

Chemokine profiling in serum from patients with ovarian cancer reveals candidate biomarkers for recurrence and immune infiltration

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Received June 11, 2018; Accepted November 8, 2018

DOI: 10.3892/or.2018.6886

Abstract. The management of advanced ovarian cancer is challenging due to the high frequency of recurrence, often associated with the development of resistance to platinum-based chemotherapy. Molecular analyses revealed the complexity of ovarian cancer with particular emphasis on the immune system, which may contribute to disease progression and response to treatment. Cytokines and chemokines mediate the cross-talk between cancer and immune cells, and therefore, present as potential biomarkers, reflecting the tumor microenvironment. A panel of circulating C-C motif chemokine ligand (CCL) and C-X-C motif chemokine ligand (CXCL) chemokines were examined in the serum of 40 high-grade patients with ovarian cancer prior to primary surgery. The level of immune infiltration in tumors was also analyzed. The preoperative levels of chemokines differ between patients. Elevated levels of circulating CXCL4+CCL20+CXCL1 combination can discriminate patients with shorter recurrence-free survival and overall survival. The presence of tumor-infiltrating T lymphocytes was detected in half of the patients. The mRNA expression analysis suggests the presence of antitumoral and immunosuppressive elements in the tumor microenvironment. The combination of circulating CXCL9+CXCL10 can distinguish immune-infiltrated tumors that will lead to shorter recurrence-free survival. The results suggest that preoperative profiling of circulating chemokines in patients with ovarian cancer may provide valuable information regarding tumor recurrence and immune infiltration. The findings demonstrate

that combinations have better prognostic utility than single chemokines, and may serve as patient stratification tools.

Introduction

The current situation in ovarian cancer (OC) management remains unsatisfactory, as the overall survival has hardly improved in the past decades (1,2). High-grade serous ovarian carcinoma (HGSOC), the most commonly diagnosed histological subtype, nearly always presents as advanced disease, and therefore, is associated with low overall survival (3). The standard treatment for primary OC is cytoreductive surgery followed by six cycles of adjuvant chemotherapy with carboplatin and paclitaxel. HGSOC tumors are initially responsive to platinum-based chemotherapy; however, the majority of patients eventually experience tumor recurrence (4). Platinum-free interval (PFI), the period from completion of primary chemotherapy to disease recurrence, serves as measure to further subdivide OC patients into the following groups based on their response to platinum: Refractory (PFI <1 month), resistant (PFI 1-6 months), par-sensitive (PFI 6-12 months) and sensitive (PFI >12 months) (5). Although currently this grouping serves as a practical guideline for secondary chemotherapy selection, it has limitations. Multiplex categorization of patients, incorporating the recent discoveries of molecular genetics, is suggested for future patient stratification in clinical trials (6).

Molecular analysis of HGSOC revealed its underlying genomic instability, DNA repair defects and copy number alterations (7). Furthermore, several independent groups were classified as HGSOC based on gene and microRNA expression patterns, with four largely overlapping molecular subtypes: C1/mesenchymal, C2/immunoreactive, C4/differentiated and C5/proliferative (7,8). Later studies emphasized an active role of the stromal tumor microenvironment in the pathogenesis of HGSOC and presented evidence for association between molecular subtypes and survival (9,10), in particular that the immunoreactive subtype-bearing patients, characterized by

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Key words: ovarian neoplasms, recurrence, chemokines, biomarkers, immunity

the elevated mRNA expression of chemokines, major histocompatibility complex class I/II, programmed cell death 1 ligand 1 (PD-L1), and interferon regulatory factor 7, have a better prognosis than the other subtypes (11). These findings emphasize the role of the immune system in OC, previously suggested in the study by Hwang *et al* (12), which demonstrated that tumor infiltrating lymphocytes (TILs) were correlated with increased overall survival in patients with OC. Recently, an immune score for OC was proposed, linking the compendium of immune marker genes with response to chemotherapy and survival. The infiltration of the majority subpopulations associated adaptive immunity [except regulatory T cells (Tregs)] was associated with favorable prognosis (13).

The role of the immune system in the development of chemoresistance and recurrence of OC remains elusive. The primary evidence for association of immune cells and chemosensitivity in OC was recently reported from *in vitro* and mouse model studies, which demonstrated the ability of T-cell surface glycoprotein CD8 (CD8)⁺ T cells to alter the metabolism of cytostatic drugs in fibroblasts (14) or negative regulation of PD-L1 on CD8⁺ T cells (15) to abrogate chemoresistance. However, the dual nature of the immune system is often exploited by tumor cells to create local immune suppression (16) and promote chemoresistance (17), as demonstrated in carcinomas of various origins (18,19). The nature of the cross-talk between cancer and other stromal cells is orchestrated by a cytokine and chemokine network, which can act both locally and systemically (20). The circulating cytokines and chemokines reflect the tumor microenvironment (21) and, therefore, are convenient candidates for the discovery of novel biomarkers as the basis for rational treatment decisions.

Despite frequent recurrence and limited effective treatment options, the selection of reliable prognostic and predictive biomarkers in HGSOC remains limited (22). The present study aimed to evaluate whether the levels of circulating signaling molecules can distinguish patients that are less likely to respond to chemotherapy. The immune profiles of HGSOC tumors were also evaluated to select circulating chemokines that reflect the immune infiltration.

Materials and methods

Patient cohort. A total of 40 patients with confirmed diagnosis of HGSOC of III-IV International Federation of Gynecology and Obstetrics stage (23) with no prior cancer history or immune disorders were involved in this study. All patients (age range 32-76 years) underwent primary cytoreductive surgery and completed six cycles of adjuvant carboplatin-based chemotherapy between April 2013 and April 2015 at the National Cancer Institute (Vilnius, Lithuania). For each patient, a pre-operative serum sample and surgically removed tumor specimen was collected. Clinical data were obtained from the patients' medical records. Patients were followed up until April 2018 to determine platinum status and recurrence. This study was approved by the Lithuanian Bioethics Committee (approval no. 158200-06-500-147). All patients signed informed consent form.

Sample preparation. Serum was centrifuged at 2,000 x g for 10 min at 22°C, aliquoted and stored at -80°C prior to

analysis. Tumor tissue was collected during surgery. Fresh tissue was immediately divided into four parts for enzymatic dissociation, protein extraction, RNA extraction and a fresh-frozen backup sample. All samples were processed on the same day. For preparing single cell suspensions, tumor tissue was incubated in digestion solution [5 mg/ml collagenase II, 5 mg/ml collagenase IV and 1 mg/ml DNase, all Sigma-Aldrich (Merck KGaA, Darmstadt, Germany)] for 30 min at 37°C. Following gentle pipetting, the solution was filtered and washed with PBS. For red blood cell lysis, the cell pellet was treated with 5 ml 1X BD FACS Lysing solution (BD Biosciences, San Jose, CA, USA) for 10 min at 22°C and washed with PBS. For protein extraction, tumor tissue was homogenized and lysed using T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc.), followed by 15 min centrifugation at 10,000 x g at 4°C and debris removal. For RNA extraction, tumor tissue was homogenized with TRIzol reagent from the TRIzol Plus RNA Purification kit (Thermo Fisher Scientific, Inc.) and RNA was purified according to the manufacturer's protocol.

Cytokine and chemokine measurement. Serum samples were analyzed using the Proteome Profiler Human Cytokine Array kit (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions. Membranes with cytokine expression signal were scanned with LI-COR C-DiGit blot scanner (LI-COR Biosciences, Lincoln, NE, USA) and analyzed with ImageJ software (v1.50f; National Institute of Health, Bethesda, MD, USA).

A panel of 13 chemokines [C-C motif chemokine ligand (CCL) 2, -3, -4, -5, -11, -17 and -20; C-X-C motif chemokine ligand (CXCL) 1, -5, -8, -9, -10 and -11) was measured in all patients serum and tumor lysate samples using LEGENDplex Human Proinflammatory Chemokine Panel (BioLegend, Inc., San Diego, CA, USA), a bead-based immunoassay, according to the manufacturer's guidelines. Samples were assayed in duplicates in 96-well plates, collected with BD LSR II flow cytometer (BD Biosciences) and analyzed with LEGENDplex data analysis software (V7.1; BioLegend, Inc.).

Evaluation of gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For cDNA synthesis, 500 µg RNA from each sample was reverse transcribed using Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed in duplicate in Eco Real-Time thermocycler (Illumina, Inc., San Diego, CA, USA). The reaction volume of 10 µl contained 5 µl Maxima SYBR-Green qPCR Master Mix 2X (Thermo Fisher Scientific, Inc.), 2.5 µl 0.8 µmol/l sequence-specific forward and reverse primers mix, 1 µl cDNA reaction product, and 1.5 µl nuclease-free water. The reaction was started by 5 min at 95°C and continued with 40 cycles of 10 sec denaturing at 95°C and 30 sec of annealing/extension at 60°C. Primer sequences are listed Table I. The expression level of selected genes was evaluated with EcoStudy software (Illumina, Inc.), using GAPDH and ribosomal protein L13 as the reference genes. The analysis was performed using $\Delta\Delta Cq$ relative quantitation method (24) with Pfaffl correction for PCR

Table I. Primer sequences.

Gene ID	Protein alias	Forward primer (5'-3' sequence)	Reverse primer (5'-3' sequence)
<i>CD163</i>	CD163	CGAGTTAACGCCAGTAAGG	GAACATGTCACGCCAGC
<i>CD274</i>	PD-L1	TATGGTGGTGCCGACTACAA	TGGCTCCCAGAATTACCAAG
<i>CD4</i>	CD4	TGCCTCAGTATGCTGGCTCT	GAGACCTTTGCCTCCTTGTT
<i>CD68</i>	CD68	TGGGGCAGAGCTTCAGTTG	TGGGGCAGGAGAACTTTGC
<i>CD8A</i>	CD8	ACTTGTGGGGTTCCTTCTCCT	GTCTCCCGATTTGACCACAG
<i>CTLA4</i>	CTLA-4	TGCAGCAGTTAGTTCGGGGTTGTT	CTGGCTCTGTTGGGGGCATTTTC
<i>CXCL10</i>	CXCL10	AAGGATGGACCACACAGAGG	ACCCTTGGAAGATGGGAAAG
<i>CXCL11</i>	CXCL11	ATGAGTGTGAAGGGCATGGC	TCACTGCTTTTACCCAGGG
<i>EOMES</i>	EOMES	AGCTCTCCAAGGAGAAAGTG	GCCTTCGCTTACAAGCACTG
<i>FCGR2A</i>	CD32	TTTGAGATGAGTAATCCCAGCCA	TCAGGCCAGTCTCCATTTTA
<i>FOXP3</i>	FOXP3	GAACGCCATCCGCCACAACCTGA	CCCTGCCCCACCACCTCTGC
<i>GAPDH</i>	GAPDH	AATCCCATCACCATCTTCCA	TGGACTCCACGACGTACTCA
<i>GZMB</i>	Granzyme B	GAAACGCTACTAACTACAGG	CCACTCAGCTAAGAGGT
<i>IDO1</i>	IDO	GGCAAAGGTCATGGAGATGT	CAGGACGTCAAAGCACTGAA
<i>IFNG</i>	IFN γ	TGGAAAGAGGAGAGTGACAGAA	TCTTTTGGATGCTCTGGTCAT
<i>IL10</i>	IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
<i>PDCD1</i>	PD-1	ACCCTGGTCATTCACCTGGG	CATTTGCTCCCTCTGACACTG
<i>PSMB9</i>	PSMB9	GCACCAACCGGGGACTTAC	CACTCGGGAATCAGAACCCAT
<i>RLP13</i>	RLP13	GAGGTATGCTGCCCCACAA	GTGGGATGCCGTCAAACA
<i>TAP1</i>	TAP1	TGCCCCGCATATTCTCCCT	CACCTGCGTTTTTCGCTCTTG
<i>TAP2</i>	TAP2	TGGACGCGGCTTTACTGTG	GCAGCCCTCTTAGCTTTAGCA
<i>TIGIT</i>	TIGIT	TCTGCATCTATCACACCTACCC	CCACCACGATGACTGCTGT

efficiency (25). Relative gene expression was z-transformed, visualized and clustered using K-means clustering by Euclidean distance for selection of non-inflamed (z-score negative) and inflamed patient (z-score positive) clusters with Morpheus software (Broad Institute, Cambridge, MA, USA).

Flow cytometry. Single tumor cell suspension was stained for 20 min at 4°C with pre-titrated amounts of monoclonal mouse anti-human antibodies protein tyrosine phosphatase, receptor type C (CD45)-V450 (cat. no. 560367; BD Biosciences) at 1:20 dilution and T-cell surface glycoprotein CD3 (CD3)-allophycocyanin (cat. no. 300312; BioLegend, Inc.) at 1:50 dilution. Cells were washed, stained with 5 μ g/ml viability dye 7-aminoactinomycin D (BD Biosciences) for 5 min at 22°C, collected with BD LSR II flow cytometer (BD Biosciences), and analyzed using BD FACSDiva software (v6.2; BD Biosciences).

Histological assessment of tumor tissue. Tumor tissue was fixed in 4% paraformaldehyde (Sigma-Aldrich; Merck KGaA) for 24 h at 22°C. Tissue sections from paraffin-embedded blocks (4 μ m-thick) were stained with Mayer's hematoxylin solution for 40 sec and 1% eosin for 30 sec (H&E; all Sigma-Aldrich; Merck KGaA) at 22°C. Tumor type and grade were assessed (26,27). Qualitative evaluations for the presence of either intraepithelial or stromal T lymphocytes within tumor tissue were conducted by the pathologist.

Statistical analysis. Data were analyzed and visualized using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla,

CA, USA) statistical software. The associations between systemic and local chemokine expression and clinical characteristics were evaluated by the Spearman rank correlation coefficient. Differences in chemokine levels between patients were determined with a Mann-Whitney U test. Differences in patient characteristics between groups were identified by Kruskal-Wallis test. Receiver operator characteristic (ROC) curves were created to determine the predictive performance of the cytokines and their combinations. The area under curve (AUC), sensitivity and specificity were calculated from ROC curves. Performance metrics and clinical utility were calculated and converted into qualitative grades: Excellent utility, ≥ 0.81 ; good, ≥ 0.64 ; fair, ≥ 0.49 ; and poor, < 0.49 , as suggested previously (28). To combine chemokines, logistic regression was applied. The best combination in each setting was selected based on the AUC and sensitivity. For combinations with equally good characteristics, a classifier with the least number of elements was chosen. The Kaplan-Meier survival curves and hazard ratios were analyzed with a log-rank test. Cohort clinicopathological features were compared with the χ^2 test. $P < 0.05$ was considered to indicate a statistically significant difference. Given the exploratory nature of this study, P-values were not adjusted for multiple testing.

Results

Patient characteristics. All patients involved in this study (n=40) were diagnosed with stage III (90%) or IV (10%) HGSOC. Following the complete resection of tumor foci, all patients completed six cycles of adjuvant chemotherapy with

Table II. Overview of patient characteristics (n=40).

Feature	Pt-resistant (PFI <6 months)	Pt-par-sensitive (PFI 6-12 months)	Pt-sensitive (recurrent PFI >12 months)	Pt-sensitive (non-recurrent)	P-value
N	15	7	7	11	
Age					0.335
Median (years)	62	69	60	67	
Range	53-76	53-75	32-72	32-74	
Stage					0.639
III	13 (87%)	6 (86%)	6 (86%)	11 (100%)	
IV	2 (13%)	1 (14%)	1 (14%)	0 (0%)	
RFS (median months)	4.1	9.7	21.0	NR	<0.0001
OS (median months)	27.5	21.0	NR	NR	0.0024

Differences between groups were identified by Kruskal-Wallis test. Pt, platinum; PFI platinum-free interval; RFS, recurrence-free survival; OS, overall survival; NR, not reached.

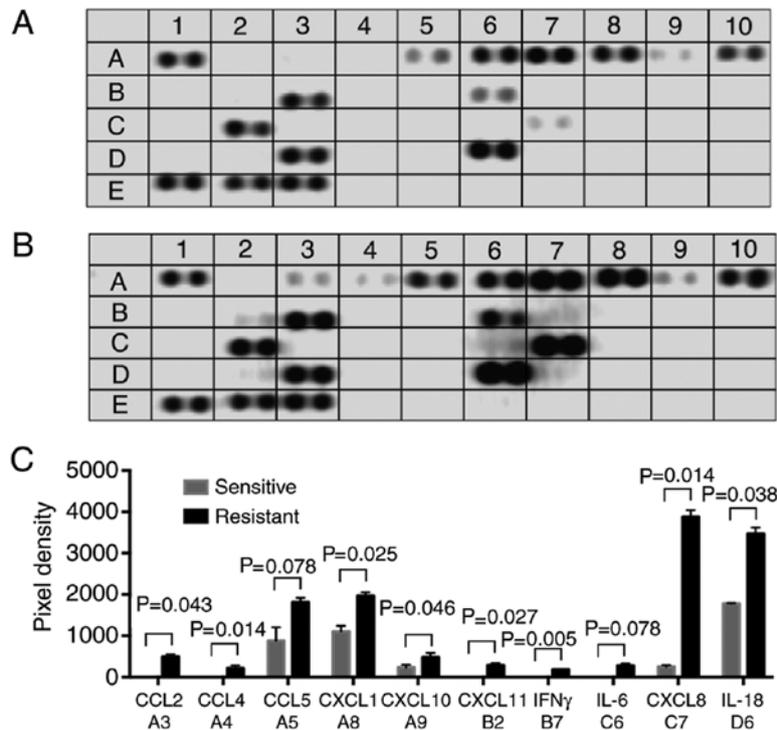


Figure 1. Serum cytokine levels in patients with ovarian cancer. The representative cytokine array membrane scans of (A) sensitive and (B) resistant patients (n=4 for each group) are presented. (C) Semi-quantitative results of differentially expressed cytokines, presented as mean with standard deviation and compared using Mann-Whitney U test. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

carboplatin and paclitaxel. The median follow up time was 46 months. The patients were regularly tested for the blood CA125 level. Recurrence was confirmed by radiological imaging. Recurrence of primary disease occurred in 73% of patients (n=29) during the follow-up period, with median recurrence-free survival (RFS) of 11.1 months. Recurrent patients were classified as platinum-resistant (PFI <6 months), par-sensitive (PFI 6-12 months) or sensitive (recurrent PFI >12 months or non-recurrent) based on the duration of PFI. An overview of patient clinical characteristics is presented in Table II.

Inflammatory serum protein profiling reveals differential chemokine expression among HGSOc patients. It was hypothesized that profiling of multiple inflammatory markers could help unveil the role of inflammation in ovarian cancer progression. The relative level of 36 different cytokines, chemokines and acute phase proteins was analyzed in the serum of platinum-resistant (Fig. 1A) and non-recurrent platinum-sensitive patients (Fig. 1B; n=4 for each group). Out of 18 proteins detected, 10 proteins were to be more abundant in sera of platinum-resistant patients compared with platinum-sensitive patients (Fig. 1C). The majority of the proteins were members of the CC and CXC chemokine families.

Table III. Correlations between circulating preoperative serum chemokine concentrations and their gene and protein expression level in tumor, tumor infiltration with immune cells and patient survival.

Serum chemokine	Relative gene expression of respective chemokine in tumor	Respective chemokine concentration in tumor lysate	Total immune tumor infiltration (CD45)	Immune infiltration with T lymphocytes (CD45 ⁺ /CD3 ⁺)	RFS	OS
CCL2	0.206	0.196	0.302	0.327	-0.220	-0.115
CCL3	0.658	0.202	0.200	0.214	-0.384	0.118
CCL4	0.175	0.245	0.095	0.234	-0.210	-0.162
CCL5	0.194	0.175	-0.096	-0.077	-0.120	-0.115
CCL11	0.235	0.153	0.309	0.120	-0.256	0.027
CCL17	0.246	0.261	-0.034	-0.121	0.024	0.060
CCL20	0.596	0.391	0.029	0.008	-0.320	-0.154
CXCL1	0.390	0.410	0.118	0.017	-0.106	-0.022
CXCL5	0.296	0.234	-0.002	-0.048	-0.170	-0.264
CXCL8	0.196	0.105	0.151	0.051	-0.129	0.096
CXCL9	0.423	0.257	0.378	0.458	-0.455	-0.295
CXCL10	0.607	0.511	0.475	0.393	-0.379	0.067
CXCL11	0.335	0.119	0.179	0.238	-0.194	-0.172

Correlations evaluated by Spearman rank correlation. Data in bold show significant differences with $P < 0.05$. CD45, receptor-type tyrosine-protein phosphatase C; CD3, T-cell surface glycoprotein CD3; RFS, recurrence-free survival; OS, overall survival; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

Therefore, the panel of interest was expanded and the concentration of thirteen selected CC and CXC family chemokines was quantified (CCL2, -3, -4, -5, -11, -17 and -20, CXCL1, -5, -8, -9, -10 and -11) in patient serum samples. To examine how the serum chemokine levels reflect the tumor microenvironment and disease course, the correlation with intratumoral chemokine expression, immune cell infiltration, RFS and overall survival (OS) were assessed (Table III). Although all serum chemokines were positively associated with gene and protein expression in tumors, their correlation coefficients varied in magnitude. Among serum chemokines, CXCL9 and CXCL10 levels were positively correlated with the intratumoral infiltration of CD45⁺ cells (total leukocytes) and CD45⁺/CD3⁺ cells (total TILs), as measured by flow cytometry. These two chemokines, along with CCL3 and CCL20, were significantly negatively associated with RFS. Serum chemokines did not have any significant correlations with OS.

Combination of circulating CCL4 + CXCL1 + CCL20 predicts recurrence-prone tumors. Due to the association of preoperative circulating chemokine levels with RFS, the patients were subsequently grouped based on their recurrence status and the serum chemokine expression patterns were analyzed. Significantly higher levels of CCL3, CCL4, CCL20 and CXCL1 were present in recurrent patient serum compared with non-recurrent patients (Table IV). To evaluate the predictive value of each chemokine, ROC curve analysis was performed to calculate the cut-off, area under curve (AUC) and sensitivity at clinically relevant specificity. Among single chemokines, CCL4 had the best sensitivity (62%) and fair positive clinical utility (CUI) at a cut-off of 20 pg/ml. Subsequently, the performance of single chemokines was compared with their

multiple combinations in order to select the best biomarkers that may potentially identify the patients in a higher recurrence risk group. All possible double, triple and quadruple combinations were assessed, and then the combination with the highest AUC and least number of elements was selected. In the recurrent cancer setting, combining CCL4, CXCL1 and CCL20 into a single classifier resulted in 81% sensitivity. The combination of CCL4 + CXCL1 + CCL20 also had good positive and fair negative clinical utility. ROC curves for CCL4 and CCL4 + CXCL1 + CCL20 classifier highlight the advantage of the chemokine combination (Fig. 2A). The combination of CCL4 + CXCL1 + CCL20 could also predict RFS and OS (Fig. 2B and C). The individuals with elevated serum levels of these three markers had greater chances of disease recurrence (HR=4.08) and worse survival prognosis (HR=5.10).

Subsequently, it was sought to determine whether preoperative circulating chemokine levels were able to predict the PFI. There was no difference in serum chemokine concentrations in patients with PFI <6 months compared with PFI >6 months. However, CCL2, CXCL9, CXCL10 and CCL11 concentrations were higher in the serum of patients with PFI <12 months compared with PFI >12 months (Table IV). The sensitivity of single markers at 94% specificity varied between 26 and 40%, resulting in the unsatisfactory clinical utility of these markers. Combining CXCL9 + CXCL10 or CXCL9 + CXCL10 + CCL11 resulted in increased sensitivity (44 and 50%, respectively). However, the AUC values of either single chemokines or their combinations did not surpass 0.750 (Fig. 2D). Nevertheless, patients with higher preoperative levels of CXCL9 + CXCL10 + CCL11 were more likely experience recurrence within 12 months (HR=3.05; Fig. 2E). No significant association with OS was observed (Fig. 2F).

Table IV. Overview of circulating chemokine levels, performance metrics and prognostic impact in recurrent vs. non-recurrent and PFI <12 months vs. PFI >12 months.

A, Recurrent vs. non-recurrent														
Chemokine	Recurrent (n=29)		Non-recurrent (n=11)		Cut-off (pg/ml)	AUC	P-value	SE (%)	SP (%)	RFS			OS	
	[mean (range) pg/ml]	[mean (range) pg/ml]	[mean (range) pg/ml]	[mean (range) pg/ml]						CUI +	CUI -	HR	P-value	HR
CCL20	46 (3-357)	12 (3-61)	32	0.794	0.006	31	91	0.267	0.303	1.486	0.363	2.855	0.041	
CXCL1	2,058 (118-7,542)	1,105 (46-3632)	2,028	0.762	0.015	42	91	0.388	0.339	1.549	0.267	1.854	0.350	
CCL3	9.0 (3-31)	3.8 (2-6)	5	0.787	0.007	54	91	0.501	0.387	2.740	0.007	2.567	0.064	
CCL4	28 (8-126)	14 (7-20)	20	0.758	0.016	62	91	0.582	0.422	3.162	0.002	2.594	0.072	
CCL4+CCL20+	-	-	-	0.854	-	81	91	0.763	0.583	4.077	0.002	5.100	0.016	
CXCL1														

B, PFI <12 months vs. PFI >12 months														
Chemokine	PFI <12 months (n=22)		PFI >12 months (n=18)		Cut-off (pg/ml)	AUC	P-value	SE (%)	SP (%)	RFS			OS	
	[mean (range) pg/ml]	[mean (range) pg/ml]	[mean (range) pg/ml]	[mean (range) pg/ml]						CUI +	CUI -	HR	P-value	HR
CCL2	1,710 (374-3,744)	1,015 (193-3,021)	2,409	0.701	0.023	26	94	0.222	0.501	1.656	0.303	1.501	0.591	
CXCL9	178 (10-953)	66 (10-287)	179	0.699	0.039	26	94	0.222	0.521	2.811	0.027	1.762	0.375	
CXCL10	1,495 (363-4,988)	869 (124-2,684)	1,916	0.707	0.034	33	94	0.288	0.547	1.813	0.224	1.519	0.575	
CCL11	224 (50-651)	159 (38-415)	221	0.704	0.036	40	94	0.363	0.547	3.010	0.003	1.681	0.330	
CXCL9+CXCL10	-	-	-	0.721	-	44	94	0.401	0.582	2.588	0.024	1.473	0.517	
CXCL9+CXCL10+	-	-	-	0.746	-	50	94	0.445	0.600	3.051	0.002	1.824	0.273	
CCL11														

Differences in cytokine levels were identified by Mann-Whitney U test. AUC and sensitivity at given specificity were calculated from the receiver operating characteristic curve. HR and their P-values were derived from log-rank test. PFI, platinum-free interval; AUC, area under curve; SE, sensitivity; SP, specificity; CUI, clinical utility; HR, hazard ratio; RFS, recurrence-free survival; OS, overall survival; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

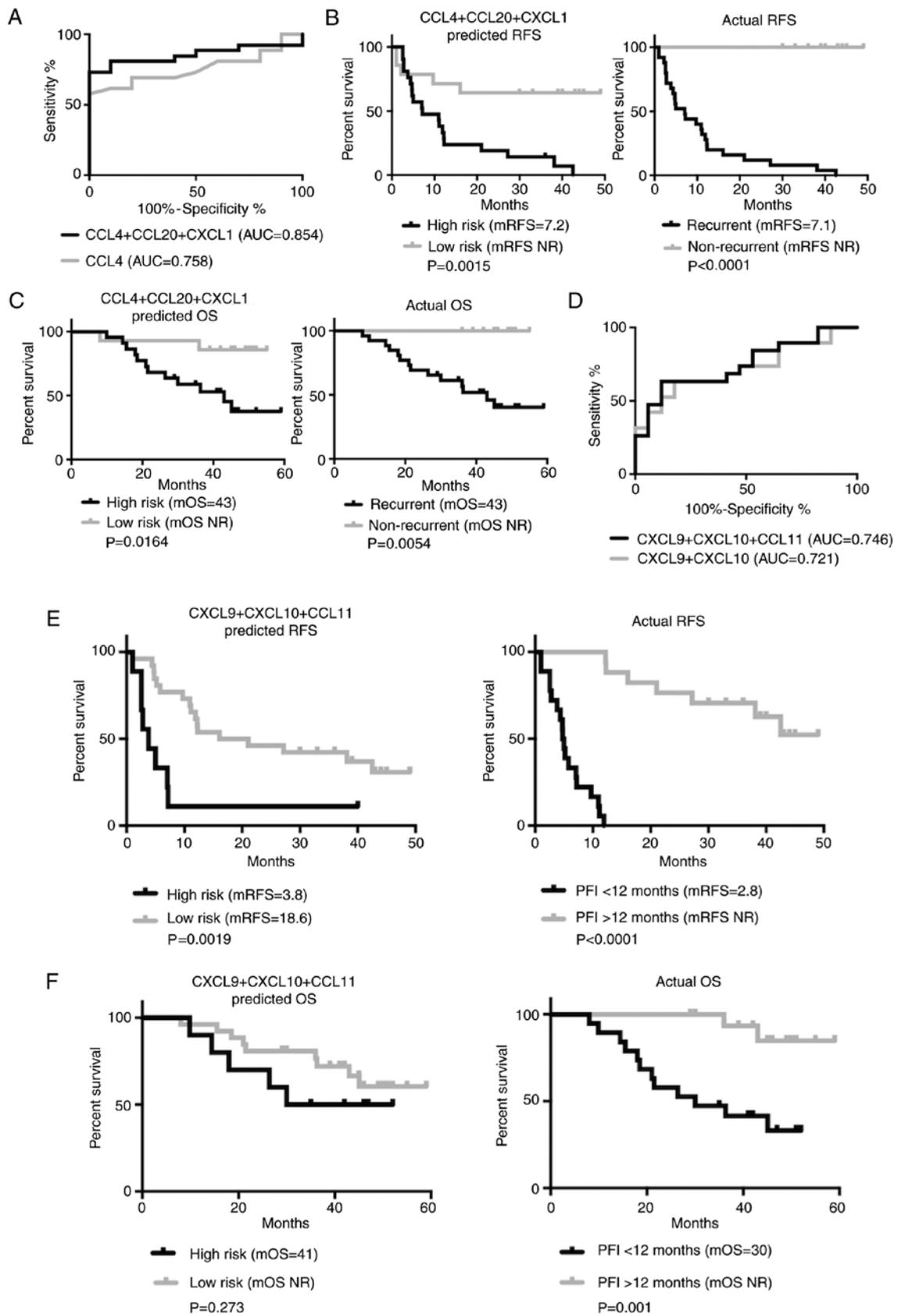


Figure 2. Performance and prognostic impact of CCL4+CCL20+CXCL1 (upper panels) and CXCL9+CXCL10+CCL11 (lower panels). (A) ROC curves of CXCL4+CCL20+CXCL1 and CCL4 alone in detecting recurrence-prone patients. (B) RFS and (C) OS estimates for patients with high risk or low risk of recurrence as predicted with CCL4+CCL20+CXCL1, compared to actual survival in recurrent vs. non-recurrent patients. (D) ROC curves of CXCL9+CXCL10 and CXCL9+CXCL10+CCL11 in detecting patients with PFI<12 months. (E) RFS and (F) OS survival estimates for patients with high risk or low risk of early (<12 months) recurrence as predicted with CXCL9+CXCL10+CCL11, compared to actual survival in patients with PFI<12 months vs. PFI>12 months. mRFS and mOS were calculated from Kaplan Meier survival curves. Survival curves were compared with log-rank test. ROC, receiver operating characteristic; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; AUC, area under curve; RFS, recurrence-free survival; mRFS, median RFS; NR, not reached; OS, overall survival; mOS, median OS; PFI, platinum-free interval.

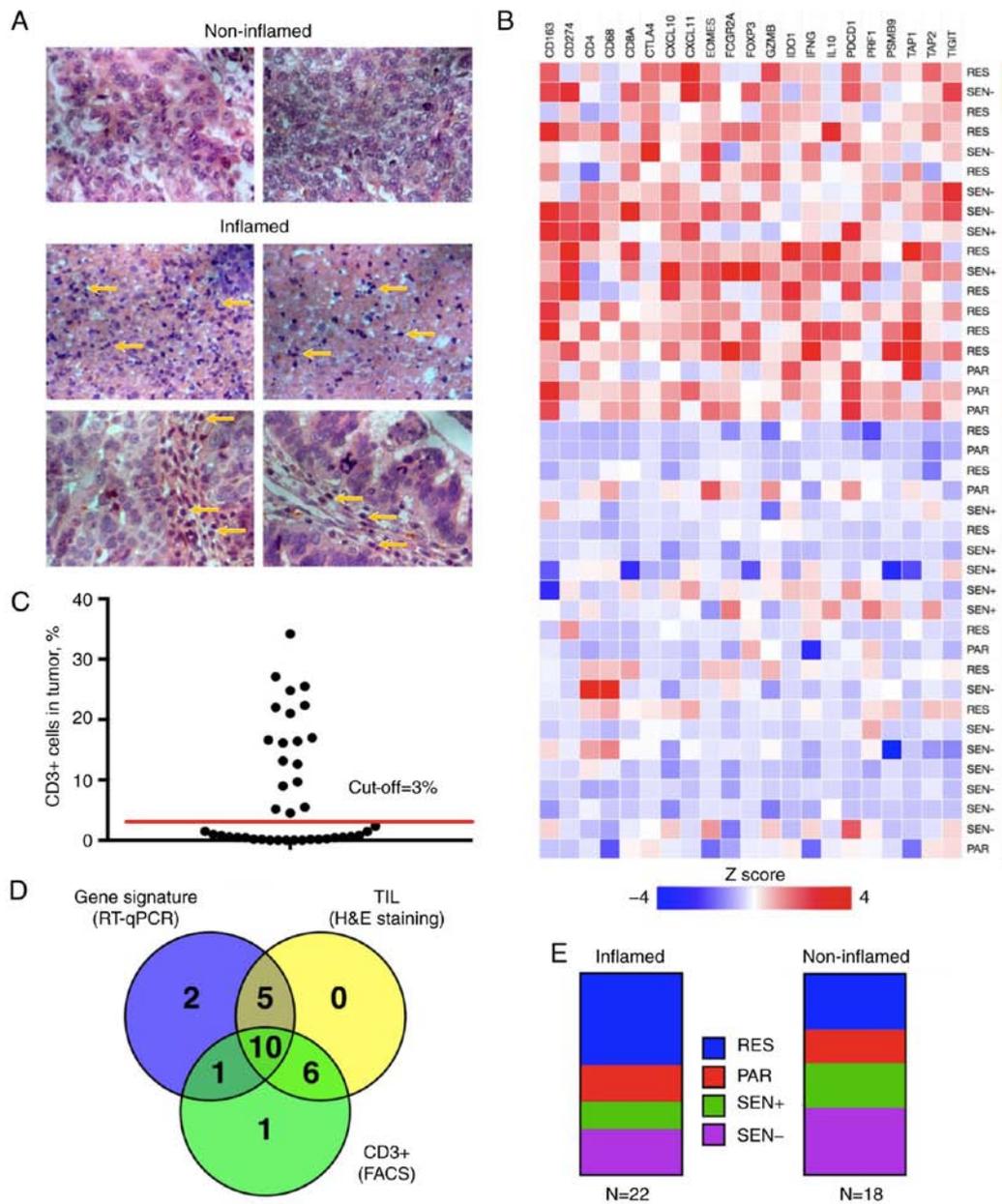


Figure 3. Selection of inflamed tumors. Patients were grouped based on three independent evaluations. (A) Tumors were classified as inflamed if intraepithelial or stromal T lymphocytes (indicated by arrows) were detected in H&E-stained tissues. (B) Z-transformed tumoral mRNA expression levels of immune response-associated genes, as measured with RT-qPCR, served as a basis for unsupervised clustering of patients into non-inflamed (z score below 0) and inflamed (z score above 0) groups (C) Level of CD3⁺ cells in tumor samples was evaluated using flow cytometry and a cut-off of 3% was applied to distinguish immune-infiltrated tumors. (D) Patients were assigned to the inflamed group if positive for at least two factors. (E) Inflamed and non-inflamed groups had a different proportional distribution of patients, based on their platinum sensitivity and recurrence status. CD3, T-cell surface glycoprotein CD3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; TIL, tumor infiltrating lymphocyte; H&E, hematoxylin and eosin; RES, platinum-resistant; PAR, partially platinum-sensitive; SEN+, recurrent platinum-sensitive; SEN-, non-recurrent-platinum-sensitive.

Immune-infiltrated tumors express more intratumoral chemokines. The patient cohort was divided into inflamed and non-inflamed tumor groups, based on three parameters: TILs in tumor sections, immune response-related gene expression in tumor tissue and CD3⁺ positive cell count in the tumor (Fig. 3). H&E-stained tumor tissue sections were evaluated by a pathologist for the presence or absence of both intraepithelial and stromal TILs (Fig. 3A), resulting in 21 TIL-positive and 19 TIL-negative samples. Immune response-associated gene expression was measured with RT-qPCR, and clustered into high-expression (18 samples) and low expression (22 samples)

clusters (Fig. 3B). The percentage of CD3⁺ cells in tumors was evaluated with flow cytometry analysis of freshly digested tumor tissue samples. Gating for live cells and CD45⁺ was applied. A cut-off of 3% CD3⁺ cells was applied to divide the study population into 18 samples with high and 22 samples with low immune infiltration in the tumor (Fig. 3C).

The distribution and overlap of samples, identified as TIL-positive, >3% CD3⁺ or high expression of immune-associated genes, is presented as a Venn diagram in Fig. 3D. Patients were assigned to the inflamed group if they were positive for at least two factors. Out of 25 samples, characterized by at

Table V. Overview of circulating chemokine levels, performance metrics, and prognostic impact in inflamed vs. non-inflamed, and inflamed PFI <12 months vs. other patients.

A, Inflamed vs. non-inflamed												
	Inflamed (n=22) [mean (range) pg/ml]	Non-inflamed (n=18) [mean (range) pg/ml]	P-value	AUC	Cut-off (pg/ml)	SE (%)	SP (%)	CUI +	CUI -	HR	RFS	P-value
CXCL11	870 (120-5,740)	406 (36-1,457)	0.033	0.709	983	30	94	0.263	0.486	2.234		0.0581
CCL2	1,796 (649-3,744)	864 (193-2,269)	0.001	0.825	2,134	30	94	0.263	0.486	1.660		0.3032
CXCL10	1,625 (217-4,988)	667 (124-2,187)	<0.001	0.841	1,410	40	94	0.355	0.521	0.949		0.9117
CXCL9	190 (10-953)	44 (10-174)	0.001	0.846	147	45	94	0.411	0.544	2.369		0.0347
CXCL9+CXCL10	-	-	-	0.900	-	70	94	0.751	0.736	2.444		0.0183
B, Inflamed PFI<12 months vs. other												
	Inflamed PFI<12 (n=14) [mean (range) pg/ml]	Other (n=26) [mean (range) pg/ml]	P-value	AUC	Cut-off (pg/ml)	SE (%)	SP (%)	CUI +	CUI -	HR	RFS	P-value
CXCL10	1,706 (530-4,988)	877 (124-2,648)	0.012	0.750	2,194	29	96	0.227	0.653	1.386		0.589
CXCL5	4,527 (1208-8,904)	3,066 (1,016-10,591)	0.016	0.740	5,590	36	96	0.302	0.667	2.136		0.091
CCL2	2,035 (712-3,744)	966 (193-3,021)	<0.001	0.854	2,409	36	96	0.302	0.667	1.871		0.239
CXCL11	1,116 (351-5,740)	376 (36-1,457)	<0.001	0.867	983	43	96	0.365	0.688	2.230		0.058
CCL3	11 (3-22)	5 (3-31)	0.002	0.808	9	64	96	0.580	0.768	2.704		0.010
CXCL9	253 (17-953)	44 (10-174)	<0.001	0.912	147	64	96	0.580	0.768	2.366		0.034
CXCL9+CXCL10	-	-	-	0.945	-	86	96	0.788	0.872	2.721		0.009

Differences in cytokine levels were identified by Mann-Whitney U test. AUC and sensitivity at given specificity were calculated from receiver operating characteristic curve. HR and P-values were derived from log-rank test. PFI, platinum-free interval; AUC, area under curve; SE, sensitivity; SP, specificity; CUI, clinical utility; HR, hazard ratio; RFS, recurrence-free survival; CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

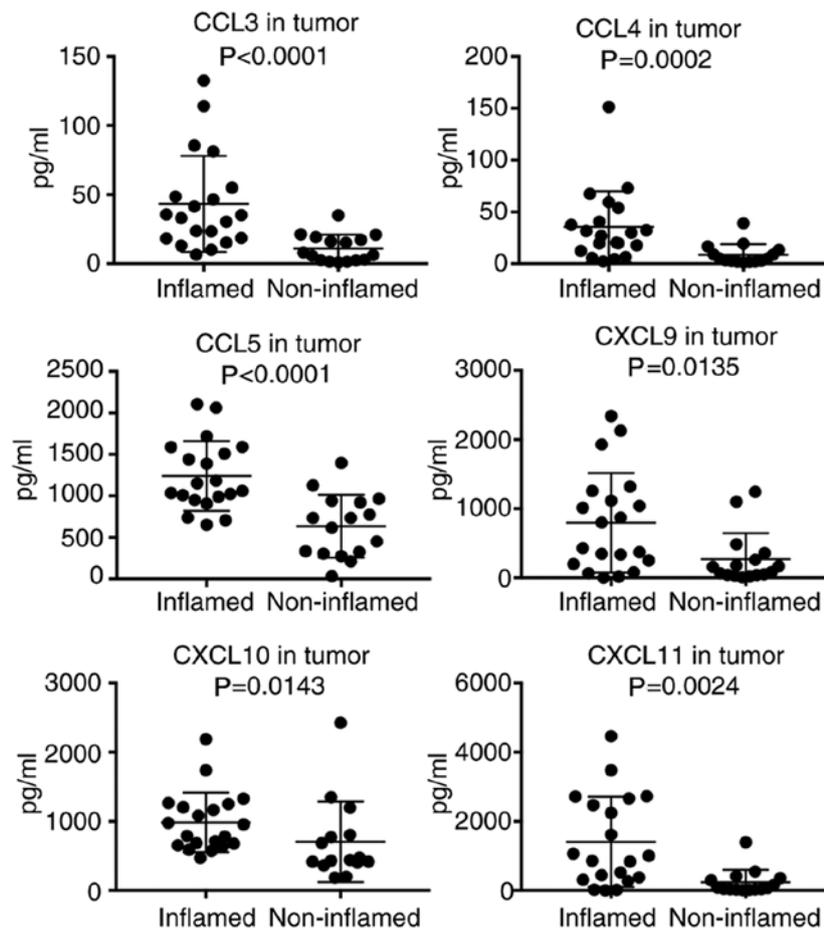


Figure 4. Inflamed tumors have increased levels of intratumoral chemokines. The concentration of chemokines was normalized to total protein concentration in tumor lysate. Scatter plots include mean \pm standard deviation. Differences in cytokine levels were identified by Mann-Whitney U test. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand.

least one positive inflammation-related factor, only 12% did not overlap with other factors, and thus were assigned to the non-inflamed group, together with the rest of the samples, which did not qualify for the inflamed group. Finally, 22 patients were allocated to the inflamed and 18 in the non-inflamed group. The proportional distribution of patients based on their platinum-resistance level and recurrence is presented in Fig. 3E.

This classification allowed identification of other significant differences between the groups, including the increased level of CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL11 (Fig. 4) in tumors from the inflamed group compared with the non-inflamed group.

Combination of circulating CXCL9 + CXCL10 detects inflamed tumors. Following grouping of the patients based on their tumor inflammation level, differences in serum level of CXCL11, CCL2, CXCL10 and CXCL9 were observed. The levels of these chemokines were significantly higher in the serum of patients in the inflamed group compared with the non-inflamed group (Table V). At 94% specificity, the sensitivity of single chemokines varied between 30 and 45%. Taken alone, these cytokines exhibited poor positive and fair negative clinical utility, despite good AUC values (>0.8). Following screening of all possible multiple chemokine combinations, the CXCL9 + CXCL10 classifier

performed best, with 70% sensitivity and 94% specificity it resulted in good positive and good negative clinical utility for discrimination of patients with inflamed tumors. CXCL9 + CXCL10 had improved AUC (0.900) compared with CXCL9 alone (0.846; Fig. 5A). Although the actual RFS curves of patients identified as inflamed or non-inflamed did not differ significantly (p=0.4102), patients with elevated serum levels of CXCL9 + CXCL10 had worse RFS (HR=2.44; Fig. 5B).

Circulating CXCL9 and CXCL10 were the two cytokines able to distinguish between inflamed and non-inflamed tumors, and patients with PFI < 12 months and PFI > 12 months, which encouraged assessment of whether circulating chemokines are able to detect immune-infiltrated tumors, which are more likely to recur within 12 months after completion of chemotherapy. For this, inflamed tumors group was narrowed into inflamed with PFI < 12 months (n=14). The patients in the inflamed PFI < 12 months group exhibited higher serum levels of CXCL10, CXCL5, CCL2, CXCL11, CCL3 and CXCL9 compared with the remaining patients, with the sensitivity of single chemokines varying from 29-64 at 96% specificity (Table V). CCL3 and CXCL9 alone had positive and good negative clinical utility. However, the AUC of CXCL9 was the best out of all single markers analyzed. Despite numerous potential multiple chemokine combinations, CXCL9 + CXCL10

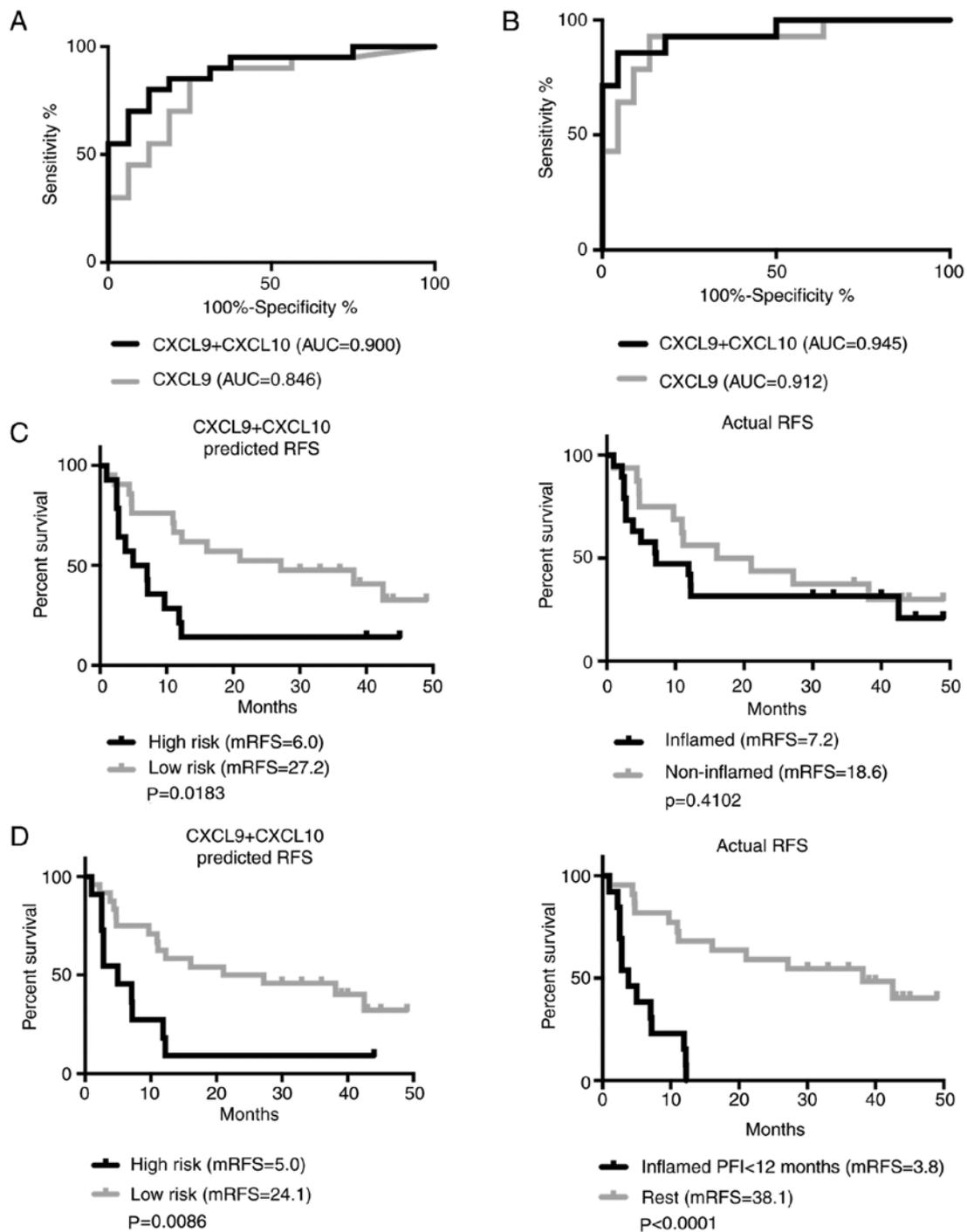


Figure 5. Performance and prognostic impact of CXCL9+CXCL10. ROC curves of CXCL9+CXCL10 and CXCL9 alone in detecting (A) inflamed or (B) inflamed with PFI<12 months patients. Survival estimates for patients with the high or low probability of immune tumor infiltration as predicted with CXCL9+CXCL10, compared to actual survival in (C) inflamed vs. non-inflamed patients or (D) inflamed PFI<12 months vs. remaining patients. mRFS was calculated from Kaplan Meier survival curves. Survival curves were compared with log-rank test. CCL, C-C motif chemokine ligand; CXCL, C-X-C motif chemokine ligand; AUC, area under curve; RFS, recurrence-free survival; mRFS, median RFS; PFI, platinum-free interval.

again had the most promising characteristics, reaching 86% sensitivity and 96% specificity. This combination had good positive and excellent negative clinical utility. Although AUC of CXCL9 alone was 0.912, the combination with CXCL10 performed even better with AUC of 0.945 (Fig. 5C). Patients with high preoperative CXCL9+CXCL10 levels were likely to belong to the inflamed group and recur within 12 months after completion of chemotherapy (Fig. 5D).

Discussion

Multidirectional cross-talk between various cell types in the tumor microenvironment and recruitment of immune cells is often mediated by the secretion of chemotactic factors (20). Chemokine signals may exhibit pleiotropic effects that go beyond their originally described function as chemoattractants, and can directly and indirectly participate in shaping the

immune contexture of the tumor (29). Studies demonstrated that the signaling components and metabolites of the tumor microenvironment can gain access to the bloodstream (21,30). Therefore, measuring chemokines in serum of patients with cancer presents as a relevant and convenient approach for identification of novel biomarkers, which may be beneficial for the personalized management of complex diseases, such as recurrent ovarian cancer.

In this study, CC and CXC family chemokines were differentially expressed in sera of patients with HGSOC. Their levels, negatively associated with RFS, reflect the corresponding intratumoral gene and protein expression. A combination of circulating preoperative CCL4 + CXCL1 + CCL20 chemokines was associated with shorter RFS and OS. The immune contexture of tumors was also highlighted and it was proposed that the CXCL9 + CXCL10 combination may be applicable for detecting immune-infiltrated tumors, including those which are more likely to recur within 12 months after completion of first-line chemotherapy.

Resistance to platinum-based chemotherapy remains the major cause of HGSOC recurrence. The second-line chemotherapy regimen decision is usually made according to the duration of PFI (5). There are no clinically useful biomarkers to predict the chemotherapy outcome ahead of treatment. As OC is often driven by somatic and germline mutations, attempts to classify tumors as platinum-sensitive or platinum-resistant have typically focused on tumor gene expression profiling and resulted in multiple predictive gene expression algorithms (31-33). Among circulating proteins, the classical OC marker CA-125 was previously demonstrated to be a positive response predictor if present at lower levels at the time of diagnosis (34); however, other studies contradict this finding (35) or emphasize another OC marker, HE4, as more specific in preoperative prediction of platinum sensitivity (36,37).

The development of chemoresistance is a complex process, depending on multiple factors, including intrinsic genetic and epigenetic alterations, cell metabolism, and even the tumor immune infiltration (38,39). The idea that the elements of host immunity may contribute to responsiveness and resistance to chemotherapy suggests the rationale for systemic analysis of soluble mediators. For example, platinum-based drugs and mitotic inhibitors are able to induce nuclear factor κ B (NF κ B)-mediated chemoresistance in cancer cells, which relies on the production of proinflammatory chemokines (40,41). We hypothesized that recurrence-prone patients may already exhibit altered levels of serum chemokines at diagnosis. Increased levels of CCL4, CXCL1 and CCL20 chemokines were detected in patients that later experienced disease recurrence. These cytokines act in multiple ways during tumor development and response to treatment. Increased levels of circulating CXCL1 in ovarian carcinomas compared with benign pelvic masses imply its potential role as a marker in early OC detection (42), which may be attributed to its ability to induce the proliferation epithelial OC cells by transactivation of epidermal growth factor (EGF) receptor and induction of mitogen-activated protein kinase signaling, as demonstrated *in vitro* (43). CXCL1 participates in an endothelial-carcinoma-myeloid signaling network through its ability to recruit neutrophils/myeloid-derived suppressor cells that promote angiogenesis *in vivo* and promote cancer cell survival (44).

Chemotherapy-induced tumor necrosis factor- α (TNF- α) increases the expression of CXCL1, amplifying the signaling loop and causing NF κ B-mediated chemoresistance (45). CCL20, similarly to CXCL1, is also expressed in response to EGF and TNF- α (46), and acts pro-metastatically, supporting proliferation, migration and adhesion of tumor cells (47,48). Additionally, CCL20 recruits CD34⁺-derived dendritic cells and Tregs (49,50). An interesting mechanism was proposed in a study of esophageal squamous cell carcinoma, which suggested that CCL4 and CCL20 recruit functionally different T lymphocyte subsets, cytotoxic T lymphocytes (CTLs) and Tregs, respectively. A high level of CCL20 was associated with worse prognosis, whereas increased CCL4 was associated with better OS (51). Correlation between increased intratumoral CCL4 and CD8⁺ TILs was also reported in OC (52). Nevertheless, the present study identified a negative association between cumulative preoperative circulating CCL4 + CXCL1 + CCL20 levels and response to chemotherapy, which may suggest the presence of multiple immune milieu-driven chemoresistance mechanisms in HGSOC.

The discovery of an immunoreactive molecular subtype of HGSOC underlined the complexity of the ovarian tumor microenvironment (TME), indicating that immune effector and inhibitory molecules are expressed in tumors. The presence of CXCL9 and CXCL10 is another characteristic of immunoreactive subtypes (7,8). Together with their receptor C-X-C motif chemokine receptor 3, these cytokines can indirectly act as both tumor-suppressing and tumor-promoting factors depending on their source. CXCL9 and CXCL10 recruit T and B lymphocytes, natural killer (NK) cells and NK T cells (53). However, their prognostic impact is rather contradictory. We propose the circulating preoperative CXCL9 + CXCL10 chemokine classifier as an auxiliary marker for microinvasive discrimination of patients with stronger immune infiltration in tumors. Notably, in the cohort of the present study, there was a negative association between systemic CXCL9 + CXCL10 and RFS (but not OS), which contradicts reports linking intratumoral CXCL9 and CXCL10 with better OS in HGSOC to some extent (13,54). However, the levels of these chemokines were also demonstrated to be associated with worse prognosis in several other cancer types (55-60). The complexity of surrounding TME can explain the differences in observed outcomes, as the tumor-dependent factors can shift the microenvironment from immune-activating to immune-suppressing. Additionally, CXCL9 and CXCL10 chemokines can recruit both CTLs and Tregs in ovarian cancer, as demonstrated *in vitro* and in murine models (61,62), and the CTL/Treg ratio can also determine the prognosis of patients with OC (63). In the present study, elevated circulating chemokine levels reflected the overall T lymphocyte infiltration. However, gene expression analysis revealed the presence of antitumoral [increased expression of CD8, interferon γ (IFN γ), granzymes, eomesodermin] and immunosuppressive [increased expression of forkhead box P3, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), PD-L1, interleukin-10 (IL-10), CD163] T cell-associated processes in inflamed tumors. These findings suggest the presence of CTLs and Tregs in the same tumor. Another reason for the contradictory prognostic role of CXCL9 and CXCL10 are their splice variants, which can act antagonistically, as previously reported in hepatocellular carcinoma and HGSOC (64-66).

Discovery of the immunosuppressive mechanisms of OC encourages the development of novel treatment strategies. The goal of immunotherapy is to generate long-term protective T cell immunity to promote tumor eradication. The cancer-immunity cycle summarizes the stepwise processes required to yield an anticancer T cell response. One of the crucial stages, trafficking and retaining of effector T cells in the tumor, is mediated by CXCL9 and CXCL10 (67). Furthermore, the expression of these cytokines, together with IFN γ and granzymes, is strongly correlated with the expression of PD-L1 tumors, suggesting the potential benefit of checkpoint blockade (68). Successful melanoma and renal cell carcinoma clinical trials reported the association of intratumoral CXCL9 and CXCL10 in TILs with clinical benefit from adoptive T cell therapy (69), ipilimumab (70,71) and pembrolizumab (72), as well as the increase of circulating CXCL9 and CXCL10 during treatment with nivolumab (73,74). The early phase checkpoint inhibitor trials in OC demonstrated a durable antitumor response in some patients [reviewed in (75)]. The mRNA expression of CTLA-4, PD-L1, IFN γ , granzyme B and CXCL10 were also increased in immune-infiltrated tumors of patients, characterized by the increased serum CXCL9+CXCL10 level. The relatively short RFS of these patients indicates them as potential candidates for immunotherapy. Thus far, the results of checkpoint inhibition in OC are promising, taking into consideration the poor sensitivity of platinum-resistant HGSOE to other conventional chemotherapy agents (75,76). Therefore, modulation of TME in patients selected with the help of accurate predictive biomarkers may be an encouraging means for improving OC management and survival. A recent study demonstrated that the expression of CXCL9 and CXCL10, and other genes of IFN γ pathway, can be stimulated by chemotherapeutic agents, suggesting that chemotherapy may benefit patients with non-inflamed HGSOE tumors (13). Such pre-treatment may therefore be advantageous for T cell recruitment and sensitizing tumors to immune modulation. However, the presence of potentially recruited Tregs has to be acknowledged too, as they may impact the choice and strategy of further immunotherapy treatment.

The unsatisfactory clinical outcome of patients with advanced HGSOE urges the search for novel prognostic and predictive biomarkers, and therefore, dictated the exploratory nature of this study. The systemic preoperative chemokine profile was evaluated in patients with OC and associated them with the immune infiltration of tumors and response to treatment. There are limitations caused by the relatively small sample size and homogeneous ethnicity of the participants in the present study. Also, the study focused on the pre-operative chemokine level measurements and did not follow their dynamics during the primary chemotherapy, recurrence-free period and at the point of recurrence. Longitudinal assessment may provide more an accurate serum chemokine profile in response to treatment, as demonstrated by Coosemans *et al* (77), which reported the significant decrease in levels of metabolites, including CCL2, IL-10, vascular endothelial growth factor and transforming growth factor- β , following debulking surgery and paclitaxel/carboplatin-based chemotherapy. Nevertheless, this study provides primary evidence for the combined use of circulating CCL4, CXCL1 and CCL20 for predicting recurrence, and CXCL9 and CXCL10 for detecting immune-infiltrated HGSOE at diagnosis, prior to of treatment. Further validations

on a larger scale are required to confirm that these chemokine combinations successfully model the outcome in other patient populations. Additionally, determining the exact source and function of these chemokines in OC setting is necessary for dissecting and targeting the tumor microenvironment.

In conclusion, the findings of the present study demonstrate that preoperative levels of serum chemokines vary among patients with advanced HGSOE. Although the predictive value of single chemokines is modest, their combinations have a potential prognostic use. The results suggest that an increased concentration of circulating CCL4+CXCL1+CCL20 in patients with OC serum is associated with shorter RFS and OS. Furthermore, the elevated levels of CXCL9+CXCL10 mark immune infiltrated tumors, and reflect the increased intratumoral gene and protein expression of other inflammatory chemokines. Despite the discussed limitations and descriptive nature of this study, the findings provide background for further investigations of the clinical performance of multiple chemokine combinations as patients stratification tools for improved HGSOE management.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets and analyses of this study are available upon request from the corresponding author.

Authors' contributions

AM, MS, JAK and VP contributed equally to the conception and design of this study. BI selected the patients and provided their follow-up clinical information. AM, GS, KZ, SG and ND processed the patient samples and performed the experimental procedures. AB performed the histological tumor examination. GL contributed to statistical data analysis. AM interpreted the data and wrote the manuscript. MS, JAK and VP critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Lithuanian Bioethics Committee (approval no. 158200-06-500-147). All patients signed an informed consent form.

Patient consent for publication

All patients signed an informed consent form allowing for publication of depersonalized data.

Competing interests

The authors declare that they have no competing interests.

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