Immunosuppressive parameters in serum of ovarian cancer patients change during the disease course

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ABSTRACT

Neoplastic cells can escape immune control leading to cancer growth. Regulatory T cells (Treg), myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) are crucial in immune escape. TAM are divided based on their immune profile, M1 are immunostimulatory while M2 are immunosuppressive. Research so far has mainly focused on the intratumoral behavior of these cells. This study, on the other hand, explored the systemic changes of the key metabolites [IL-4 (interleukin), IL-13, arginase, IL-10, VEGF-A (vascular endothelial growth factor), CCL-2 (chemokine (C-C) motif ligand 2) and TGF-β (transforming growth factor)] linked to Treg, MDSC and TAM during the course of the disease in ovarian and fallopian tube cancer patients. Serum samples were therefore analyzed at diagnosis, after (interval)-debulking surgery and after chemotherapy (paclitaxel–carboplatin). We also determined galectin-1 (gal-1), involved in angiogenesis and tumor-mediated immune evasion. We found significantly lower levels of IL-10, VEGF-A, TGF-β and arginase and higher levels of gal-1 after chemotherapy compared to diagnosis. After debulking surgery, a decrease in IL-10 was significant. Gal-1 and CCL-2 appeared independent prognostic factors for progression-free and overall survival (OS) (multivariate analysis). These results will help us in the decision making of future therapies in order to further modulate the immune system in a positive way.

Abbreviations: CBA, Cytometric Bead Array; CCL-2, chemokine (C-C) motif ligand 2; CD, cluster of differentiation; DAMPs, damage-associated molecular patterns; ELISA, Enzyme-Linked Immuno Sorbent Assay; FIGO, International Federation of Gynecology and Obstetrics; Gal-1, galectin-1; IDO, indoleamine 2,3 dioxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; OS, overall survival; PFS, progression-free survival; STIC, serous tubal intraepithelial carcinomas; TAA, tumor associated antigens; TAM, tumor-associated macrophages; TGF-β, transforming growth factor β; Treg, regulatory T cells; VEGF, vascular endothelial growth factor

Introduction

Ovarian cancer is the second most frequent pelvic gynecological cancer and the most common cause of gynecological cancer-associated death among women. In most women, the disease is diagnosed in an advanced stage, which correlates with a poor prognosis and a high recurrence risk. The standard of care remains debulking surgery in combination with platinum-based chemotherapy. This consists of either primary debulking surgery and adjuvant chemotherapy or neoadjuvant chemotherapy followed by interval debulking surgery, depending on FIGO stage and predictive factors concerning residual macroscopic disease after surgery. Tubal cancer on the other hand, is very rare with an incidence of 0.41 cases per 100,000 women in the US. Since the discovery of the serous tubal intraepithelial carcinomas (STIC) and a recent review discovering only few differences between primary fallopian tube cancer and primary ovarian cancer, tubal cancer was and still is treated like ovarian cancer (For a review see refs 3–4).

Current evolutions in anticancer research have confirmed that the immune system can control cancer. If cells transform into (pre-) cancerous cells the host responds to the expressed tumor antigens and damage-associated molecular patterns (DAMPs) with an innate and adaptive immune response. This often leads to elimination of the neoplastic cells or to equilibrium. In this situation, tumor cells are not eliminated by the immune system, but reside in a dormant state. Due to the continuous immune pressure, more immune-resistant tumor cells will arise. A myriad of events will occur: (1) tumor associated antigens (TAA) and major histocompatibility complex (MHC) molecules are lost; (2) chronic inflammation at the tumor site leads to continuous activation of peripheral T cells and induces the development of Treg. In the tumor system, but reside in a dormant state.
microenvironment, certain chemokines such as CCL-2 and CCL-22 lead to the trafficking of Treg, MDSC and monocytes into the tumor. Further expansion of the Treg population is enhanced (5) through the presence of several immunosuppressive factors such as indoleamine 2,3 dioxygenase (IDO) and transforming growth factor β (TGF-β). (3) MDSC accumulate in the tumor microenvironment through the presence of VEGF, CCL-2, TGF-β and other chemokines7,28 (4) monocytes infiltrate into the tumor and differentiate into TAM. Initially, they will present an M1 phenotype (CD86+, MHCII+), leading to antitumor immunity by initiating the adaptive immune response. Once hypoxia and immunosuppression take the upper hand, there is a switch to the M2 phenotype (CD163+, CD206+). Although this creates new points of action for immunotherapy, this switch will lead to further immunosuppression and promotion of tumor growth, through the production of several immunosuppressive cytokines, such as interleukin (IL)-4, IL-10, IL-13, VEGF, CCL-2 and TGF-β.10 In the end, this combination will result in a strong immune suppressive environment, leading to immune escape. Tumor cells can proliferate and the tumor becomes clinically apparent.

Until now, ovarian cancer research has primarily focused on tumor tissue, with a large focus on genetic changes. Moreover, immunological changes so far have only been studied in tumor tissue. Nevertheless, since ovarian cancer is a widespread metastatic disease, one can appreciate that the analysis of the systemic immune changes is crucial. One way to look at the changes in the immune suppressive milieu is by looking at the metabolites produced by tumor cells and immune suppressive cells. Table 1 gives an overview on what is currently known about a selection of them. Additionally, we analyzed gal-1, a glycan-binding protein. It has a natural immunosuppressive

### Table 1. Overview on immunologic metabolites that can be detected in serum.

| Metabolite          | Origin and function                                                                 | Evidence in ovarian cancer                        |
|---------------------|-------------------------------------------------------------------------------------|---------------------------------------------------|
| IL-4                | Th2 immune response, leading to M2 type macrophages33                                | No literature data for ovarian cancer             |
| IL-10               | Production: almost all immune cells, including Treg and TAM Antitumoral effects by downregulating proinflammatory cytokine expression and by inducing NK-mediated tumor cell lysis Pretumoral effects by immunosuppressive effect on DC and macrophages34 | Higher serum levels in advanced disease stages    |
| IL-13               | Th2 immune response, leading to M2 type macrophages35                                | Decreased after debulking surgery16,17            |
| IL-17               | Pro-inflammatory cytokine Produced mainly by activated T cells and macrophages Induces secretion of other cytokines and chemokines, causing accumulation of neutrophils and monocytes | High levels of pro-inflammatory cytokines are believed to correlate with tumor progression and a negative prognosis16,37 |
| IFNγ                | Th1 immune response, leading to M1-type macrophages, that stimulate the cell-mediated immunity | IL-17 is elevated in ovarian tumor tissue and higher levels are described to correlate with improved PFS in advanced disease stage36 |
| Arginase-1          | MDSC are a heterogeneous group of cells that act immune suppressive and tumor promoting through secretion of inflammatory mediators, such as ROS and NO, IDO and arginase. Arginase-1 causes depletion of L-arginine, which results in T cell anergy49 | Increased plasma arginase has been observed in EOC patients18 |
| TGF-β               | Can convert tumor infiltrating leucocytes into Treg. Acts immune suppressive, increases proteolytic activity of cells, adhesion and directly stimulates angiogenesis48 Inhibits the function of CD8 cytotoxic T cells and Th1 cells Inhibits the development of M1 macrophages from monocytes and plays an indispensable role in tumor development and progression45 | TGFβ1 mRNA expression is an indicator of tumor sensitivity to standard therapy that it can identify biologically aggressive and highly malignant tumors and can predict the prognosis of patients with ovarian cancer46 Chemotherapy (paclitaxel–carboplatin) can upregulate CCL-2 expression50 Elevated CCL-2 expression by ovarian cancer cells is reported to be associated both with a better chemotherapy (paclitaxel–cisplatin) responses51 as with chemotherapy resistance51 CCL-2 levels in serum of ovarian cancer patients compared to healthy controls are both described to be lower58,59 as higher60,61 Higher levels are associated with advanced disease60,61 |
| CCL-2 /MCP-1        | One of the key chemokines that regulate migration and infiltration of monocytes, memory T cells, NKC and DC to sites of inflammation Has both tumor growth-promoting as growth-inhibiting influences46,49 | Elevated VEGF in serum of ovarian cancer patients is correlated with a poor prognosis20,23 |
| VEGF                | Cytokine expressed by macrophages in the hypoxic tumor microenvironment and by fibroblasts in tumor stroma. Stimulates vasculogenesis and angiogenesis in response to HIF-1α. VEGF-A shows chemotactic properties for macrophages, granulocytes, Treg, MDSC53 Overexpression of VEGF in the tumor microenvironment will lead to dilated leaky vessels, which are inefficient in the transport of oxygen, immune cells and chemotherapy into the tumor14 VEGF concentrations in serum increase after surgery9 | |

Legend: IL (interleukin); CCL-2 (chemokine (C-C) ligand-2); gal-1 (galectin-1); TGF-β (tumor growth factor β); VEGF (vascular endothelial growth factor); IFNγ (interferon gamma); DC (dendritic cells); PFS (progression-free survival); MDSC (myeloid derived suppressor cell); ROS (reactive oxygen species); NO (nitric oxide); IDO (indoleamine 2,3-dioxygenase); EOC (epithelial ovarian cancer); Treg (regulatory T cell); mRNA (mRNA); NKC (natural killer cells); HIF (hypoxia inducible factor).
function and a pivotal role in the maintenance of self-tolerance and T cell homeostasis. Via interaction with β-galactoside expressing glycoproteins on the T cell surface, gal-1 can negatively regulate T cell survival, antagonize T cell signaling and block pro-inflammatory cytokine secretion. Furthermore, gal-1 blunts T cell responses via promoting accumulation and expansion of Tregs. It is overexpressed by numerous malignant cell types, including ovarian cancer, by activated vascular endothelial cells, by normal activated T cells and by Treg. In anti-VEGF refractory tumors, gal-1 has been documented to bind VEGF receptor 2 and to maintain angiogenesis. The role of gal-1 has been studied in ovarian cancer and is associated with a poor prognosis and it accelerates the proliferation and invasive capacity of the tumor cells.

**Results**

**Patient characteristics**

An overview of the patient characteristics and outcome is given in Table 2 and Fig. 1. The majority (90%) was diagnosed with serous ovarian carcinoma at an advanced stage (FIGO stage IIIC and IV) and 79% of patients had one or more relapses. The median follow up time was 47 months. The median PFS was 16 months, the median OS was 50 months (Fig. 1). We can therefore conclude that our study population was a representative group.

**Table 2. Overview on patient characteristics (n = 80).**

| Characteristics          | Results |
|--------------------------|---------|
| Age (mean, range) (years)| 61.9 (27–87) |
| FIGO (%)<sup>5</sup>     |         |
| I                        | 7.5     |
| II                       | 2.5     |
| III                      |         |
| IIIB                     | 6       |
| IIIC                     | 50      |
| IV                       | 34      |
| Histology (%)            |         |
| Clear cell carcinoma     | 1       |
| Carcinosarcoma           | 2       |
| Endometrioid             | 3       |
| Mucinous                 | 3       |
| Serous                   | 90      |
| Serous + endometrioid    | 1       |
| Tumor grade (%)          |         |
| Well differentiated/low grade | 9 |
| Moderately differentiated | 1       |
| Poorly differentiated/high grade | 90 |
| Remaining tumor after radical surgery (%) |         |
| Yes                      | 21      |
| No                       | 78      |
| Unknown                  | 1       |
| Number of recurrences (%)<sup>6</sup> |         |
| 0                        | 21      |
| 1                        | 27.5    |
| 2                        | 17.5    |
| 3                        | 20      |
| ≥ 4                      | 14      |
| Platin-free interval (months)<sup>7</sup> |         |
| Median, range            | 10 (0–49.5) |
| Mean, range              | 15.62(0–49.5) |
| Outcome (%)              |         |
| No evidence of disease   | 27.5    |
| Alive with evidence of disease | 25 |
| Death of disease         | 47.5    |

<sup>5</sup>during the total follow-up time.

<sup>6</sup>three patients did not receive platin-based chemotherapy.

**Immunosuppression at diagnosis of ovarian cancer patients versus healthy controls**

First, we compared the metabolite values between naïve samples (diagnosis of ovarian cancer without invasive procedure, most commonly by diagnosis at ultrasound (n = 32) and samples taken after diagnostic laparoscopy (n = 23). There were no significant differences in the values between these two time points (Table 3). Therefore, we will combine the two groups in further analyses and we will refer to them as one group “at diagnosis.” In case we had patients with measurements at both occasions, the average value was used (this was the case in five patients). Two metabolites (TGF-β and arginase) could not be measured in two samples (naïve and laparoscopy) because of the small sample volume.

Serum samples from 50 patients “at diagnosis” were compared with serum samples from 10 healthy donors. IL-10 (p < 0.001) and TGF-β (p = 0.021) were significantly higher in patients compared to controls. We could not observe a decrease change of gal-1 with increasing age of healthy controls (p = 0.135).

**Immunosuppression in ovarian cancer patients at diagnosis vs. after three chemotherapy cycles**

A total of 37 patients received three cycles of paclitaxel–carboplatin and three patients received three cycles of carboplatin in monotherapy. We found significant lower levels of IL-10 (p < 0.001), VEGF (p = 0.040), TGF-β (p < 0.001) and arginase (p < 0.001) and higher levels of gal-1 (p = 0.016) after chemotherapy compared to diagnosis (Table 3). After exclusion of the seven patients who received AMG 386 or placebo together with carboplatin–paclitaxel in study (BGOG-ov7), statistical results did not change (data not shown). After exclusion of patients treated with carboplatin only (since this is not the standard of care in ovarian cancer treatment), IL-10, TGF-β, arginase and gal-1 kept their statistical significance.

**Longitudinal evolutions in metabolite values**

Of 40 patients, we gathered more than one sample during their disease course, enabling us to measure longitudinal evolutions in metabolite values. The composition of the groups is presented in Table 4. We can discriminate three groups: group 1/17 samples from patients at diagnosis and after three cycles of paclitaxel–carboplatin. Here, we found significant lower levels of IL-10 (p = 0.0005), VEGF (p = 0.0079), TGF-β (p = 0.0092), arginase (p = 0.0093) and CCL-2 (p = 0.0093). There was a trend for increasing gal-1 levels (p = 0.0797); group 2/11 and seven samples from patients at diagnosis and respectively after primary debulking surgery and interval debulking surgery.
Comparable to the whole group of samples, IL-10 showed decreased levels ($p = 0.0049$ and $p = 0.0781$); group 3/from four patients we gathered measurements taken after treatment (one patient after primary debulking and adjuvant chemotherapy, one patient after three cycles of neoadjuvant chemotherapy, after interval debulking and after three cycles of adjuvant chemotherapy and two patients after adjuvant chemotherapy) and at recurrence. No systematic differences in metabolite values were found between these two groups.

### Immunosuppressive metabolites and tumor grade

Metabolite values at diagnosis did not differ significantly between high grade and low grade ovarian cancers.

### Progression free and overall survival

The association between metabolite values and PFS and OS was studied in a multivariable (including FIGO stage and residual disease after cytoreductive surgery as prognostic variables) analysis. Gal-1 and CCL-2 appeared to be independent prognostic factors for both PFS and OS. In detail, higher values of gal-1 were associated with an increased risk of progression ($p = 0.0293$) and death ($p = 0.0096$). For CCL-2, a quadratic effect appeared, implying that both lowest and highest values of CCL-2 were associated with increased risk of progression ($p = 0.0294$) and death ($p = 0.0377$) (Fig. 2).

### Discussion

The role of the immune system in the development and recurrence of cancer is crucial. In ovarian cancer, studies so far have investigated the intratumoral presence of immune suppressive cells. This study is the first one to suggest an important systemic role for Treg, MDSC and TAM, based on the presence of their metabolites in serum allowing us to gain insight in overall immunosuppression. Moreover, we could demonstrate that conventional standard therapies (radical debulking surgery and paclitaxel–carboplatin based chemotherapy) significantly reduce these metabolite levels and that gal-1 and CCL-2 independently worsened the PFS and OS.

As demonstrated in Table 1, the existing immunological studies in ovarian cancer are scarce, do not cover the total immune suppressive repertoire and are limited in sample size (mean 61.5, range 16–130 patients). However, our results certainly confirm previous findings: decrease of IL-10 after cytoreduction and an increase of IL-10, TGF-β and arginase in ovarian cancer patients at diagnosis. In contrast to reported findings on VEGF, we could not correlate the presence of VEGF to prognosis nor did we see an increase after surgery.

We found that gal-1 serum levels increased after three cycles of paclitaxel–carboplatin. Similar finding have already been described for glioblastoma, where gal-1 expression increased in endothelial and glioma cells after radiotherapy and after treatment with temozolomide. This seems contradictory, however, in lung and ovarian cancer, gal-1 overexpression appears to promote chemotherapy resistance and downregulation of gal-1 expression can sensitize tumor cells to platin-based chemotherapy. In ovarian cancer, gal-1 could possibly mediate these effects through activation of the H-Ras/Raf-1/ERK pathway. The group of Le Mercier et al. suggested that increased gal-1 levels therefore seem to be representative of defense mechanisms against cytotoxic drugs, such as chemotherapy, and that gal-1 could consequently be of major importance in chemotherapy resistance. Both our results in gal-1 (increase after chemotherapy and being an independent prognostic factor) support this theory.

Literature provides mixed data about CCL-2 levels in the serum of ovarian cancer patients. Compared to healthy controls, both lower levels as higher levels of CCL-2 are reported. Some studies claim that higher levels are associated with advanced disease. In our study population, we showed that both the lowest as well as the highest serum levels of CCL-2 were independently associated with a poor prognosis. A possible explanation might lay in the findings that CCL-2 can act...
Table 3. Overview on the presence of metabolites in serum of patients with ovarian cancer at different time points during the course of the disease (comparison of cohorts of patient samples, n=135).

| Metabolites       | IFN-γ (pg/ml) | IL-4 (pg/ml) | IL-10 (pg/ml) | IL-13 (pg/ml) | IL-17 (pg/ml) | CCL-2 (pg/ml) | VEGF (pg/ml) | TGF-β (pg/ml x10pg/ml) | Arginase (U/L) | Gal-1 (pg/ml) |
|-------------------|---------------|--------------|---------------|--------------|--------------|--------------|-------------|------------------------|----------------|--------------|
| **Sample occasions** | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value | **Mean** (range), p-value |
| **Diagnosis** | | | | | | | | | | |
| Naive            | 32 (11-14)   | 1.4 (0.7-2.9) | 3.7 (0.1-5.5) | 0.154 (0.01-0.58) | 15.0 (0.8-35.8) | 0.5 (0.0-1.3) | 137 (0.05-9.25) | 46.08 (0.17-6.34) | 0.174 (0.012) | 447.8 (0.253) |
| After diagnostic laparoscopy | 23 (10-12)   | 2.3 (0.3-6.4) | 3.4 (0.3-6.4) | 0.091 (0.01-0.39) | 13.6 (0.04-6.2) | 0.4 (0.01-0.7) | 152 (0.05-1.04) | 5.03 (0.12-6.24) | 0.174 (0.012) | 508.8 (0.194) |
| Diagnosis = naive + after diagnostic laparoscopy | 50 (11-14)   | 1.6 (0.3-5.6) | 3.4 (0.3-6.4) | 0.2 (0.01-0.39) | 12.2 (0.04-6.2) | 0.4 (0.01-0.7) | 136 (0.05-1.04) | 47.43 (0.124) | 0.174 (0.012) | 474.6 (0.246) |
| **At diagnosis vs Healthy controls** | | | | | | | | | | |
| Healthy controls | 10 (1.9-3.3) | 0.317 (0.01-0.7) | 0.5 (0.01-0.7) | <0.001 | 0.2 (0.01-0.7) | 0.788 (0.01-0.7) | 14.8 (0.01-0.7) | 30.01 (0.01-0.7) | 0.21 (0.01-0.7) | 3686 (0.238) |
| Surgery debulking | 15 (3.0-3.0) | 0.7 (0.01-0.7) | 1.3 (0.01-0.7) | <0.001 | 0.2 (0.01-0.7) | 0.646 (0.01-0.7) | 10.5 (0.01-0.7) | 132 (0.05-9.5) | 0.174 (0.012) | 540.9 (0.174) |
| Surgery debulking | 19 (3.0-3.0) | 0.6 (0.1-1.0) | 0.9 (0.01-0.7) | <0.001 | 0.1 (0.01-0.7) | 0.414 (0.01-0.7) | 9.4 (0.01-0.7) | 153 (0.05-9.5) | 0.143 (0.012) | 572.2 (0.166) |

Legend: N (number), IL (interleukin), CCL-2 (chemokine (C-C) ligand-2), Gal-1 (galectin-1), TGF-β (tumor growth factor β), VEGF (vascular endothelial growth factor), IFN-γ (interferon gamma). 1 n=31; 2 n=22; 3 n=48; 4 n=17; 5 n=18. Bold (significant values).
Table 4. Overview on the presence of metabolites in serum of patients with ovarian cancer at different time points during the course of the disease (comparison of consecutive samples taken from the same patient).

| Sample occasions | N | Mean (range) | p-value | Mean (range) | p-value | Mean (range) | p-value | Mean (range) | p-value | Mean (range) | p-value | Mean (range) | p-value | Mean (range) | p-value |
|------------------|---|--------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|---------|--------------|---------|
| Diagnosis        |   |              |         |              |         |              |         |              |         |              |         |              |         |              |         |
| Naïve            | 5 | 0.48 (0.96)  | 0.0125  | 3.98 (0.1297)| 1.0000 | 5.38 (2.47) | 0.8750 | 0.55 (0.274) | 1.0000 | 46.36 (10.36) | 0.6250 | 199 (10.199) | 0.1250 | 115 (0.115)  | 1.0875 |
| After laparoscopy|   | 0.66 (1.12) | 2.64    | 0.66 (1.12) | 0.0005 | 0.10 (0.01) | 0.3750 | 0.10 (0.01) | 0.3750 | 10.23 (0.1023)| 0.0794 | 135 (0.135) | 0.0093 | 78 (0.078)  | 0.0079 |
| vs Chemotherapy | 17| 0.61 (1.23) | 0.4263 | 1.01 (0.314) | 0.0005 | 0.10 (0.01) | 0.3750 | 0.10 (0.01) | 0.3750 | 10.23 (0.1023)| 0.0794 | 135 (0.135) | 0.0093 | 78 (0.078)  | 0.0079 |
| Chemo-therapy    |   | 0.34 (1.08) | 1.78    | 0.68 (0.17) | 0.05   | 0.05 (0.05) | 0.05   | 12.60 (0.126) | 0.05   | 0.05 (0.05) | 0.05   | 12.60 (0.126) | 0.05   | 0.05 (0.05) | 0.05   |
| vs Primary debulking surgery | 11| 2.43 (0.11) | 0.5771 | 4.32 (0.2622)| 0.0004 | 0.35 (0.28) | 1.0000 | 20.38 (0.2038)| 0.2324 | 14.2 (0.142) | 0.9658 | 87 (0.087)  | 0.0413 | 4889 (0.0488) | 0.0461 |
| Primary debulking surgery | | 0.81 (0.70) | 1.46    | 0.63 (0.335)| 0.21   | 0.21 (0.21) | 0.21   | 13.65 (0.1365)| 0.7169 | 14.1 (0.141) | 0.7169 | 14.1 (0.141) | 0.7169 | 14.1 (0.141) | 0.7169 |
| vs Interval debulking surgery | 7 | 0.36 (0.18) | 0.8750 | 1.52 (0.314) | 0.0006 | 0.10 (0.01) | 1.0000 | 17.68 (0.1768)| 0.2188 | 115 (0.115) | 0.5781 | 64 (0.164)  | 0.9375 | 4314 (0.4314) | 0.5625 |
| Interval debulking surgery | | 0.89 (0.89) | 1.71    | 0.49 (0.125) | 0.17   | 0.17 (0.17) | 0.17   | 13.87 (0.1387)| 0.2646 | 112 (0.112) | 0.3257 | 62 (0.362)  | 14.13 | 3072 (0.3072) | 5.62   |
| After treatment vs Recurrence  | 4 | 0.81 (0.15) | 2.3000 | 1.89 (0.378) | 0.005 | 0.28 (0.092) | 0.9000 | 12.57 (0.1257)| 0.1250 | 139 (0.139) | 0.3265 | 58 (0.158)  | 0.6250 | 3304 (0.3304) | 0.6250 |
| Recurrence       |   | 0.06 (0.02) | 0.12    | 0.76 (0.127) | 0.00  | 0 (0.0)  | 2.72  | 125 (0.125) | 0.9010 | 79 (0.079)  | 0.1910 | 3269 (0.3269) | 0.1910 | 6064 (0.6064) | 0.1910 |

Legend: N (number); IL (interleukin); CCL-2 (chemokine (C-C) ligand-2); Gal-1 (galectin-1); TGF-β (tumor growth factor β); VEGF (vascular endothelial growth factor); IFN-γ (interferon gamma); Bold (significant values). Chemotherapy (3 cycles neoadjuvant paclitaxel-carboplatin). Diagnosis (naïve + after diagnostic laparoscopy). Treatment (cf. text longitudinal evolutions).
dichotomously. In a mammary carcinoma model for example, Li et al. found that CCL-2 seemed to stimulate immunosurveillance of developing malignancies and metastatic cells. However, after a long-term inhibition of CCL-2 they observed an increase of metastatic burden. On the other hand, CCL-2 also appeared to enhance the progression of primary lesions that had already reached a “critical mass”. This finding might explain the measurements of CCL-2 in our study, however, it also implies cautiousness when it should be used in a diagnostic or therapeutic setting.

This is—to the best of our knowledge—the first study in serum that explores the different aspects of immune suppression at diagnosis and after standard treatment in ovarian cancer patients. The next step to study the systemic changes in the immune system in ovarian cancer is a prospective inclusion of ovarian cancer patients from the moment of diagnosis until palliation, not only at the serum level but also at the cellular level. This type of study will be able to reveal what type of immune suppressive cells/systemic immune suppression will be most crucial during what point in the disease course. Hopefully, this insight can help us to better optimize and time the best therapy at the best moment in the future.

Materials and methods

Serum samples

After approval of the local ethical committee, a total of 135 serum samples, obtained in 80 patients with the histopathological diagnosis of ovarian/tubal cancer, were analyzed. Samples were collected from 2010–2014, after written informed consent. They were gathered at diagnosis (n = 32), after diagnostic laparoscopy (n = 23), after primary debulking (n = 15) [all without macroscopic tumor post-surgery], after three neoadjuvant cycles of paclitaxel–carboplatin (n = 40), after interval debulking (n = 19) [17] had no macroscopic remaining tumor post-surgery, two had an unresectable metastasis of 1–2cm post-surgery] and at diagnosis of recurrent disease (n = 6). In seven patients, neoadjuvant paclitaxel–carboplatin was given in the BGOG-OV7 study, implying that the chemotherapy was associated with the simultaneous administration of AMG386 (a selective angiopoietin-1/-2 neutralizing peptibody) or placebo. At present, the study has not been unblinded yet. Samples after laparoscopy, chemotherapy, debulking or interval debulking were collected respectively 13, 33, 26.5 and 21 d (median) after surgery/chemotherapy. Of 40 patients, two or more consecutive samples were available. In addition, serum was collected prospectively after approval of the local ethical committee from 10 healthy age-matched controls, without ovarian pathology.

Serum was collected in BD Vacutainer® Serum Tubes containing silica (ref 369032 and 367896, BD) and kept at 4°C until centrifugation. Samples were centrifuged at 2700–3000 rpm during 10 min. This was done in the majority of samples within 48 h after prelevation. However, 12 samples (8%) could only be processed 3–8 d after prelevation (mean 4.5 d). Resulting serum was collected and stored in aliquots at −80°C until further analysis.

Cytometric bead assay (CBA)

All serum samples were analyzed on the presence of IL-4, IL-10, IL-13, IL-17, IFNγ, VEGF-A, TGF-β and CCL-2 by the use of CBA flex sets (ref respectively 558272, 558274, 558450, 562151, 561515, 558336, 560429, 558287—BD), according to the firms’ guidelines in 96-well plates. Samples were acidified prior to the analysis for TGF-β; samples (except for TGF-β)
were used undiluted. Samples were analyzed by the LSR For
tessa flow cytometer (BD). Analysis was performed by FLOWJO software.

**Enzyme-linked immunosorbent assay (ELISA)**

All serum samples were analyzed for the presence of gal-1 by
ELISA (anti-gal-1 from R&D, ref AF1152 and a biotinylated
antibody (R&D with ref BAF1152). Our protocol was published earlier.19

**Arginase-1 activity assay**

Arginase-1 was determined to give an impression of MDSC and
TAM activity. L-arginine is a substrate for two enzymes, iNOS
(that generates nitric oxide) and arginase-1 (that converts L-argi
nine in urea and L-ornithine). MDSC show an increased activity of
arginase-1 and iNOS, resulting in a relative depletion of L-arginine
in the micro-environment and a relative increase in NO. This
results in the inhibition of T cell proliferation and function. In all
serum samples, arginase-1 activity was measured, through determi
nation of the urea content using the QuantiChrom™ Arginase
Assay Kit (ref DARG-200—Bioassay Systems) following the manu
facturer’s protocol.

**Statistical methodology**

Normality was assessed by visual inspection of the histograms of
metabolite values. The Mann–Whitney U test was used to com
pare metabolite values between two groups of patients evaluated
at different measurement occasions. The Wilcoxon signed-rank
test was used to analyze evolutions of metabolites within subsets
of patients with longitudinal measurements. The Cox propor
tional hazard model was used to analyze the association between
metabolite values at diagnosis and progression-free survival
(PFS) and OS. Both linear and quadratic trends were tested.

All statistical tests are two-sided and a 5% signifi
cance level is assumed for all tests. A large number of statistical tests was per
formed. Given the exploratory nature of this study, no correction
for multiple testing was applied. All analyses have been performed
using SAS software, version 9.4 of the SAS System for Windows.

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No potential conflicts of interest were disclosed.

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