Clinical Study

Circulating CCL5 Levels in Patients with Breast Cancer: Is There a Correlation with Lymph Node Metastasis?

Ann Smeets,[1,2] **Barbara Brouwers,**[3] **Sigrid Hatse,**[3] **Annouschka Laenen,**[4]
Robert Paridaens,[1,3] **Giuseppe Floris,**[1,5] **Hans Wildiers,**[1,3] **and Marie-Rose Christiaens**[1,2]

[1] *Multidisciplinary Breast Center, KU Leuven, University Hospitals, Herestraat 49, 3000 Leuven, Belgium*

[2] *Department of Oncology, KU Leuven, Surgical Oncology, University Hospitals, Herestraat 49, 3000 Leuven, Belgium*

[3] *Laboratory of Experimental Oncology (LEO), Department of Oncology, KU Leuven and Department of General Medical Oncology, University Hospitals Leuven, Leuven Cancer Institute, Herestraat 49, 3000 Leuven, Belgium*

[4] *Interuniversity Institute for Biostatistics and Statistical Bioinformatics, KU Leuven, Kapucijnenvoer 35, 3000 Leuven, Belgium*

[5] *Department of Pathology, KU Leuven, University Hospitals, Minderbroederstraat 12, 3000 Leuven, Belgium*

Correspondence should be addressed to Ann Smeets; ann.smeets@uzleuven.be

CC-chemokine ligand 5 (CCL5) was measured in plasma of 238 patients with breast cancer and in serum of 149 of these patients. Mean circulating CCL5 levels tended to be higher in patients with lymph-node-positive breast cancer, larger tumour sizes, the presence of lymphovascular invasion, and multifocal tumours. Additionally, circulating CCL5 levels were higher in the order of stages III, II, and I. The addition of circulating CCL5 concentration to known clinicopathological predictors for lymph node involvement did not allow more precise prediction of the lymph node status. These results suggest that CCL5 is a biomarker for tumour load rather than for lymph node involvement. As such, it might be helpful to identify patients with escape from immunosurveillance who will benefit from therapies to restore immune function.

1. Introduction

CC-chemokine ligand 5 (CCL5), previously called RANTES (Regulated on Activation, Normal T cell Expressed and Secreted), belongs to the family of the CC chemokines. The major role of chemokines is to act as chemoattractants to guide the migration of cells. Some chemokines control cells of the immune system during processes of immune surveillance. Others are inflammatory and mainly attract leukocytes to sites of inflammation and/or infection. Their release is stimulated by pro-inflammatory cytokines. Thus, chemokines coordinate intricate leukocyte trafficking patterns that regulate immune responses against cancer. These are multistep processes that involve localization of immune effectors to appropriate sites, antigen presentation, and optimal triggering of specific T cells.

It is increasingly clear that cancer-mediated modulation of the host immune response contributes to tumour progression and correlates with patient outcome [1]. Indeed, tumours can evade immunosurveillance through altering the properties and functions of host stromal and/or immune cells. They polarize the tumour microenvironment towards chronic inflammatory states, leading to impaired tumor cell killing and to tumour escape.

The exact functions of CCL5 in tumour biology are still unclear. On the one hand, CCL5 is important to trigger and amplify the anti-tumour host response. Besides its role as a potent chemoattractant, CCL5 plays an important role in T-cell responses. Therefore, production of CCL5 is important for inducing proper immune responses against tumours [2]. On the other hand, it has been reported that CCL5 is associated with cancer progression and metastasis because it promotes tumour cell survival, proliferation, and invasion [3].

CCL5 can be expressed and secreted either by breast cancer cells or by non-malignant stromal cells at the primary or metastatic sites, such as macrophages, activated natural killer cells, and T cells [2].

Metastasis to tumour draining lymph nodes is a crucial step in breast cancer progression and is used to predict patient outcome and survival. It is mainly determined by tumour characteristics such as the size of the primary tumour, the presence of lymphovascular invasion, and multifocality. However, it has been hypothesized that the host immune response may play an essential role in the process of lymph node metastasis. It might be that the functioning of the immune system in node-negative patients is less impeded and can thus prevent tumour invasion more effectively compared to that of node-positive patients. As such, lymph node metastasis requires breakdown of the host immune response in addition to the escape of cancer cells from the tumour.

We previously compared gene expression profiles of primary tumour from node-negative and node-positive patients and found a significant downregulation of genes associated with immune-related pathways, including CCL5 and its cellular receptor CCR5, in node-positive patients (unpublished data).

We therefore hypothesized that CCL5 may play a role in regulating lymph node involvement in breast cancer. To test this hypothesis, we examined the association between the levels of circulating CCL5 in plasma and serum of patients with breast cancer and the presence of lymph node metastasis. Additionally, we evaluated whether the addition of circulating CCL5 to known independent clinicopathological predictors of lymph node involvement in breast cancer allowed for a more precise prediction of the presence of lymph node metastasis.

2. Patients and Methods

2.1. Patients. Since 2003, the Leuven Multidisciplinary Breast Centre (University Hospitals Leuven) has systematically collected plasma and serum from all consenting (±75%) breast cancer patients at the time of diagnosis, before the start of any local or systemic therapy. All patient data have also been included in a clinical database, containing extensive general and tumour-related information, as well as clinical follow-up data. For the present study, eligible patients were selected from this database based on the following inclusion criteria: (i) patients were diagnosed with primary early invasive breast cancer between June 2003 and February 2010, (ii) patients received primary surgery and pathological confirmation in our institution, and (iii) plasma and serum were collected at the time of diagnosis. For the present study, 244 patients were selected. Written informed consent was obtained from all patients. The University Hospitals Ethical Committee approved the study. Patients' clinicopathological characteristics are shown in Table 1.

2.2. Plasma and Serum Samples. Plasma and serum samples were obtained following our standard procedures: a 4 mL K2E (EDTA) blood sampling tube and a 4 mL SSTII (serum separator tube) blood sampling tube were used for collection of plasma or serum, respectively (both BD vacutainers, Plymouth, UK). The blood sampling tubes were centrifuged (1300 ×g, 10 min at 4°C) within 1 hour. Plasma and serum

Table 1: Clinicopathological characteristics.

Variable	$N = 244$
Size, mean (SD)	29.2 (22.5)
pT (3cat.), no. (%)	
1	90 (42%)
2	110 (51%)
3/4	15 (7%)
pN (3cat.), no. (%)	
0	127 (59%)
1	57 (26%)
2/3	33 (15%)
Grade, no. (%)	
1	37 (15%)
2	115 (48%)
3	89 (37%)
LVI, no. (%)	
No	156 (68%)
Yes	73 (32%)
Histological subtype, no. (%)	
IDC	190 (78%)
ILC	33 (14%)
Mixed	1 (0%)
Other	20 (8%)
Multifocal tumour, no. (%)	
No	202 (83%)
Yes	42 (17%)

were then isolated and immediately frozen in aliquots at −20°C. Samples were transferred to −80°C for long-term storage.

Plasma CCL5 levels were successfully measured in 238 patients. Serum CCL5 levels were measured in a subgroup of 149 patients.

2.3. ELISA. Circulating CCL5 levels were assessed by enzyme-linked immunosorbent assay (ELISA) using the Quantikine ELISA kit for Human CCL5/RANTES (R&D Systems, Minneapolis, USA, cat no. DRN00B) following manufacturer's instructions. Absorbance was measured at 450 nm. Readings at 570 nm were subtracted from these at 450 nm, to correct for plate imperfections. All samples were assessed in duplicate. On each microplate, a new standard curve was established by diluting a standard with known concentration.

Mean absorbance for the duplicate wells was used to determine the chemokine concentration for each sample, using a logistic curve-fitting algorithm. With appropriate dilutions, all absorbance values were within the linear portion of the standard curve. Concentrations read from the standard curve were multiplied by the dilution factor. The results were expressed as ng/mL.

2.4. Statistical Analysis. The association between CCL5 and categorical variables was tested by the Kruskal Wallis test. The Mann-Whitney U test was used for post-hoc pairwise

comparisons. The choice of these tests was motivated by a skew distribution of the observations on several markers. The association between CCL5 and continuous variables was tested by the Spearman correlation coefficient.

A multivariable linear model was used for testing the presence of an interaction effect between the different categories of lymph node involvement (pN) and tumour size (either categorical (pT) or continuous) on CCL5. The patients were classified into four stages according to TNM classification. Samples of patients with a pN2 or pN3 tumour were analysed together because of small numbers of samples of patients in these categories. Similarly, samples of patients with a pT3 or pT4 tumour were analysed together.

A multivariable logistic regression model was used to determine the area under the receiver operating characteristic curve (AUC) for the prediction of positive lymph nodes (pN+). Firstly, we determined the AUC for the predictors tumour size, lymphovascular invasion, and multifocality. Secondly, we determined the AUC if we additionally included CCL5 in the model.

All analyses were performed using SAS software, version 9.2 of the SAS system for Windows.

3. Results

3.1. CCL5 Levels in Plasma. Mean plasma CCL5 level was 81,37 ng/mL (SD 34,68). Mean plasma CCL5 levels tended to be higher in patients with node-positive breast cancer compared to these of patients with node-negative breast cancer. Moreover, the mean CCL5 levels increased with increasing nodal stage. However, the difference was not statistically significant (P 0.215). Next, the association between CCL5 levels in plasma and other known independent predictors of lymph node metastasis was calculated. Mean plasma CCL5 levels increased with increasing tumour size, measured as the categorical variable pT (P 0.068). The results are shown in Table 2. A similar trend was observed when tumour size was considered as a continuous variable (P 0.0686, Spearman correlation coefficient 0.120).

To investigate whether the association between pN and CCL5 was different for distinct categories of tumour size, we allowed an interaction effect between pN and pT. However, the result was not significant (Table 4(a)). After correction for pT, there was no evidence of a residual main effect of CCL5 on pN (Table 4(b)).

Plasma CCL5 levels were higher in patients with lymphovascular invasion (P 0.087), multifocal tumours (P 0.364), and higher tumour stages (P 0.168).

Based on tumour size, lymphovascular invasion, and multifocality, the presence of lymph node metastasis could be predicted with an AUC of 0.74. Addition of plasma CCL5 levels to these variables had no impact on the AUC.

3.2. CCL5 Levels in Serum. Mean serum CCL5 level was 66,83 ng/mL (SD 20,89). Mean serum CCL5 levels were higher in patients with node-positive breast cancer compared to those in patients with node-negative breast cancer, although this was not statistically significant (P 0.098). Next,

TABLE 2: Association between plasma CCL5 levels (ng/mL) and tumour characteristics.

Variable	Statistics				
	N	Mean ± SD	Median	Range	P value
CCL5 plasma					
pN	**238**	**81,37 ± 34,68**	**78,16**	**1,72–208,12**	
0	123	79,13 ± 33,87	73,40	5,76–194,20	
1	56	83,27 ± 37,01	84,22	1,72–189,28	0.215
2/3	32	88,34 ± 33,19	89,70	25,32–208,12	
CCL5 plasma					
pT	**238**	**81,37 ± 34,68**	**78,16**	**1,72–208,12**	
1	87	76,16 ± 33,27	73,40	1,72–178,00	
2	107	83,22 ± 32,80	84,11	23,29–189,28	0.068
3/4	15	104,06 ± 47,52	96,86	43,89–208,12	
CCL5 plasma					
LVI	**238**	**81,37 ± 34,68**	**78,16**	**1,72–208,12**	
No	151	79,24 ± 35,21	74,24	1,72–194,20	0.087
Yes	72	87,00 ± 35,26	83,62	10,93–208,12	
CCL5 plasma					
Multifocal	**238**	**81,37 ± 34,68**	**78,16**	**1,723–208,12**	
No	194	80,36 ± 35,52	77,48	1,723–208,12	0.364
Yes	41	85,14 ± 31,39	78,48	24,85–178,00	

P values are based on a Kruskal Wallis test (no. of levels > 2).
Min: the lowest value, max: the highest value, Q1: percentile 25, Q3: percentile 75, SD: standard deviation.

the association between CCL5 levels in serum and other known independent predictors of lymph node metastasis was calculated. Mean serum CCL5 levels increased with increasing tumour size (P 0.009), measured as the categorical variable pT. These results are shown in Table 3(a). Pairwise comparisons of the 3 pT categories with and without correction for multiple testing are shown in Table 3(b). When tumour size was considered as a continuous variable, the P value for correlation between CCL5 levels and tumour size was 0.0194. The Spearman correlation coefficient was 0.196.

Again, the interaction effect between pN and pT was not significant (Table 4(a)), indicating that the observed association between pN and CCL5 was not different for distinct categories of tumour size. After correction for pT, there was no evidence for a main effect of CCL5 on pN (Table 4(b)).

Serum CCL5 levels were higher in patients with lymphovascular invasion, (P 0.408), multifocal tumours (P 0.319), and higher tumour stages (P 0.231).

Based on tumour size, lymphovascular invasion, and multifocality, the presence of lymph node metastasis could be predicted with an AUC of 0.68. The AUC with addition of serum CCL5 levels was 0.69.

4. Discussion

We observed that mean CCL5 concentrations in plasma and serum of patients with breast cancer are elevated in patients with lymph node-positive breast cancer compared to those in

TABLE 3: (a) Association between serum CCL5 levels (ng/mL) and tumour characteristics. (b) Pairwise comparison between serum CCL5 levels (ng/mL) and pT.

(a)

Variable		Statistics			
	N	Mean ± SD	Median	Range	P value
CCL5 serum					
pN	**149**	**66,83 ± 20,89**	**64,37**	**19,72–145,36**	
0	78	64,40 ± 22,02	60,91	31,89–145,36	
1	39	68,90 ± 17,97	72,31	30,69–104,37	0.098
2/3	16	67,49 ± 17,09	66,31	32,45–102,75	
CCL5 serum					
pT	**149**	**66,83 ± 20,89**	**64,37**	**19,72–145,36**	
1	51	60,23 ± 19,24	59,77	30,69–121,86	
2	70	67,02 ± 16,73	66,34	38,94–120,93	0.009
3/4	10	82,40 ± 28,93	76,17	45,27–145,36	
CCL5 serum					
LVI	**149**	**66,83 ± 20,89**	**64,37**	**19,72–145,36**	
No	101	66,98 ± 20,20	64,27	3189–145,36	
Yes	38	63,02 ± 19,54	63,41	19,72–104,19	0.408
CCL5 serum					
Multifocal	**149**	**66,83 ± 20,89**	**64,37**	**19,72–145,36**	
No	120	67,46 ± 21,75	64,32	19,72–145,36	
Yes	26	61,68 ± 15,19	63,87	33,97–87,83	0.319

P values are based on a Kruskal Wallis test (no. of levels > 2).
P values of pairwise comparisons are shown in next table.
Min: the lowest value, max: the highest value, Q1: percentile 25, Q3: percentile 75, SD: standard deviation.

(b)

Variable	pT (3cat.)		P values	
			Raw	Bonferroni
CCL5 serum	—	—	**0.009**	—
	1	2	0.027	0.082
		3/4	0.013	0.039
	2	3/4	0.077	0.231

P value in bold refers to the result of the Kruskal Wallis test.
Mann-Whitney U tests are used for pairwise comparisons. Raw P values as well as P values corrected for multiple testing are given.

TABLE 4: (a) Interaction effect between pN and pT. (b) Effect of pN after correction for pT.

(a)

Variable	Model deviance		Chi^2	DoF	P value
	Reduced	Full			
CCL5 plasma	4930.08	4926.33	3.75	4	0.4408
CCL5 serum	2945.16	2940.84	4.33	4	0.3635

Result of likelihood ratio test comparing the deviance (minus 2∗loglikelihood) of two nested models. Chi^2: chi-square statistic, DoF: degrees of freedom.

(b)

Variable	Model deviance		Chi^2	DoF	P value
	Reduced	Full			
CCL5 plasma	4930.79	4930.08	0.71	2	0.7003
CCL5 serum	2947.64	2945.16	2.48	2	0.2899

Result of likelihood-ratio test comparing the deviance (minus 2∗loglikelihood) of two nested models. Chi^2: chi-square statistic, DoF: degrees of freedom.

First, discrepancies in the reported effect of CCL5 in breast cancer may be due to inclusion of different tumour subtypes. It has been shown that CCL5 signalling is preferentially active in the basal and Her2 subtypes [4]. Additionally, it is possible that the sample size in our study is too small to measure small, but clinically relevant differences.

Secondly, tumour-derived CCL5 expression alone may not result significantly in breast cancer progression [3].

Thirdly, the levels of CLL5 in peripheral blood might not be comparable to CCL5 levels measured in tumour tissue.

Finally, CCL5 could be a marker of tumour load rather than of lymph node involvement. Our finding that CCL5 levels are correlated with tumour stage supports this finding.

To date, there are no reports of the association between CCL5 levels in serum or plasma and the presence of lymph node involvement in breast cancer, nor has the correlation between circulating CCL5 levels and breast tumour size been previously described. Concerning the association of CCL5 levels in blood with breast cancer progression, Niwa et al. [5] reported elevated levels of CCL5 in patients with stage II, III, or IV breast cancer. In addition, they demonstrated that the correlation of elevated CCL5 content in diseased tissue was much stronger than the correlation of elevated plasma CCL5, which suggests some fundamental role of CCL5 in the lesions. Kim et al. reported elevated levels of circulating CCL5 in plasma of patients with metastatic gastric cancer [6]. Furthermore, Tsukishiro et al. showed that elevated serum CCL5 levels in patients with ovarian cancer correlate with the extent of the disorder [7].

Interestingly, Lin et al. recently reported that CCR5 and CCL5 were highly expressed locally in tissue of breast cancer lymph node metastases [8].

Also, numerous studies have demonstrated that elevated levels of CCL5 correlate with progression of breast cancer [9–11], pancreatic cancer [12], colorectal cancer [13, 14], prostate cancer [15], and oral cancer [16]. Most of these studies measured CCL5 expression on tissue and compared

patients with lymph node-negative breast cancer. However, this difference was not statistically significant. Moreover, we observed that CCL5 levels significantly increased with increasing tumour size.

Addition of circulating CCL5 concentration to known clinicopathological predictors for lymph node involvement did not allow more precise prediction of the lymph node status.

Nevertheless, CCL5 levels in plasma and serum are correlated with tumour progression. CCL5 levels in patients with stage I disease were lower compared to those in patients with stages II and III disease (plasma P 0.168, serum P 0.231).

There may be different reasons why the mean CCL5 levels are only slightly elevated in blood of node-positive patients.

the expression between patients with benign lesions, early invasive cancer, and metastatic cancer.

Growing evidence indicates that tumour development and progression not only rely on cancer cells but also on cancer-related inflammation generated within the tumour microenvironment. Interactions between inflammatory factors, tumour cells, and immune system components recruited into the tumour microenvironment are crucial for disease progression [17].

Accumulated clinical and experimental data indicate that immune cells influence cancer development and progression. Acute tumour-directed immune responses involving cytolytic T lymphocytes appear to protect against tumour development, whereas immune responses involving chronic activation of humoral immunity, infiltration by Th2 cells, and pro-tumour polarized innate inflammatory cells result in the promotion of tumour development and disease progression.

It has been suggested that CCL5 expression by breast tumour cells results not only in monocyte migration to the tumour site, but also in increased production and recruitment of pro-inflammatory cytokines as well as recruitment and activation of inflammatory cells that may facilitate metastasis formation [4].

Zuckerman et al. [1] recently suggested that alterations in overall immune function underlie the risk for lymph node metastasis in patients with breast cancer.

These findings confirm the importance of studying immune cell function in the setting of breast cancer. A better understanding of the immunosuppressive environment is needed, in order to monitor and manipulate the individual's immune status in an appropriate way to ensure optimal treatment. Reactivation of anti-tumour adaptive immune responses could be of clinical benefit, either alone or in combination with conventional cancer therapies [18].

5. Conclusion

These data suggest that CCL5 is a biomarker for tumour load rather than for lymph node involvement. It might be helpful to identify patients with escape from immunosurveillance who benefit from therapies to restore their immune function.

Authors' Contribution

A. Smeets and B. Brouwers contributed equally to this paper.

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