ANTICANCER RESEARCH *39*: 5953-5962 (2019)
doi:10.21873/anticanres.13800

Increased Immunosuppression Is Related to Increased Amounts of Ascites and Inferior Prognosis in Ovarian Cancer

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Abstract. *Background/Aim: The presence of ascites in ovarian cancer patients is considered a negative prognostic factor. The underlying mechanisms are not clearly understood. Materials and Methods: The amount of ascites was evaluated, preferably, using diffusion-weighted MRI at primary diagnosis in a retrospective cohort of 214 women with ovarian cancer, in an ordinal manner (amount of ascites: none, limited, moderate, abundant). In a prospective cohort comprising 45 women with ovarian cancer, IL-10 (interleukin), VEGF (vascular endothelial growth factor), TGF-β (transforming growth factor) and CCL-2 [chemokine (C-C) motif ligand 2] were measured at diagnosis (and at interval debulking, when available). Results: Gradually increasing amounts of ascites were correlated significantly, even after correction for FIGO stage, with reduced survival ($p<0.0001$) and stronger immunosuppression (IL10 and VEGF). Neoadjuvant chemotherapy reduced immunosuppression, which was observed as a reduction in CCL-2, IL-10 and VEGF. Conclusion: The amount of ascites is an independent predictor of survival and correlates with increased immunosuppression.*

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Key Words: Ovarian cancer, ascites, immunosuppression.

Ovarian cancer has the fifth highest mortality rate among women diagnosed with cancer in Europe (1). Ovarian cancer is a silent killer, metastasizing throughout the abdomen before causing symptoms. Consequently, 63% of patients are detected at FIGO stage III or IV. Patients with advanced stage ovarian cancer have an overall 5-year survival of only 20% (2, 3). The majority of women is diagnosed with high grade serous ovarian cancer (HGSOC). Radical debulking surgery in combination with platin-based (neo-)adjuvant chemotherapy (4) is the gold standard for ovarian cancer treatment. If the tumor relapses within six months after initial treatment with platin-based chemotherapy, there are little effective therapeutic options and prognosis is very poor (5). Borderline ovarian tumors (BOT), or low malignant potential tumors, represent 10-15% of all epithelial ovarian malignancies (6).

Ovarian cancer is the most common cause of ascites in women (7). Approximately 70% of patients with epithelial ovarian cancer will develop ascites during the disease course (8). Malignant ascites is defined as the pathological buildup of free fluid within the peritoneal cavity of patients with peritoneal carcinomatosis. In contrast to ascites due to portal hypertension, the protein content of malignant ascites is high (9). It indicates a pathological imbalance between the production/increased filtration and absorption/drainage of intraperitoneal fluid. Impaired drainage of free fluid in the peritoneal cavity due to tumor buildup in the lymphatic system, is a contributing factor for malignant ascites in ovarian cancer, however, malignant ascites can also occur in the absence of mechanical obstruction (10). In addition, an

inflammatory state in the tumor microenvironment induced by cytokines and chemokines is linked to the production of ascites in ovarian cancer (11). Increased capillary permeability and oncotic pressure, orchestrated by vascular endothelial growth factor (VEGF), leads to increased filtration of ascites and seems to be an important factor (11). Ascites can cause debilitating symptoms such as early satiety, abdominal pain and respiratory and gastro-intestinal problems (11). In patients with ovarian cancer, ascites often resolves early as a result of the underlying tumor response to chemotherapy. However, once chemotherapy resistance develops, current treatment options for malignant ascites are limited. Both pharmacological and non-pharmacological options have been suggested. A few targeted therapies have been tested in clinical trials with fairly good success rates: bevacizumab (anti-VEGF-A) (12), aflibercept (anti-VEGF-A, anti-VEGF-B and anti-PlGF) (13), catumaxomab (anti-EpCAM and anti-CD3) (since 2014 no longer marketed in the EU) (14), HEA125xOKT3 (bispecific antibody to redirect T cells towards carcinoma cells and to induce tumor cell lysis *in vitro*) (15), an intraperitoneal alpha-2B-interferon (16), tumor necrosis factor alpha (17) or matrix metalloproteinase inhibitor (18). Next in line are the diuretics with only weak evidence for their use (19) and a somatostatin analogue to increase the glomerular filtration rate (20). However, once chemotherapy resistance has developed, several patients will be subjected to frequent paracenteses to temporary alleviate the symptoms (21). In certain cases, a peritoneovenous shunt or an intraperitoneal catheter can be placed to reduce the repeated drainages and hospital admissions (22).

Although some studies have suggested ascites to be a poor prognostic factor, most reports have not differentiated between histological subtypes or tumor grade, leading to an important bias (23). Furthermore, the origin of ascites in ovarian cancer and the mechanisms by which the presence of ascites affects overall survival have not been clarified (24). As the majority of therapies tested to treat ascites influence the immune system, we hypothesize that the immune system might be an important driver in ascites development. Several papers have indeed measured interleukins (IL), chemokines and cytokines in ascites at the protein or genetic level. All types of immune cells have been described in ascites, ranging from T effector cells, regulatory T cells (Tregs) to innate immunosuppression such as myeloid derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs). However, neither a clear overview, nor a study that combines several markers in one profile exist. Literature data is rather overwhelming and currently not taken into account in clinical practice.

In this study, we evaluated the prognostic significance of increasing amounts of ascites in ovarian cancer patients in a retrospective cohort. In addition, we examined the ascites composition at the protein level in a prospective cohort in ovarian cancer patient groups, presenting with different amounts of ascites.

Materials and Methods

Study population. For the retrospective study, patients diagnosed with ovarian cancer and BOT and with an accurate reporting of the presence or absence of ascites, between 2009 and 2015 in UZ Leuven, were consecutively included. The study was approved by the local ethical committee (S58468). The need for informed consent was waived. For the prospective study, consecutive patients diagnosed with ovarian cancer/BOT (with ascites expected based on preoperative imaging) between 2014 and 2017 in UZ Leuven were included. This study was approved by the local ethical committee (S56311). After signing an informed consent, ascites was evaluated during the clinical diagnostic work up of the patient, which included a diagnostic laparoscopy. Next, ascites was centrifuged and the supernatant was frozen at –80˚C.

Inclusion criteria for both the retrospective and the prospective study were women diagnosed with epithelial ovarian cancer, a follow-up of at least 12 months after diagnosis, diagnosis by computed tomography (CT), whole body diffusion-weighted magnetic resonance imaging (WB-DWI/MRI), gynecological ultrasound and/or laparoscopy. For the prospective study, patients were excluded in case of a concomitant second tumor, presence of immune disease, treatment with immunomodulators, pregnancy at the moment of diagnosis, surgical removal of the primary tumor prior to inclusion and/or infectious serology (HIV, HepB, HepC).

For each patient, we recorded: age at diagnosis, FIGO stage (25), tumor grade, histology, BRCA/CHECK status, primary treatment strategy (primary debulking surgery (PDS) *versus* neo-adjuvant chemotherapy (NACT) with interval debulking surgery (IDS)), residual tumor after debulking surgery and history of prior abdominal surgery, diagnostic procedure (CT, MRI, diagnostic laparoscopy, gynaecologic ultrasound) and survival.

Assessment of ascites. All CT and WB-DWI/MRI images were assessed by a radiologist (Prof. Dr. Vincent Vandecaveye) for the presence of ascites. All CT-scans were performed with intravenous and oral contrast. WB-DWI/MRI scans included diffusion-weighted sequences obtained in the transverse plane and reconstructed in the coronal plane, coronal T2 weighted sequences and transverse gadolinium enhanced T1-weighted sequences. All sequences covered the body from the head to below the pelvis. The presence of ascites was evaluated using the T2-weighted images. To grade the amount of ascites systematically, the abdomen was divided in four quadrants (right upper, left upper quadrant, right lower and left lower quadrant). The presence of ascites was graded as follows: absent ascites, limited ascites only involving the pelvic cavity, moderate ascites involving maximum three quadrants and abundant ascites filling all quadrants.

If preoperative radiological imaging was not available, the amount of ascites was evaluated based on the findings during diagnostic laparoscopy or gynecologic ultrasound. Drained ascites volumes during diagnostic laparoscopy above 1000 ml were categorized as abundant ascites, below 1000 ml but above 100 ml as moderate, below 100 ml as limited and if no ascites was present it was categorized as absent. A specialized gynaecologist reviewed gynaecologic ultrasound images. If there was no free fluid in the pouch of Douglas, ascites was absent, if there was free fluid only in

the pouch of Douglas, according to IOTA terms and definitions (26) free fluid was present but not ascites, but for the consistency of this study, it was defined as limited ascites. If free fluid was present outside the pouch of Douglas but still within the pelvis, ascites was moderate and if free fluid was present outside the pelvis between the bowels and towards the diaphragm, ascites was called abundant.

Reference standard. Disease specific survival (DSS) was calculated as the time between diagnosis and death of the patient due to disease. Progression free survival (PFS) was calculated as the time between diagnosis and first relapse. Patients were censored at their last follow-up or death of other cause. Patients lost to follow-up were included if the available follow-up was at least 12 months.

Cytometric bead array (CBA). To determine immune related proteins in ascites of ovarian cancer patients CBA flex sets were used as described earlier by our group (27). Both thawed and unthawed samples were analysed for the presence of interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-17, C-C motif chemokine ligand 2 (CCL-2), VEGF, Fas ligand, granulocyte-macrophage colony-stimulating factor (GM-CSF) and transforming growth factor beta (TGF-β).

Statistical analysis. For the retrospective part, logistic regression models were used to estimate the effect of predictor variables on ascites (present/absent). Cox models were used to estimate the effects of ascites on time-to-event variables. The Fisher's exact test was used to study the association between ascites as binary variable and treatment. The Kruskal-Wallis test was used to study the association between ascites as ordinal variable and treatment. The comparability of the retrospective and the prospective cohort was tested with an exact multinomial test. For the prospective study, Mann-Whitney *U*-test was used to test the differences in immune marker levels between the two groups, Kruskal-Wallis test was used to compare multiple groups. The association between immune marker levels and continuous or ordinal clinical parameters was tested by the Spearman correlation coefficient (a positive correlation means that higher marker levels are associated with a higher amount of ascites, a negative correlation means that higher marker levels are associated with a lower amount of ascites). The association between immune marker levels and survival outcomes (DSS, PFS, OS) was analyzed using Cox regression models.

All tests were two-sided and a 5% significance level is assumed. All analyses were performed using SAS software, version 9.4 of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Patient characteristics. After review of patient files, 214 patients were included for retrospective analysis. Table I provides an overview of the main patient characteristics. Ascites was present in 71% of patients. The amount was graded as follows: 60 patients (28%) had a minimal amount of ascites, 27 (12.5%) moderate and 50 patients (23.5%) abundant. In 15 cases (7%) the information necessary to determine the amount of ascites was missing. These patients were excluded from all analyses with ascites as ordinal variable. MRI whole body was used to score the presence and amount of ascites at diagnosis in 140 patients (65%). In 32 cases, the amount of ascites was determined based on the

Table I. *Patient characteristics of the retrospective study cohort analysis (N=214).*

Characteristic	Result
Mean age at diagnosis [yrs (range)]	58.4 (19-88)
Median follow up period [m (range)]	35 (0.5*-88)
FIGO [N (%)]	
I	62 (29)
II	9 (4)
III	72 (33.5)
IV	70 (33)
Unknown	1 (0.5)
Histologic subtype [N (%)]	
Mucinous	34 (16)
Serous	159 (74)
Clear cell	8 (4)
Endometrioid	13 (6)
Grade [N (%)]	
Borderline tumor (BOT)	46 (21.5)
High grade (HG)	136 (63.5)
Low grade (LG)	20 (9)
Moderately differentiated	1 (0.5)
Undetermined	11 (5)
Amount of ascites [N (%)]	
None	62 (29)
Limited	60 (28)
Moderate	27 (12.5)
Abundant	50 (23.5)
Present but not otherwise defined	15 (7)
Previous abdominal surgery [N (%)]	
No	113 (53)
Yes	99 (46)
Unknown	2 (1)
BRCA/CHECK Mutation [N (%)]	
No	53 (25)
Yes	19 (9)
Not tested	142 (66)
Therapy sequence [N (%)]	
Upfront debulking surgery	111 (52)
Neoadjuvant chemotherapy (NACT)	62 (29)
Only chemotherapy because inoperable	33 (15.5)
Palliation	8 (3.5)
Remaining tumor after surgery [N (%)]	
No	160 (75)
Yes	11 (5)
Inoperable	33 (15.5)
Not applicable	9 (4)
Unknown	1 (0.5)
Recurrence [N (%)]	
No	96 (45)
Yes	107 (50)
Unknown	3 (1.5)
Not applicable	8 (3.5)
Outcome [N (%)]	
No evidence of disease (NED)	89 (41.5)
Alive with evidence of disease (AWED)	32 (15)
Dead of disease (DOD)	89 (41.5)
Dead not of disease (DNOD)	3 (1.5)
Unknown	1 (0.5)

*Follow up period <12 months because of early dead of disease. yrs: Years; m: months; FIGO: International Federation of Gynecology and Obstetrics; N: number.

Table II. *Patient characteristics of the prospective study cohort analysis (N=38).*

Characteristic	Result
Mean age at diagnosis [yrs (range)]	60.7 (26-89)
Median follow up period [m (range)]	31 (16-41)
FIGO [N (%)]	
I/II	9 (24)
III	14 (37)
IV	15 (39)
Histologic subtype [N (%)]	
Mucinous	4 (11)
Serous	28 (73.5)
Clear cell	2 (5)
Endometrioid	1 (2.5)
Non-epithelial	2 (5)
Unknown	1 (2.5)
Grade [N (%)]	
Borderline tumor (BOT)	6 (16)
High grade (HG)	24 (63)
Low grade (LG)	5 (13)
Moderately differentiated	1 (2.5)
Undetermined	2 (5.5)
Amount of ascites [N (%)]	
None	3 (8)
Limited	20 (52.5)
Moderate	8 (21)
Abundant	7 (18.5)
Previous abdominal surgery [N (%)]	
No	21 (55)
Yes	17 (45)
BRCA/CHECK Mutation [N (%)]	
No	22 (58)
Yes	4 (10.5)
Not tested	12 (31.5)
Therapy sequence [N (%)]	
Upfront debulking surgery	19 (50)
Neoadjuvant chemotherapy (NACT)	19 (50)
Use of Bevacizumab in first line therapy [N (%)]	
No	25 (66)
Yes	13 (34)
Remaining tumor after surgery [N (%)]	
No	27 (71)
Yes	5 (13)
Unknown	5 (13)
Not applicable	1 (3)
Recurrence [N (%)]	
No	17 (45)
Yes	21 (55)
Outcome [N (%)]	
No evidence of disease (NED)	17 (45)
Alive with evidence of disease (AWED)	11 (28.5)
Dead of disease (DOD)	10 (26.5)

yrs: Years; m: months; FIGO: International Federation of Gynecology and Obstetrics; N: number.

volume of ascites drained at diagnostic laparoscopic surgery. CT was used in 27 patients, gynecologic ultrasonography for assessing the presence of ascites was used in 15 patients.

Table III. *Patient characteristics of the prospective study cohort analysis at interval debulking (N=7).*

Characteristic	Result
Mean age at diagnosis [yrs (range)]	70 (51-85)
FIGO [N (%)]	
III	4 (57)
IV	3 (43)
Histologic subtype [N (%)]	
Serous	7 (100)
Grade [N (%)]	
High grade (HG)	5 (71.5)
Low grade (LG)	2 (28.5)
Amount of ascites [N (%)]	
Limited	4 (57)
Moderate	1 (14.5)
Abundant	2 (28.5)
Remaining tumor after surgery [N (%)]	
No	4 (57)
Inoperable	3 (43)
Recurrence [N (%)]	
Yes	7 (100)
Outcome [N (%)]	
Alive with evidence of disease (AWED)	5 (71.5)
Dead of disease (DOD)	2 (28.5)

yrs: Years; FIGO: International Federation of Gynecology and Obstetrics; N: number.

Ascites was more likely to occur in older patients ($p<0.0001$), with an invasive tumor ($p<0.0001$), a serous histology [endometrioid *vs.* serous ($p=0.004$), mucinous *vs.* serous ($p=0.022$), clear cell carcinoma (CCC) *vs.* serous ($p=0.019$)], high grade tumors ($p=0.0003$) and in an advanced stage of the disease [$p=0.0002$ (stage III *vs.* stage I/II) and $p<0.0001$ (stage IV *vs.* stage I/II)]. There was no difference between stage III and IV ($p=0.308$). No increase in ascites was observed if patients had a prior history of abdominal surgery ($p=0.536$). Also, the presence of ascites was not influenced by the BRCA/CHECK status of the patient ($p=0.409$).

Immune characteristics. We prospectively collected ascites samples and clinical data of 38 patients at diagnosis. In addition, seven patients were included at IDS. Characteristics are displayed in Tables II and III, respectively. Distribution of patient characteristics (histology, grade, FIGO, age, previous surgery) was similar in both the retrospective and prospective cohort. Of note, BRCA testing was more often defined in the prospective group, which is a reflection of the current clinical practice.

Development of ascites results in poor prognosis. Median follow-up of patients in the retrospective cohort was 46 months (Q1-Q3: 36-65). Univariable analysis revealed that the presence of ascites was significantly associated with

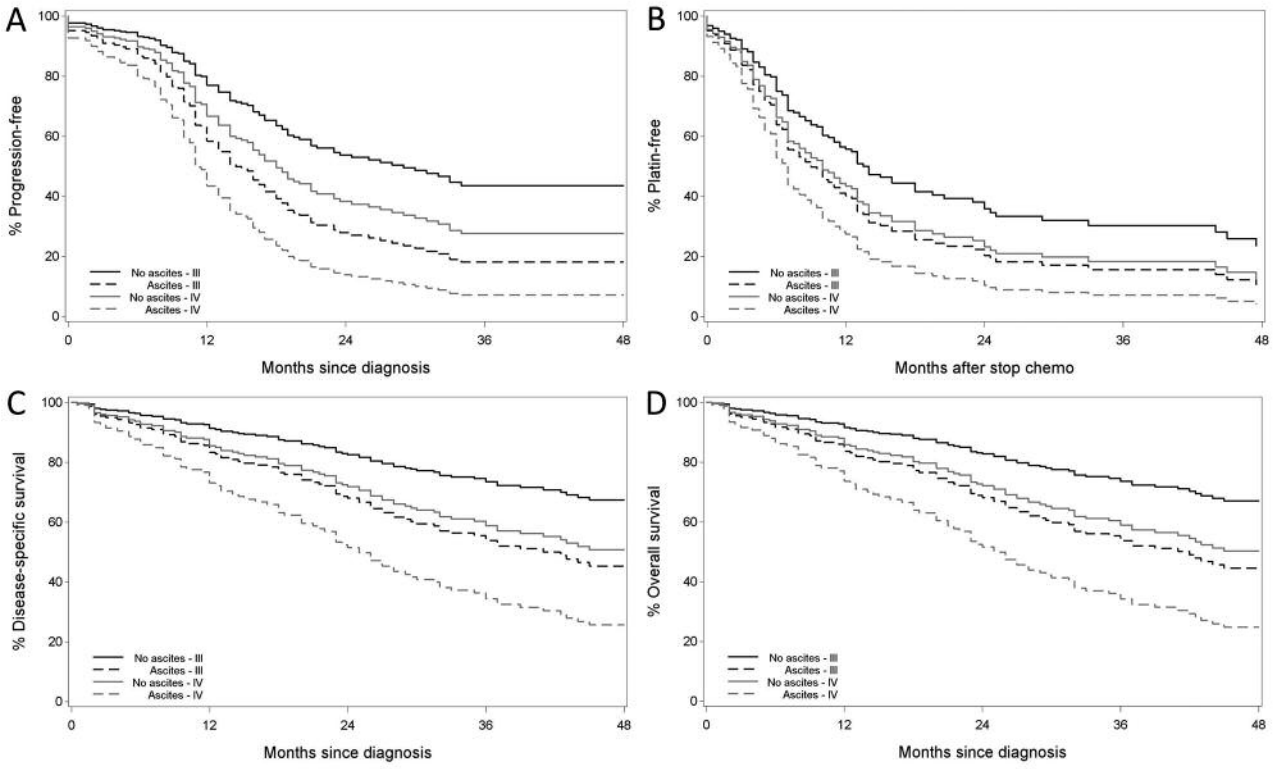

Figure 1. *Survival curves for the Cox model based on the presence or absence of ascites in stage III and IV ovarian tumors. A: Progression-free survival (PFS). B: Platin-free survival. C: Disease-specific survival (DSS). D: Overall survival (OS). Multivariable analysis, including tumor stage and presence of ascites, showed that the presence of ascites is significantly related with patients' PFS (p=0.006), DSS (p=0.032) and OS (p=0.025). A trend towards an association with the presence of ascites was seen for platin-free survival (p=0.098).*

patients' PFS, DSS and OS ($p<0.0001$) and platin-free survival ($p=0.017$). These findings were confirmed when tumor stage was added as a variable to the analysis in addition to the presence of ascites (multivariable analysis): PFS ($p=0.006$), DSS ($p=0.032$) and OS ($p=0.025$). A trend towards an association with the presence of ascites was maintained for platin-free survival ($p=0.098$) (Figure 1).

The more ascites, the worse the prognosis. In a univariable analysis of the retrospective cohort, survival worsened with increasing amount of ascites: PFS, DSS, OS and platin-free survival all $p<0.0001$ (Figure 2). The same level of significance was obtained in a multivariable analysis correcting for stage and when correcting for stage and excluding BOT. Separate results for BOT only are not available due to the limited number of patients.

Increased levels of IL-10 and VEGF were associated with increased amount of ascites and inferior prognosis. Only the results obtained for non-defrosted samples rendered reliable results. Therefore, only results for IL-10, VEGF, TGF-β and CCL-2 are available. Increased amounts of ascites estimated by MRI were associated with higher IL-10 levels in ascites ($p=0.012$; $\varrho=0.426$). Comparison of patients with limited *versus* abundant ascites revealed an increase in IL-10 ($p=0.005$) and VEGF ($p=0.041$). This was the case only for IL-10 when comparing moderate amounts to abundant amounts of ascites ($p=0.018$). Increased amounts of VEGF were associated with higher incidence of recurrence ($p=0.033$), whereas increased amounts of IL-10 were associated with worse PFS ($p=0.0499$) and OS ($p=0.046$). Given the limited number of events, it was not possible to create one large multivariable model including all confounders. Therefore, we tested the effect of IL-10 in separate bivariable models, each time correcting for one confounder. The effect of IL-10 on PFS and OS was maintained when correcting for age, previous surgery and mutation, as well as for subtype and grade in case of PFS. Residual tumor after surgery was not associated with any of these proteins.

Influence of clinical variables on the immune composition of ascites. Age, previous abdominal surgery, FIGO stage and grade were univariably evaluated as confounders for immune

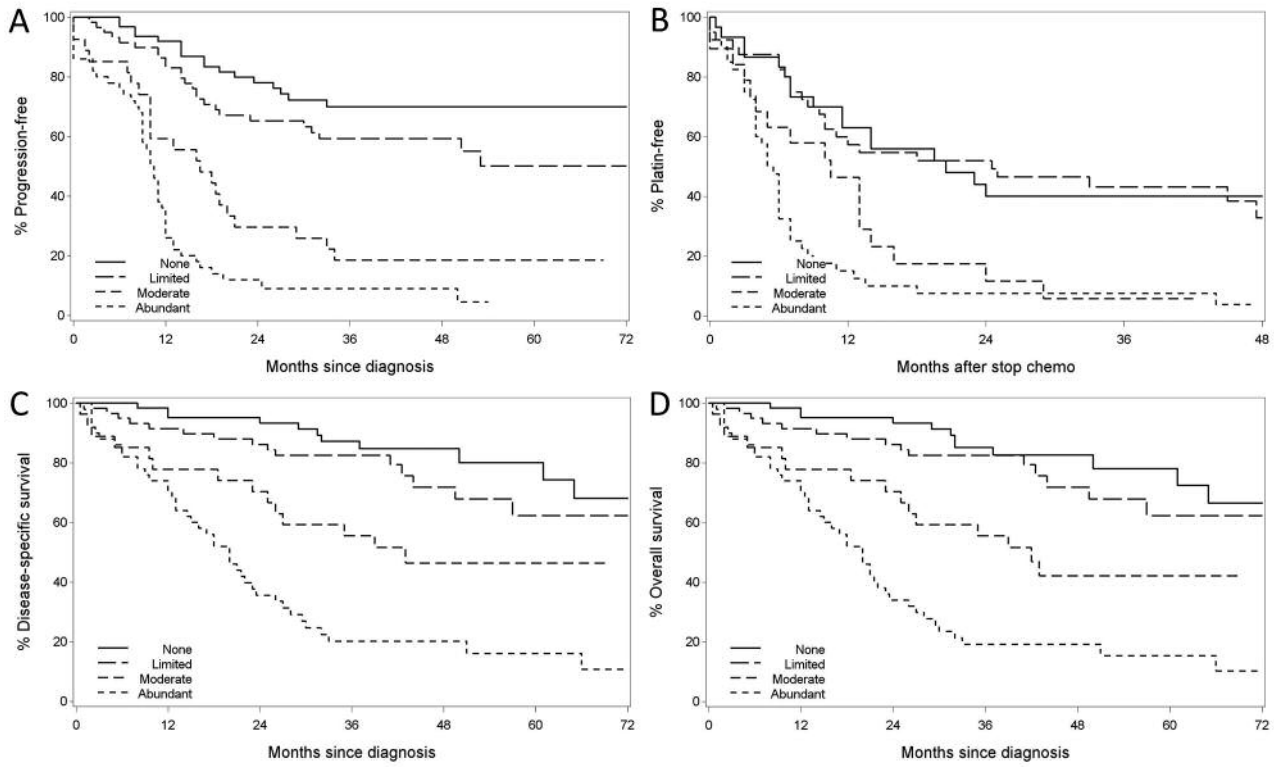

Figure 2. *Survival curves for the Cox model according to the amount of ascites (none, limited, moderate, abundant). A: Progression-free survival (PFS). B: Platin-free survival. C: Disease-specific survival (DSS). D: Overall survival (OS). Univariable analysis showed that PFS, DSS, OS and platin-free survival worsened with increasing amount of ascites (p<0.0001).*

composition of ascites. Histological subtype as well as the presence of a mutation was not taken into account because of the small number of patients in different subgroups (Table II). CCL-2 decreased with increasing age (p=0.028; ϱ=–0.354) and increasing FIGO stage (p=0.026). Similar to CCL-2, VEGF was influenced by age (p=0.024; ϱ=0.363). Also, a history of prior abdominal surgery was associated with increased VEGF levels (p=0.043). A comparison between BOT and invasive tumors revealed an increase in VEGF in case the tumor was invasive (p=0.021). TGF-β was different in ascites of low-grade invasive tumor *versus* high grade invasive tumors, showing an increase in the case of low grades (p=0.037). IL-10 was not influenced by any of these parameters.

Influence of ascites on surgical strategy and success rate. We noticed in our retrospective cohort, that if ascites, considered as an ordinary variable, was absent in stage III ovarian cancer (n = 54), patients were more likely to be selected for upfront debulking surgery (p<0.001). Moreover, if ascites was present, there was a higher chance of residual tumor after debulking surgery (p=0.037). Our prospective study revealed that with increasing amounts of ascites, VEGF and IL-10 were significantly increased (see above) and a similar trend was observed for CCL-2. No correlation was found between TGF-β and the amount of ascites. Interestingly, CCL-2, VEGF and IL-10 (all related to immunosuppression) decreased after three cycles of NACT (Figure 3). Moreover, of the seven patients selected for NACT, three had still moderate to abundant ascites at the moment of IDS, which was reflected in the highest values for CCL-2 and a short platin-free interval.

Discussion

In this study, we were able to demonstrate that the preoperative amount of ascites, as a categorical variable at diagnosis (in our study preferably measured by MRI), independently predicts the outcome of ovarian cancer patients. The higher the amount of ascites, the worse the outcome of the patient. Immunosuppressive cytokines IL-10, CCL-2 and VEGF increased with increasing amounts of ascites. NACT reduced the immunosuppressive nature of ascites.

A combined exploratory analysis of AGO-OVAR 3, 5 and 7 demonstrated a clear relationship between ascites > 500ml at primary debulking surgery and worse progression-free and

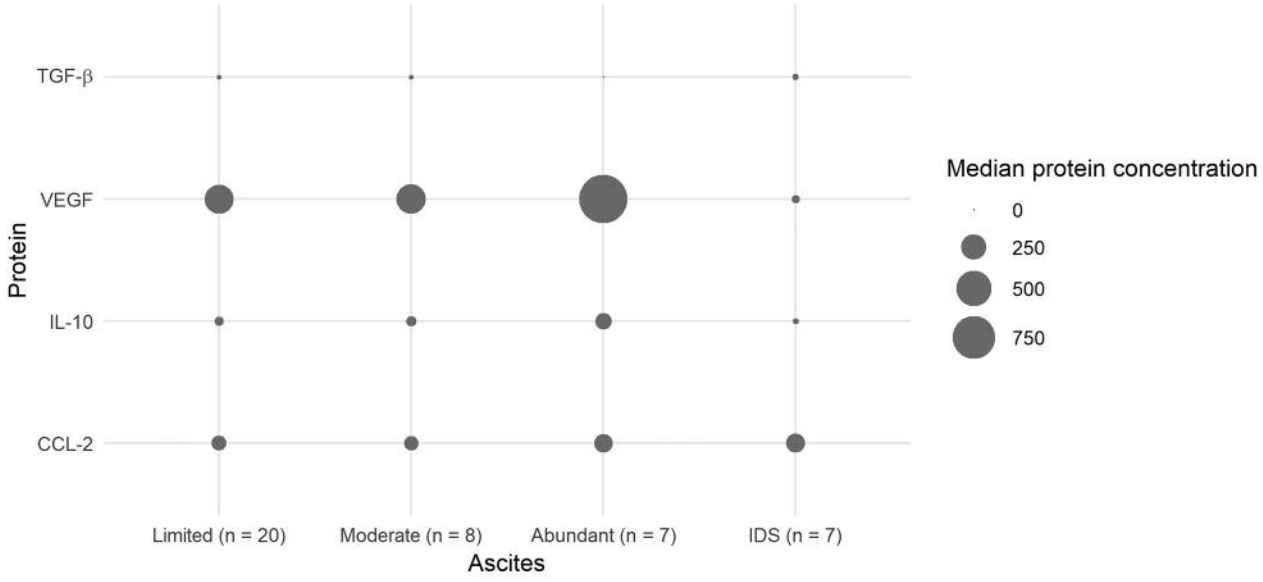

Figure 3. *Representation of TGFβ, VEGF, IL-10 and CCL-2 protein concentrations in ascites at diagnosis (based on the amount of ascites: limited, moderate or abundant) and at interval debulking surgery. TGF-β: Transforming growth factor beta; VEGF: vascular endothelial growth factor; IL-10: interleukin 10; CCL-2: C-C motif chemokine ligand 2. Protein concentrations are indicated in pg/ml.*

overall survival (OS: univariate HR=1.95, 95%CI=1.76-2.16, $p<0.0001$, multivariate HR=1.36, 95%CI=1.22-1.51, $p<0.0001$, PFS: univariate HR=1.80, 95%CI=1.64-1.98, $p<0.0001$, multivariate HR=1.28, 95%CI=1.16-1.41, $p<0.0001$) (4). In 2013, Huang *et al*. have described a relationship between the amount of ascites and prognosis of ovarian cancer in 330 patients (28). This was validated by Feigenberg *et al*. in 2014 in 149 patients (23) and by Szender *et al*. in 2017 in 685 patients (29). The study of Ayhan *et al*. in 2007 (372 patients) did not confirm these results (24). The novelty of our study is the use of MRI as a preoperative grading method for ascites and the analysis of immunologic factors in ascites, showing increased immunosuppression.

Our main clinical attitude towards the presence of ascites is that it is still considered a side phenomenon. Nevertheless, du Bois *et al*. have demonstrated that ascites remained a poor prognostic marker (OS and PFS: HR=1.92 *vs*. 1.70 respectively) even in patients with no residual disease after primary debulking surgery. The prognostic effect of ascites was lost in patients with residual disease (4). Also, the Desktop trial has demonstrated that in case of more than 500 ml ascites, the chance of successful secondary debulking surgery without residual tumor was significantly reduced (30). This was confirmed by Szender *et al*. in 2017, showing a worse chance of successful debulking during primary surgery in case of >2000 ml ascites (29). This was the first clinical suggestion that ascites should not merely be considered a side phenomenon.

Our results demonstrate also a link between ascites and an immune suppressive signature that determines the prognosis of the patient. A prior publication by Feigenberg *et al*. has demonstrated a relationship between low-volume ascites HGSOC and a more favorable immune landscape in adjacent tumor tissue (23). The fact that the immune system plays an important role in the development and progression of cancer has been established since 2011 (31). Its role in ovarian cancer has mainly been studied in tumor tissue (32). Several authors have also examined ascites fluid to find biomarkers related to the immune system. In the majority of studies, this has resulted in an enumeration of immune-related cytokines detected in ascites and their individual link with the prognosis of the patient. It has been shown that IL-10 is elevated in ascites of patients with advanced stage ovarian cancer (33, 34) and is related to poor prognosis (35-37). In cancer, IL-10 seems to originate mainly from the immunosuppressive innate immune system, specifically from TAMs and monocytes (38, 39). IL-10 is capable of inhibiting T cell proliferation, increasing regulatory T cells (Treg), skewing TAM towards an M2 phenotype and inducing monocytic myeloid derived suppressor cells (mMDSC) (35-39). All these effects will contribute to an inferior immune profile with abundant immunosuppression. Our results confirmed the importance of IL-10 in ascites, however the link between increased amounts of ascites and increased levels of IL-10 and an inferior prognosis is novel. This finding underscores the importance of 'ascites' in the

diagnostic workup. In contrast to IL-10 (as well as to all immune cells) which will only be known after invasive sampling of ascites (during surgery or by selective ultrasound guided drainage), the amount of ascites is an objective, preoperatively known, measurable biomarker, that should be considered as a poor prognostic marker. In addition, it may also be a prognostic marker for complete resection at primary debulking and it may also reflect the immunosuppressive state of the patient.

In this respect, it is interesting to observe that platin-based NACT is able to reduce IL-10. The same holds true for CCL-2 and VEGF. These results suggest a reduction of the immunosuppressive character, and possibly the necessity to combine our conventional therapies with immunotherapies in the future in highly immunosuppressive patients. This has also been suggested by Goyne *et al.* (40). Dendritic cell immunotherapy in ovarian cancer is often without success (41). However, once combined with an IL-10 antibody, its power can increase (40).

In addition to IL-10, we studied VEGF, CCL-2 and TGF-β. The importance of VEGF is not surprising (42). It has been shown in clinical trials that bevacizumab and aflibercept reduce ascites (12, 13). CCL-2 and TGF-β are much less studied, nevertheless both of them are also associated with an immune suppressive signature. The prognostic role of CCL-2 in ascites is unclear as results are sometimes conflicting, suggesting a correlation (35) or not (36) between survival and CCL-2 in ascites. A decrease of CCL-2 in ascites by paclitaxel has been confirmed in a study by Penson *et al.* (2000) (43), though the type of patients and the moment of sampling were different between our study and that of Penson. In 2011, Liao *et al.* have demonstrated that TGF-β blockade in an ovarian cancer mouse model decreased ascites (44). In our study, we could not demonstrate a link between TGF-β and amount of ascites or survival.

This study has its limitations. First, the size of the prospective cohort was rather small. Biobanking of ascites is very often neglected during surgery. Blood is certainly easier to sample at diagnosis. However, conclusions from blood samples cannot be extrapolated to ascites, since the intraperitoneal cavity seems to be unique with concentrations of proteins that are generally higher than those in blood, therefore creating an ideal environment for tumor growth (43, 45, 46). We join the plea of Grabosch *et al.* to not only systematically collect tumor tissue during surgery but also ascites fluid to monitor the immune response (45). Second, the types and amount of proteins that were measured. However, based on our analyses in the serum of ovarian cancer patients (27), we started with a larger battery of immune-related proteins. Some of them (*e.g.* IL-1β and IL-17) had to be omitted because of barely detectable values. Some of the analyses was performed on defrosted samples and therefore could not be integrated in the final analyses, as it has been demonstrated that freeze-thaw cycles can lead to clear alterations in protein concentrations (47, 48). Third, the prospective cohort specifically included patients with ascites for protein measurements. This explains the relatively large number of patients that received NACT instead of upfront debulking surgery, since the presence of ascites indicates a more advanced disease. For this reason, a possible bias should be considered when evaluating these results. Fourth, all patients that underwent IDS had ascites at the time of IDS. This might include a minor bias as well since it might be a selection of patients with an inferior prognosis.

In conclusion, preoperatively determined amounts of ascites by MRI can be correlated to the immunosuppressive cytokines present in ascites. This information should be taken into account when deciding upon the therapeutic work-up at diagnosis. Moreover, since our data suggest a decrease in immunosuppression based on neoadjuvant chemotherapy, this could provide a therapeutic window of opportunity for immunotherapies. In the future, it will be interesting to evaluate if patients with ascites have a different response rate to immunotherapy compared to patients without ascites.

Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

Authors' Contributions

AC designed the study, wrote the manuscript and performed the literature search. TB performed sample analysis and helped writing the manuscript. VD set up the database. RW helped writing the manuscript. LDL helped with the literature review. AVH and GT processed patient samples. JC and AL performed the statistical analysis. VVDC assessed CT and MRI images. IV contributed to the study design and reviewed the manuscript. All Authors agreed with all aspects of the final manuscript.

Acknowledgements

Funding: This work was supported by Kom Op Tegen Kanker (Stand up to Cancer), the Flemish cancer society (2016/10728/2603 to AC); the Olivia Fund (2017/LUF/00135 to AC); Amgen Chair for Therapeutic Advances in Ovarian Cancer (2017/LUF/00069 to IV); Research Foundation – Flanders (FWO) (12F3114N to AC); EVO-LSROC8-O2010 by the Vriendtjes tegen Kanker fund (grant number EVO-FOVTK1-O2010).

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Received September 27, 2019
Revised October 11, 2019
Accepted October 14, 2019