCL12+CA 15-3	0.6713	0.0691	0.536-0.807	0.01
CXCR4+CA 15-3	0.6811	0.0611	0.561-0.801	0.003
CXCL12+CXCR4+CA 15-3	0.7162	0.0643	0.590-0.842	<0.001
II stage of BC				
CXCL12	0.7562	0.0565	0.645-0.867	<0.001
CXCR4	0.6210	0.0561	0.511-0.731	0.03
CA 15-3	0.7163	0.0551	0.608-0.824	<0.001
CXCL12+CA 15-3	0.7702	0.0533	0.666-0.875	<0.001
CXCR4+CA 15-3	0.7583	0.0520	0.656-0.860	<0.001
CXCL12+CXCR4+CA 15-3	0.8103	0.0500	0.712-0.908	<0.001
III and IV stage of BC				
CXCL12	0.8712	0.0420	0.789-0.954	<0.001
CXCR4	0.5689	0.0711	0.430-0.708	0.33
CA 15-3	0.9098	0.0426	0.826-0.993	<0.001
CXCL12+CA 15-3	0.9545	0.0291	0.897-1.012	<0.001
CXCR4+CA 15-3	0.8817	0.0492	0.785-0.978	<0.001
CXCL12+CXCR4+CA 15-3	0.9380	0.0375	0.864-1.012	<0.001

p: Statistically significantly larger AUCs compared to AUC=0.5. AUC: Area under the ROC curve; CI: confidence interval; ROC: receiver-operating characteristics; SE: standard error.

NPV values were demonstrated for CXCR4 or CXCL12 in early stages of cancer (in stage I of BC – CXCR4, in stage II – CXCL12), while in stage III and IV for CXCL12. Increased NPV values were found when CXCL12 or CXCR4 were combined with CA 15-3 in all stages of tumor.

The ROC curve is a graphical plot used to illustrate the relationship between the sensitivity and specificity measures (Table IV). It has been stated that AUC above 0.8 indicates a good diagnostic performance. As shown in Figure 1, the AUC for CXCL12 (0.75) in BC group was higher than that for CXCR4 (0.62) or CA 15-3 (0.74). Moreover, values apparently exceed the value of 0.5 being an edge indicator for diagnostic use (*p*<0.001 in all cases, except for CXCR4, *p*=0.007). The combined analysis of CXCL12 or CXCR4 and CA 15-3 derived an increase in AUC (0.78; 0.76; respectively).

Higher BC tumor grade was associated with enhanced AUC values of CXCL12 as well as CA 15-3. In early stages of BC, the highest AUC values were observed for CXCL12 (I stage – 0.67, II stage – 0.76), and were higher than that for CA 15-3 (Figures 2 and 3). As shown in Figure 3, the AUCs for CXCL12 and CXCR4, CA 15-3, were remarkably higher than AUC=0.5 (*p*<0.001; *p*=0.03; *p*<0.001; respectively). The combination of CXCL12 or CXCR4 with the classical tumor marker showed an increase in AUC values (*p*<0.001 in both cases). In stages III and IV of BC the highest AUC was calculated for plasma CXCL12 (0.87; *p*<0.001). This value was lower than that for CA 15-3 (0.91; *p*<0.001). The combination of CXCL12 or CXCR4 with CA 15-3 resulted in an increased AUC value (Figure 4). The AUC of all studied analytes exceeded 0.8 in patients with advanced (stage III and
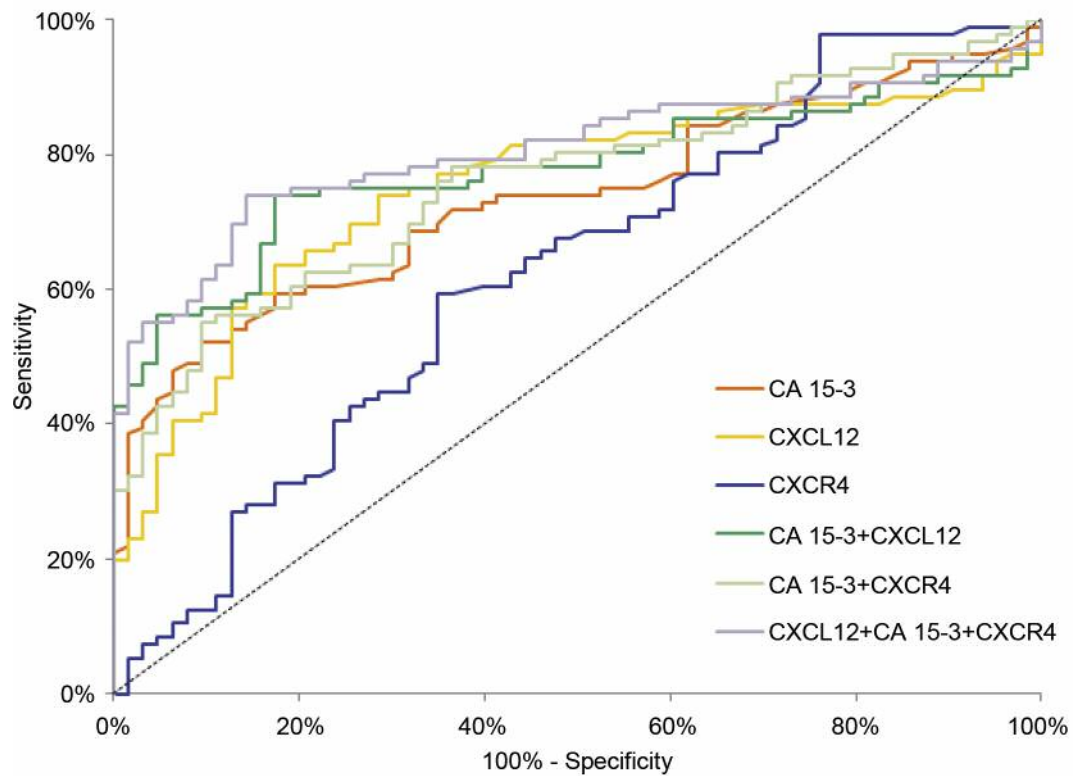
Figure 1. *Diagnostic criteria of ROC curve for CXCL12, CXCR4 and CA 15-3 and CXCL12 and CXCR4 in combination with CA 15-3 in total BC group.*
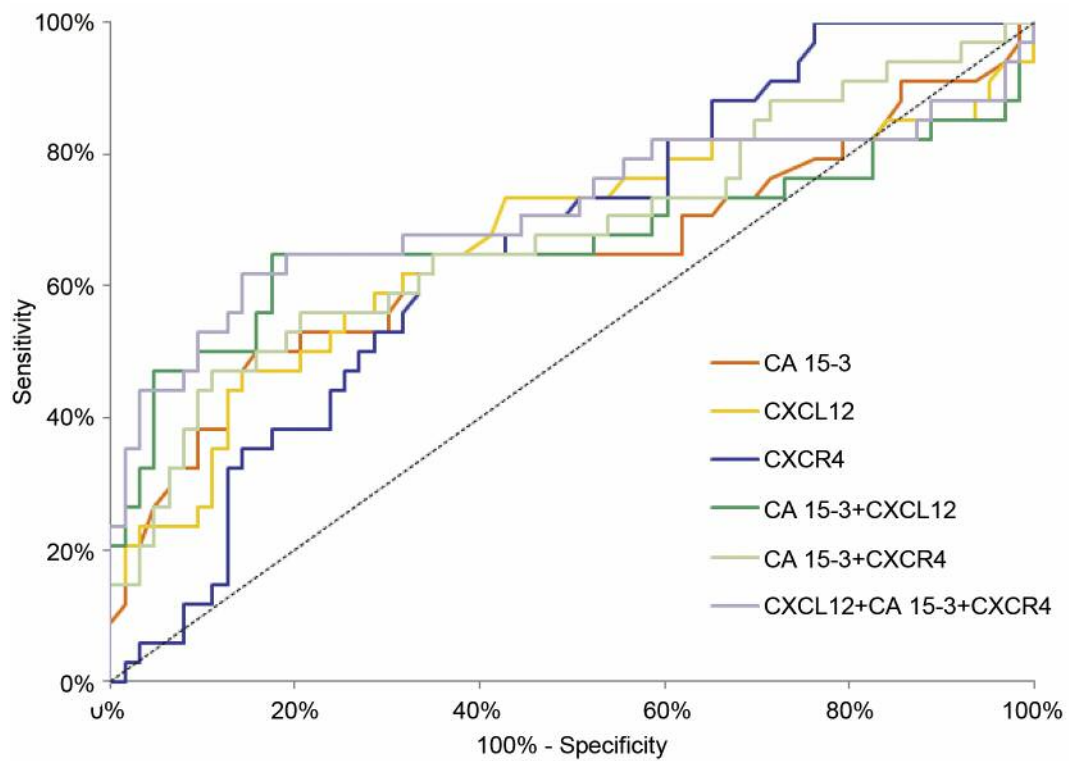

Figure 2. *Diagnostic criteria of ROC curve for CXCL12, CXCR4 and CA 15-3 and CXCL12 and CXCR4 in combination with CA 15-3 in stage I of BC.*
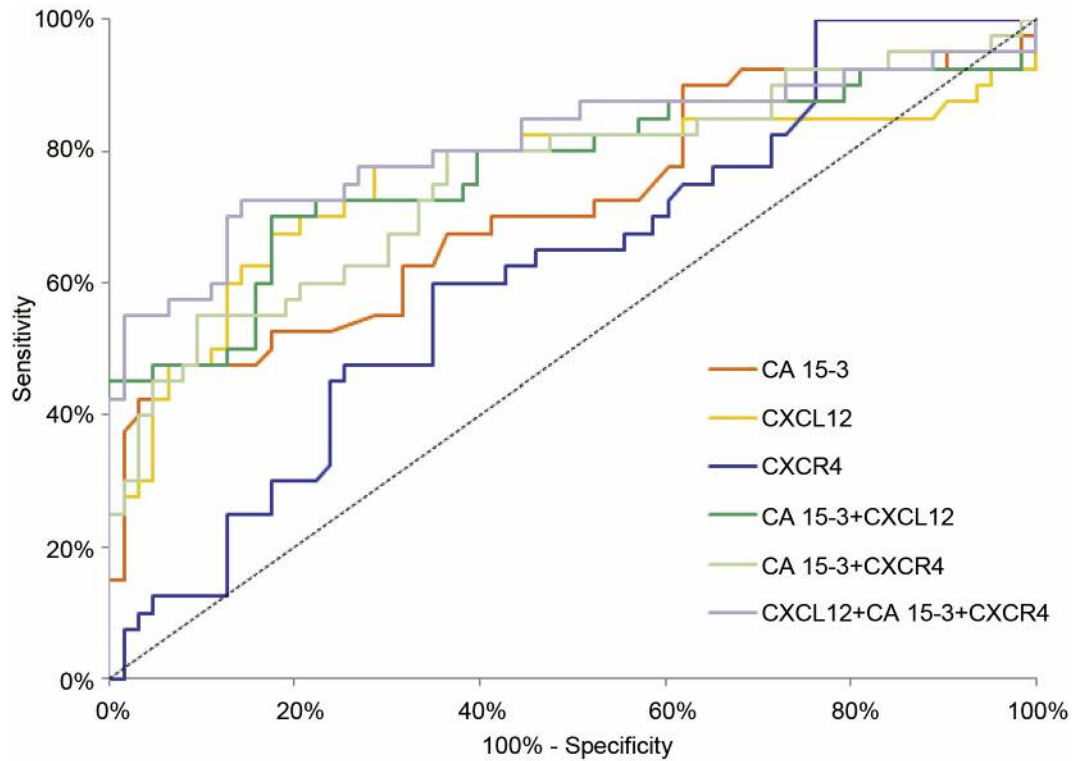
Figure 3. *Diagnostic criteria of ROC curve for CXCL12, CXCR4 and CA 15-3 tested parameters and CXCL12 and CXCR4 in combination with CA 15-3 in stage II of BC.*
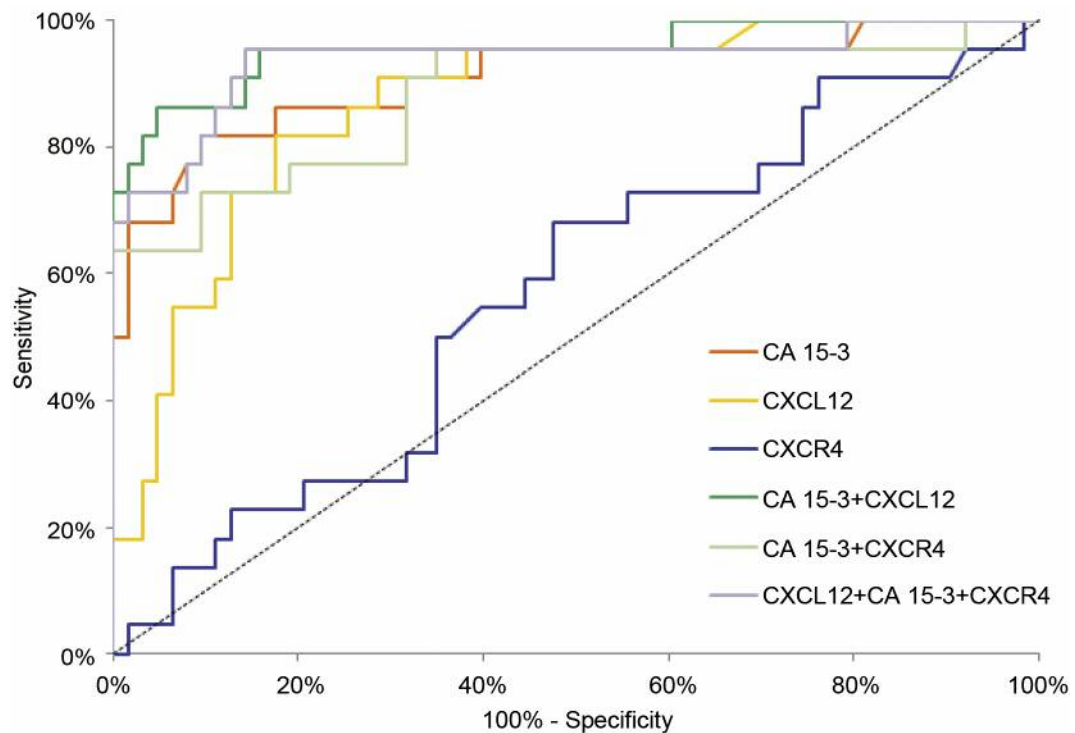

Figure 4. *Diagnostic criteria of ROC curve for CXCL12, CXCR4 and CA 15-3 tested parameters and CXCL12 and CXCR4 in combination with CA 15-3 in stages III and IV of BC.*

IV) BC. Moreover, the AUC value of the combined test (CXCL12+CXCR4+CA 15-3) exceeded the AUC of CA 15-3 alone in the whole group, stage I, II, and III and IV by 0.071, 0.071, 0.094 and 0.028, respectively.

Discussion

Several studies have suggested the relationship between the CXCR4/CXCL12 axis and other pathways that serve an important role in the promotion of invasion and metastasis of BC (16, 17). In our study, we demonstrated the diagnostic usefulness of chemokine CXCL12 and its receptor CXCR4 alone and in combination with the tumor marker CA 15-3 in BC cases.

This study reports that patients with cancerous changes in the breast have a notably higher concentration of plasma CXCL12, CXCR4 and CA 15-3 than controls. In agreement with our observations, there have been studies showing that the CXCL12 plasma levels were significantly higher in breast cancer group than in untreated controls (18). Moreover, earlier research has noted that the expression of CXCR4 and CXCR7 in BC tissues was significantly higher than in normal tissues, while the levels of CXCL12 in BC tissues did not show any significant differences (19). In other carcinomas, the serum levels of CXCL12 were significantly higher, while those of CXCR4 were significantly lower in case of esophageal cancer patients compared to healthy controls (20). An important finding of our study was the significant increase in CXCL12 levels in the BC group compared to those in the BBT group. Our observations highlight the possible role of CXCL12 as a marker, which may distinguish cancerous and benign changes in human breast. The work of Wang and co-workers (9) has shown that in consecutive stages of cancer development and progression, beginning from benign precancerous lesions through invasive cancer, the levels of CXCR4 and CXCL12 differed significantly. Guo *et al*. (21) have found that CXCL12/CXCR4 chemokine axis is engaged in the transformation of epithelial ovarian tumors. This study demonstrated significantly higher expression of CXCL12/CXCR4 in epithelial ovarian carcinomas than in benign epithelial ovarian tumors.

Of note, in our current study, we observed that the CXCL12 concentrations were noticeably different in every examined group (in relation to the stage of advancement of BC) in comparison to the control groups (benign breast disease and healthy subjects). Importantly, even in the early stages of cancer, we observed significant differences in the concentrations of CXCR4 when compared to the entire control subjects. The clinicopathological study by Sun *et al*. (22) has suggested that the CXCR4-CXCL12 axis could be a prognostic marker in the BC group. The work by the above authors has revealed that the co-expression of CXCR4 and CXCL12 correlated with lymphatic metastasis and TNM staging.

The diagnostic characteristics for tumor markers are sensitivity, specificity and AUC. The diagnostic specificity CA 15-3 and CXCL12 was 85.71% and 74.23% in entire BC group, respectively. Additionally, highest sensitivity (92.78%) was observed for the combination of CXCL12 and CXCR4 with CA 15-3. In the study by Hassan *et al*. (18), the ELISA method was also used to determine the plasma concentrations of DF-1 (CXCL12), which were defined as low and high. The authors observed that SE value of low plasma SDF-1 levels for distant metastasis was 66%, and the SP value was 53.4%. The results of the study on the role of CXCL12 and CXCR4 in different types of malignancies demonstrated that the SP and SE values were 47.00% and 80.00% for CXCL12 and 80.00% and 57.00% for CXCR4 differentiating esophageal tumor patients from the healthy volunteers (20). Moreover, our study demonstrated that the plasma concentrations of CXCL12 were markedly increased in patients with stage III and IV disease than in patients with stage I, and that these increased plasma levels of CXCL12, might be useful for identifying patients with early breast cancer. The same relationship was observed regarding the diagnostic sensitivity of CXCL12, which indicates the possible use of CXCL12 in BC staging.

The ROC curve illustrates the clinical usefulness of a tumor marker. Currently, there is lack of research articles regarding the diagnostic utility of CXCL12 and CXCR4 concentrations in women with BC. Herein, the AUC of CXCL12 was the highest of all parameters in the BC group. Of note, in our current study, we observed that the ROC area of CXCL12 was higher in the early stages of disease. AUCs between 0.62 and 0.75 found for CXCL12, CXCR4 and CA 15-3 in patients with stage I and II BC indicate poor overall diagnostic performance of these tests. with a study on lung carcinoma and lymph node tissues has also revealed that the AUC value for CXCL12 and CXCR4 was 0.64 and 0.61, respectively (23). Similar results have been presented by Łukaszewicz-Zając *et al*. (20) in patients with esophageal cancer. Additionally, the AUC of all studied markers exceeded 0.8 in patients with advanced BC which indicates the possibility of using these tumor markers in the diagnosis of early stage of breast cancer. Moreover, studies by other authors in patients with gastric cancer indicate that CXCL12 and CXCR4 expression was associated with the stage of the cancer (24). Moreover, ROC curve analysis using the combination CXCL12+CXCR4+CA 15-3 (AUC=0.8062) enhanced BC identification in comparison with CXCL21 or CXCR4 alone. The study also demonstrated that the AUC of the combined analysis (CXCL12+CXCR4+CA 15-3) exceeded the AUC of CA 15-3 alone in the all examined groups. Diagnostic power assessment, should be carried out as a combined analysis of the various parameters, as was presented in our previous study on other cytokines (M-CSF, VEGF) in BC patients (25, 26).

Our data and the findings of other researchers suggest an important role of CXCL12 and CXCR4 in BC diagnostics

(especially laboratory diagnostics). Additionally, our study is the first to indicate the diagnostic usefulness of CXCL12 chemokine and its receptor CXCR4 in the diagnosis of BC patients.

In conclusion, the present study demonstrated that the plasma concentrations of CXCL12 and CXCR4 were significantly higher in BC patients, and particularly in early stages of cancer, when compared to control groups (healthy subjects and benign breast disease). The AUC value was the highest for the combination of CXCL12 and CXCR4 with CA 15-3, which pointed out a viable clinical importance of CXCL12 and CXCR4 in the diagnosis of BC. These results suggest that CXCL12 and CXCR4 may be useful in diagnosis of BC patients. Moreover, CXCL12 and CXCR4 can be considered an additional diagnostic tool slightly improving diagnostic performance of CA 15-3 in the BC diagnosis. Unfortunately, the small number of cases in stage III and IV in this study necessitates affirmation of our results in a higher group of patients.

Conflicts of Interest

The Authors declare that there are no conflicts of interests regarding this study.

Authors' Contributions

ED, AP, SL conceived the idea for the study. ED, AP, MSZ, SL contributed to the design of the research. ED, BMP, MZ, IS were involved in data collection. ED, MZ, SL analyzed the data. ED, AP, SL obtained funding for the project. All Authors edited and approved the final version of the manuscript.

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