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Solitary ovarian cancer cells in the peritoneum: What happens below the surface?

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ABSTRACT

Background: In advanced epithelial ovarian cancer (EOC), the peritoneum is the primary site of disease recurrence which occurs in >75% of patients despite complete cytoreductive surgery (CRS) and chemotherapy. Macroscopically undetectable remaining cancer cells are deemed to be a source for recurrent disease. We investigated characteristics of occult disease in biopsies of macroscopically normal peritoneum during CRS.
Materials and methods: We included 14 patients with advanced stage high grade serous ovarian cancer (HGSOC). Eleven patients had received neoadjuvant chemotherapy (NACT) and three patients were chemotherapy naïve. Each patient underwent three study-related peritoneal biopsies: 1) of a metastasis, 2) adjacent to a metastasis and 3) at distance from metastases. Cryostat sections were immunohistochemically stained for PAX8 and PanCK as markers of EOC cells and for CD31 as a marker for vascular and lymphatic endothelium. The sections were analyzed semi-quantitatively.
Results: Macroscopically normal peritoneum showed solitary PAX8-positive cells adjacent to and at distance from metastases in all patients. Thirteen percent of these PAX8-positive cells were found to be attached to the mesothelium and are presumably spread through intra-abdominal fluid. Eighty-seven percent of the solitary PAX8-positive cells were found in the stroma underneath the mesothelium, of which 59% were firmly attached to endothelium and 33% were found in the stroma. In most cases, no sign of proliferation of the solitary cells was observed. Only a few clusters of PAX8-positive cells were found. Chemotherapy did not affect these results.
Conclusions: Solitary PAX8-positive cells are present in the macroscopically healthy-looking peritoneum of all EOC patients investigated, irrespective of the distance to macroscopically-visible metastases and of previous treatment. The majority of these solitary cancer cells were attached to endothelium of capillaries, venules or lymphatic vessels. Their solitary character and lack of proliferation suggests a dormant state, which could explain why these cells are unaffected by neo-adjuvant chemotherapy.

1. Introduction

Epithelial ovarian cancer (EOC) has the highest mortality of all gynaecological malignancies because the majority of patients present with advanced stage disease including peritoneal metastases [1,2]. Out of all EOC patients, 70% has a high grade serous (HGS) histotype [3]. Primary treatment of advanced EOC consists of cytoreductive surgery (CRS) and platinum- and taxane-based chemotherapy [4]. Despite this extensive therapy, 70–95% of all advanced stage patients develop recurrent disease [5]. These recurrences predominantly develop on the peritoneum [6,7]. The presence of occult disease in the peritoneum of patients with ovarian cancer has been reported in various studies [8–10]. However, there is little known about the characteristics and pathophysiology of these peritoneal micro-metastases in ovarian cancer.

There are various hypotheses regarding peritoneal spread of ovarian cancer cells. It is commonly accepted that intra-abdominal fluid is the predominant route of metastasizing EOC cells to the peritoneum [11]. Patients with EOC often have large volumes of ascites containing single

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EOC cells and small clusters of cancer cells called spheroids causing dissemination of EOC cells throughout the abdominal cavity [12]. EOC cells can undergo epithelial-to-mesenchymal transition (EMT) which allows them to detach and survive in hypoxic conditions [13]. Single cells as well as spheroids of EOC cells are washed through the abdomen with the physiological flow of ascites caused by gravity and diaphragmatic respiratory movements. Therefore, the complete peritoneal surface is exposed to these cancer cells [14].

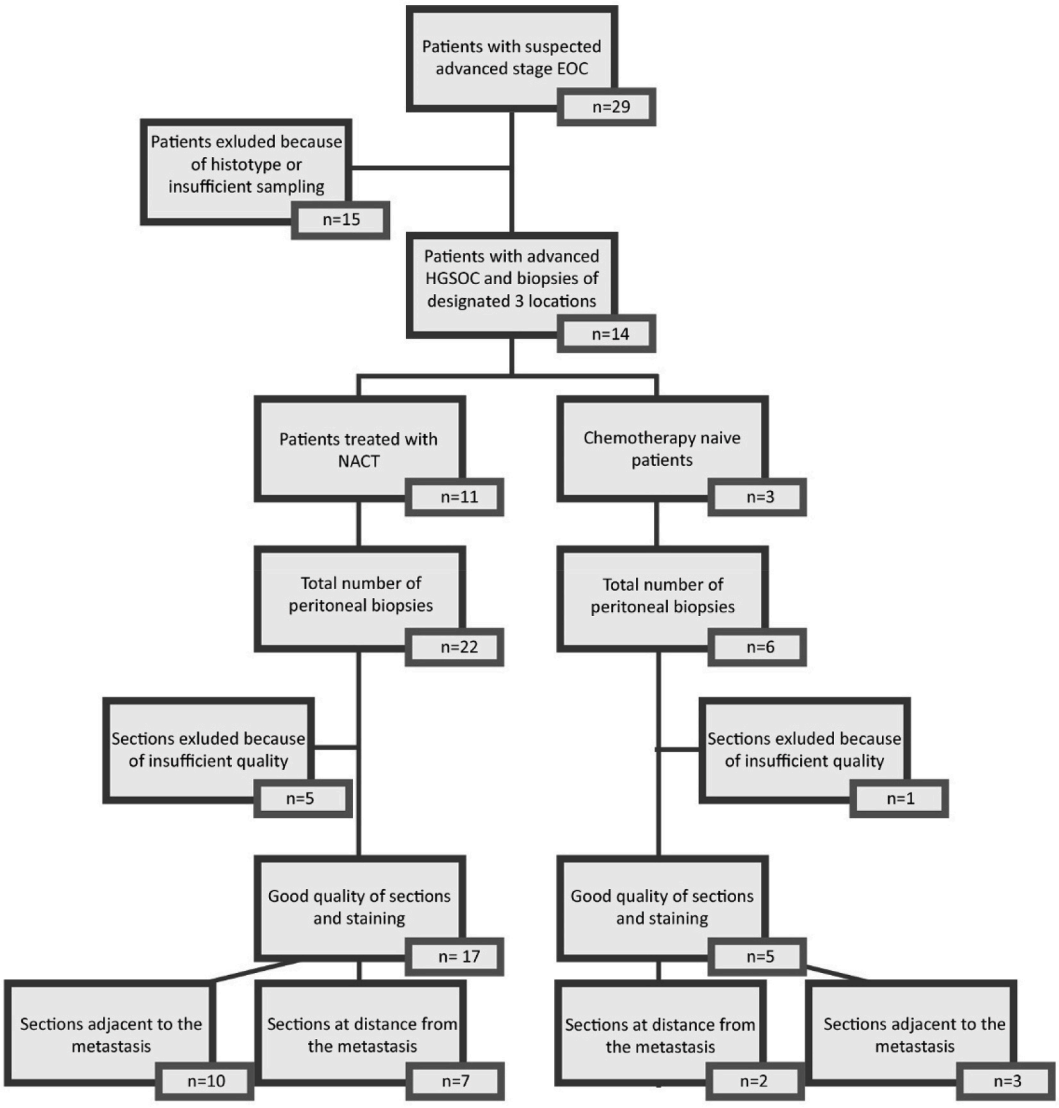
The peritoneum is a large serous membrane covering the intra-abdominal cavity and abdominal organs. It consists of a monolayer of mesothelial cells on top of stroma [14,15]. Cancer cells and spheroids can attach to the mesothelial cell layer using adhesion molecules, such as α- and β-integrins, P-cadherin and CD44 [16]. The cancer cells can penetrate this layer by using myosin to mechanically force intra-cellular gaps between mesothelial cells [12]. EOC cells have a high affinity for the submesothelial stroma and attach to the extracellular matrix (ECM) through integrins and PAX8 [17,18]. Apart from direct attachment to mesothelium, there is evidence that cancer cells enter the lymphatic system. The peritoneum is characterized by a high density network of lymphatic structures that permeate the mesothelial mono-layer with lymphatic stomata [19,20].

High recurrence rates of EOC show that current treatment strategies are not efficient in removing all EOC cells from the abdominal cavity, even when all visible disease is removed during CRS [9,21]. The most recent innovation designed to eliminate residual small or microscopic peritoneal disease is the addition of hyperthermic intra-peritoneal chemotherapy (HIPEC) to CRS [5]. This abdominal washing exposes the peritoneum to a high dose of chemotherapy combined with hyperthermia. Although HIPEC improves survival by one year, recurrence still occurs very often [5]. This suggests that there may be residual disease in the peritoneum itself unaffected by chemotherapy. Therefore, we aimed to identify the presence and characteristics of EOC cells both on the mesothelial surface and in the peritoneal stroma and the relation with vascular and lymphatic networks in the stroma. We aim to elucidate the pathophysiology of recurrent disease and to generate ideas to target elusive remaining cancer cells.

2. Materials & methods

2.1. Patients

In this prospective single-center observational study, we recruited women undergoing CRS for advanced HGSOC. This study was performed at the Department of Gynaecological Oncology of the



Flowchart 1. Flowchart of inclusions. EOC: Epithelial ovarian cancer, HGSOC: High grade serous ovarian cancer, NACT: neo-adjuvant chemotherapy.

Table 1
Characteristics of different types of vessels in peritoneal tissues [22–24].

	Lumen diameter	Appearance	Endothelium	Pericytes	Smooth muscle layer
Capillary	5–10 μm	Possible intracellular gaps	Single layer	Surrounding EC, no complete coverage	–
Venule	>20 μm	Flattened	Single layer	+	Thin layer of tunica media
Arteriole	20–130 μm	Round	Single layer	+	Thick layer of tunica media
Lymphatic vessel	10–80 μm		Single layer	–	–

Netherlands Cancer Institute. Patient characteristics were retrieved from medical charts. Written informed consent was obtained from all individual participants included in the study. The protocol was reviewed by the Institutional Review Board (IRB) of the Netherlands Cancer Institute (IRB-code IRBdm21-057) (Flowchart 1).

2.2. Tissue samples

Study-related biopsies were taken of 1) a peritoneal metastasis, 2) peritoneum adjacent to the metastasis within 1 cm of the visible metastasis, 3) macroscopically normal peritoneum not in vicinity of visible metastases. All biopsies were taken of parietal peritoneum. The distance between biopsy 1 and 3 varied due to inter-patient variability and was determined by the gynecological surgeon. After removal of the biopsies in the operating theatre, the samples were snap frozen in liquid nitrogen and stored at −80°C. Cryostat sections, 5 or 8 μm thick, were cut on the Cryostar NX50 (Thermo Scientific™) and placed on glass slides and stored at −20°C.

2.3. Immunohistochemistry

PAX8 is a gene of the paired-box gene family that plays an important role in the embryological development of the mullarian system, thyroid, kidney, brain and eye. High PAX8 expression is found in HGSOC and plays an important role in migration and adhesion to the ECM. We performed a keratin staining using PanCK antibody (clone AE1/AE3, Abcam, 1/400 for 32 min at 37°C), to confirm whether PAX8-positive cells were of epithelial origin. We used staining of the pan-endothelial marker CD31 to confirm vascular or lymphatic structures (clone JC70, ready to use for 32 min at 37°C). All staining procedures were performed on serial sections of each sample. Immunohistochemistry of cryostat sections was performed on a BenchMark Ultra autostainer. Prior to staining, sections were fixed in 4% formaldehyde for 1 h. Afterwards, sections were rinsed with Reaction Buffer (Ventana Medical Systems). PAX8 was detected using a rabbit monoclonal antibody (clone SP348, 1/200 dilution, 32 min at 37°C; Ventana Medical Systems). Bound antibodies were visualised using the UltraView Universal DAB Detection Kit (Ventana Medical Systems). Negative controls were performed in the absence of primary antibody and a positive control was performed using kidney tissue sections. Slides were scanned using a P1000 scanner (Sysmex) and uploaded on Slide Score (www.slidescore.com).

2.4. Classification

To distinguish different types of vessels in the sections, we predefined characteristics attributed to capillaries, wide capillaries (sinuses), venules, arterioles and lymphatic vessels (Table 1). The number of individual cancer cells was measured and the distance to the surface of each biopsy was calculated using Slide Score (www.slidescore.com) (Fig. 1). Cells were classified according to their location within the biopsy and vicinity to nearby tissue structures. We formulated six catagories: 1) cells on the mesothelial surface, 2) cells in stroma, 3) cells attached to endothelium (intra-luminally), 4) cells attached to endothelium (extra-luminally), 5) cells not attached but situated intraluminally), 6) unidentifiable cells.

2.5. Statistical analysis

Descriptive statistics were used to summarize the demographic variables. Non-normally distributed data were presented as medians with interquartile ranges. Statistical comparisons were not made because of the small sample size and observational nature of our study. All data were analyzed using SPSS, version 27.0 for Windows (IBM ®). We did not perform a power calculation prior to the study, since this was a

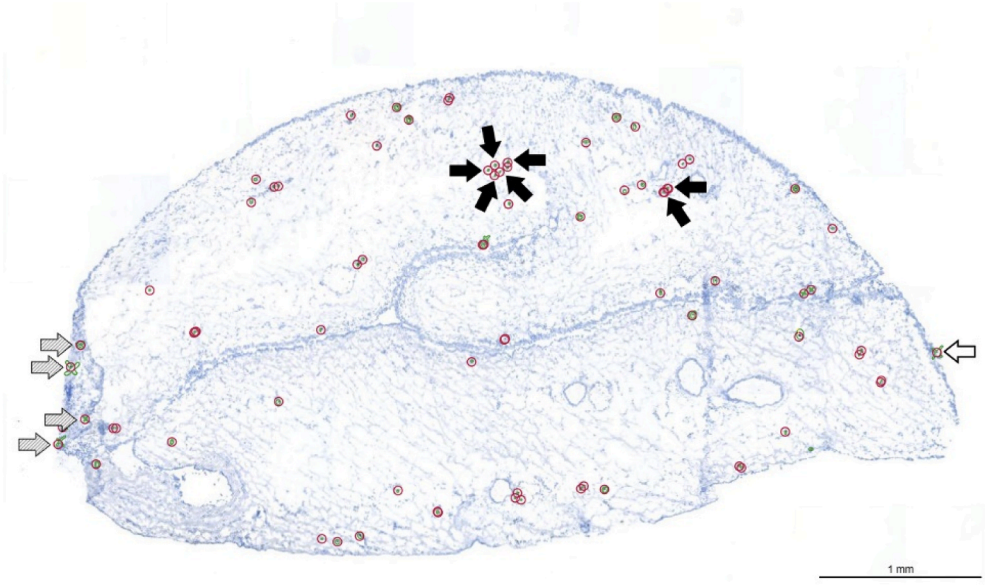


Fig. 1. Scoring of solitary cancer cells with Slide Score. This example shows a cryostat section of macroscopically healthy-looking peritoneum of a high stage HGSOC patient. Solitary PAX8-positive cells are identified with a red circle and classified. Most PAX8-positive cells were found to be attached to endothelium (black arrows). One cell was observed on the mesothelial surface (white arrow), and a few cells were deemed unidentifiable (grey arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Patient characteristics represented as percentage or as a median value with inter quartile range (IQR).

	All patients (n = 14)	Patients treated with NACT (n = 11)	Chemotherapy-naïve patients (n = 3)
Patient characteristics			
Age, yrs (median, IQR)	71 (64–76)	70 (62–76)	76 (65 – X)
Ethnicity Caucasian (n,%)	13 (93)	10 (91)	3 (100)
Previous abdominal surgery (n,%)	7 (50)	6 (55)	1 (33)
Disease characteristics			
High grade serous EOC (n,%)	14 (100)	11 (100)	3 (100)
FIGO stage			
IIIc (n,%)	12 (85)	9 (82)	3 (100)
IV (n,%)	2 (15)	2 (18)	–
Ascites (n,%)	10 (71)	8 (73)	2 (67)
Extensive peritonitis (n,%)	11 (78)	9 (82)	2 (67)
BRCA-2 mutation (n,%)	1 (7)	0 (0)	1 (33)
Treatment characteristics			
Carboplatin + Paclitaxel, 3 rounds (n,%)	6 (43)	6 (54)	–
Carboplatin + Paclitaxel, 4 rounds (n,%)	2 (15)	2 (18)	–
Carboplatin + Paclitaxel, 5 rounds (n,%)	1 (7)	1 (9)	–
Carboplatin + Paclitaxel, 6 rounds (n,%)	1 (7)	1 (9)	–
Carboplatin mono, 3 rounds (n,%)	1 (7)	1 (9)	–
Chemotherapy naïve (n,%)	3 (21)	–	3 (100)
Platinum resistant (n,%)	3 (21)	3 (27)	–
Surgical characteristics			
Complete cytoreduction (n,%)	3 (21)	2 (18)	1 (33)
Optimal cytoreduction (n,%)	7 (50)	3 (27)	1 (33)
Incomplete cytoreduction (n,%)	4 (29)	6 (54)	1 (33)

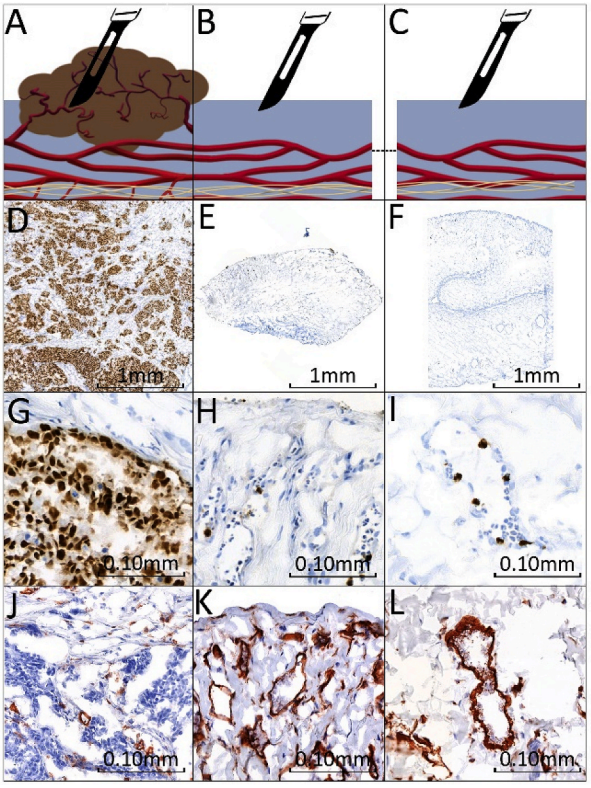


Fig. 2. Examples of biopsies taken from a patient with HGSOC.
Column 1: A,D,G,J: Biopsy of a peritoneal metastasis.
Column 2: B,E,H,K: Biopsy of peritoneum adjacent to metastasis.
Column 3: C,F,I,L: Biopsy of macroscopically healthy peritoneum at distance from any visible metastasis.
Row 1: Scheme of A) biopsy of peritoneal metastasis, B) biopsy of peritoneum adjacent to metastasis, C) biopsy of macroscopically healthy peritoneum at distance from any visible metastases.
Row 2: Low magnification of PAX8 and hematoxylin staining of D) biopsy of peritoneal metastasis with clear clusters of PAX8-positive cells, E) biopsy of peritoneum adjacent to metastasis, with no apparent clusters of PAX8-positive cells, F) biopsy of macroscopically healthy peritoneum at distance from any visible metastasis, with no apparent clusters of PAX8-positive cells.
Row 3: High magnification of PAX8 and hematoxylin staining of G) clusters of PAX8-positive cancer cells, H) solitary PAX8-positive cells attached to endothelial cells intra-luminally in a biopsy of peritoneum adjacent to metastasis, I) solitary PAX8-positive cells attached to endothelial cells intra-luminally in macroscopically healthy peritoneum.
Row 4: J,K and L; High magnification of CD31 control to confirm endothelial cells in structures described in row 1–3.

hypothesis-generating pilot study with unknown effect sizes. We aimed to include at least 10 participants with peritoneal disease and neo-adjuvant chemotherapeutic treatment.

3. Results

We included fourteen patients with advanced stage HGSOC. Anti-PAX8, anti-PanCK and anti-CD31 staining was performed on all samples of macroscopic metastatic sites to identify the presence of HGSOC cells on the peritoneal surface and in the peritoneal stroma. Fourteen patients had a PAX8-positive peritoneal metastasis and had biopsies of the macroscopically healthy peritoneum of sufficient quality. The PAX8- and PanCK-positivity of the cancer cells in the metastases was used as a positive control for the staining of the macroscopically healthy peritoneum. Patient characteristics are summarized in Table 2. An example of the assessment of three peritoneal biopsies from one patient is shown in Fig. 2.

We observed a pattern of scattered solitary PAX8-positive cells in macroscopically normal peritoneum in 100% (n = 11) of patients treated with NACT, and in 100% (n = 3) of chemotherapy-naïve patients. Out of all scored solitary PAX8-positive cells, 86% were clearly related to surrounding structures and 14% were deemed unidentifiable. Out of all identifiable PAX8-positive cells, 6% were present on the mesothelial surface, 59% were in the vicinity of endothelium, and 33% were located in submesothelial stroma not in the vicinity of a vessel or the mesothelial surface. A comparison of median values is shown in Table 3 and Fig. 3. We observed tumor clusters in three occasions of which two were near a vascular or lymphatic structure and one near the mesothelial surface. When comparing NACT-treated patients to chemotherapy-naïve patients, respectively, a median 62% vs 50% of solitary cancer cells were found to be associated with endothelium within the submesothelial stroma (Fig. 4). We did not observe

Table 3
Results represented as median percentage of attached cells with inter quartile range (IQR).

	All sections (n = 22)	Sections of patients treated with NACT (n = 17)	Sections of chemotherapy-naïve patients (n = 5)	Sections adjacent to a metastasis (n = 11)	Sections at distance from a metastasis (n = 9)
Presence of EOC cells					
Sections with solitary cells (n, %)	22 (100)	17 (100)	5 (100)	11 (100)	9 (100)
Cells per section (median, IQR)	32 (6)	31 (7)	37 (12)	23 (10–39)	30 (10–73)
Cells/mm^2 (median, IQR)	5 (2)	6 (2)	3 (2)	2 (1–8)	1 (1–3)
Location of solitary cancer cells					
% of cells attached to					
mesothelium (median, IQR)	0 (0–10)	0 (0–7)	0 (0–20)	0 (0–5)	0 (0–22)
endothelium* (median, IQR)	59 (39–74)	62 (41–79)	50 (38–74)	64 (38–78)	50 (44–78)
in stroma (median, IQR)	32 (8–54)	15 (3–54)	50 (20–77)	33 (12–59)	31 (0–50)
unidentifiable (median, IQR)	8 (0–21)	10 (0–21)	6 (1–24)	13 (2–9)	9 (2–8)
** composed of:*					
attached to endothelium intra-luminally	51 (16–100)	40 (21–93)	90 (0–100)	40 (9–95)	61 (21–100)
attached to endothelium extra-luminally	7 (0–20)	8 (3–18)	6 (0–38)	7 (3–28)	13 (0–22)
unattached in lumen	0 (0–2)	0 (0–4)	0 (0–3)	0 (0–2)	0 (0–7)

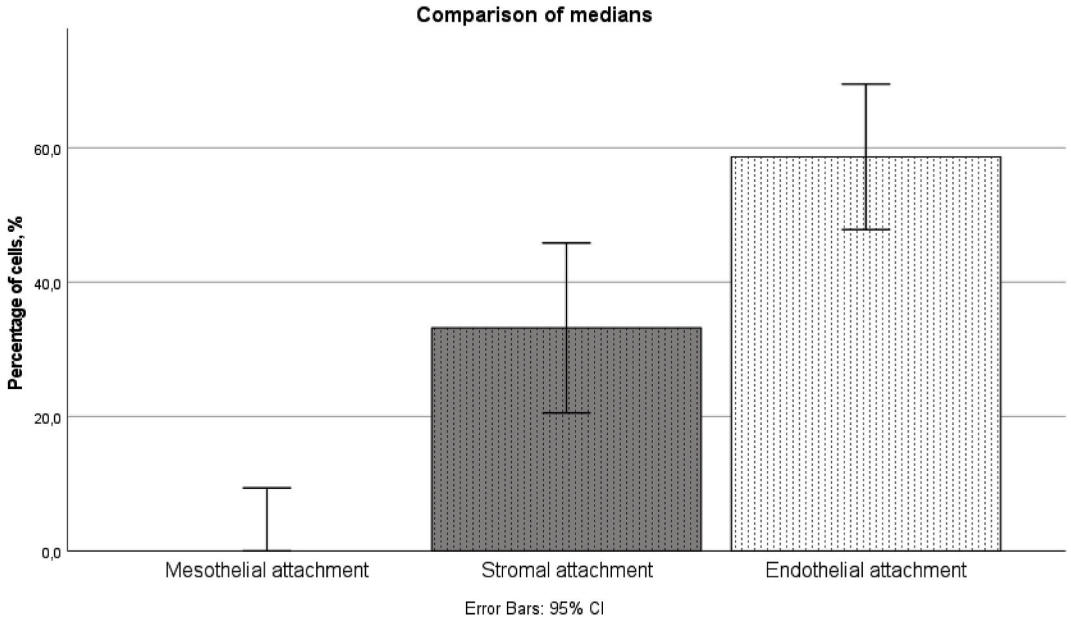


Fig. 3. Median percentage of cells attached to different structures in the peritoneum.

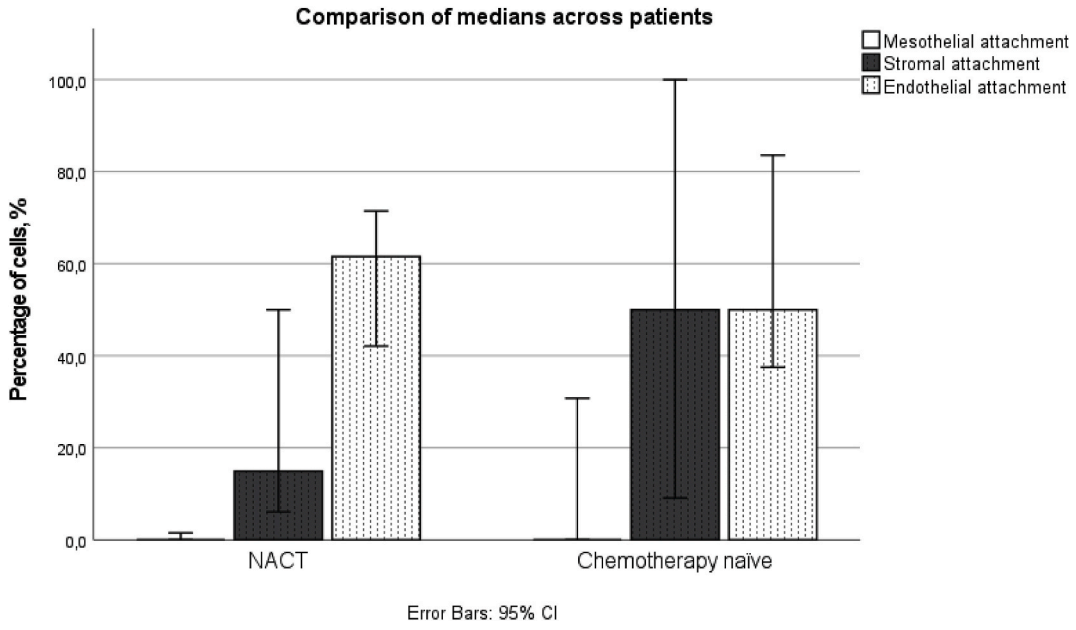


Fig. 4. Median percentage of cells attached to the different structures in the peritoneum. Comparison between patients treated with neo-adjuvant chemotherapy (NACT) and chemotherapy-naïve patients.

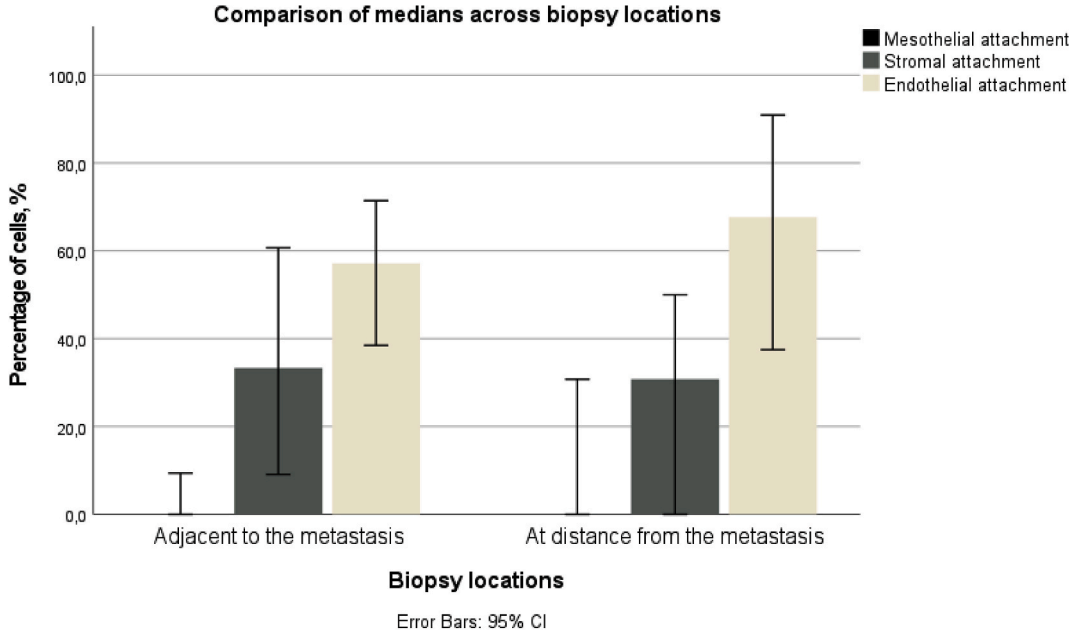


Fig. 5. Median percentage of cells attached to the different structures in the peritoneum. Comparison between peritoneal biopsies taken adjacent to a metastasis and at distance from the metastasis.

differences between biopsies taken adjacent to a metastasis and biopsies of peritoneum at distance from the metastasis (Fig. 5).

All solitary PAX8-positive cells that showed attachment to the endothelium were found around lymphatic vessels, and vascular capillaries and venules only. We did not find any solitary PAX8-positive cell associated with the endothelium of arterioles.

4. Discussion

Our results show that in patients with advanced HGSOC, solitary PAX8-positive cells are present in the submesothelial stroma of the peritoneum and often show attachment to vascular or lymphatic endothelium. This was observed in macroscopically healthy peritoneum in the vicinity of a metastasis as well as in areas at distance of a peritoneal metastasis.

Hypothetically, if ascites-mediated dissemination is the predominant route of peritoneal metastasizing cancer cells, EOC cells would be found attached to the mesothelial cell layer of the peritoneum, or directly below the mesothelial monolayer. Our results suggest that lymphatic and hematological spread of PAX8-positive cells occurs and causes a pattern of disseminated cancer cells in the submesothelial peritoneum.

Circulating cancer cells can be found in peripheral blood of EOC patients and are correlated to poor progression free survival (PFS) and poor overall survival (OS) [25]. Although extra-abdominal and hematogeneous metastasis are rare in HGSOC, translational research has

shown that hematological and lymphatic spread of EOC cells should not be overlooked [19,20]. Pradeep et al. connected the vascular system of two mice, one with ovarian cancer and one without. In this parabiosis model the unaffected mouse developed ovarian cancer in the ovaries and on the peritoneum. This development of EOC has to be attributed to hematological dissemination [26]. Similarly, Coffman et al. developed three mouse models that show hematogeneous spread of OC leading to recurrences in the ovaries and peritoneum [27]. Lymphatic involvement of EOC can be observed in the pelvic and paraaortic lymphnodes and can also present as mediastinal lymph node metastasis [28,29]. It seems that EOC cells both in ascites, and in the vascular and lymphatic circulation, favor the peritoneum as a metastatic site and are able to invade its ECM. Our data implicates that the vascular and lymphatic network of the peritoneum are involved in the spread of solitary EOC cells through the peritoneum.

Most HGSOC patients respond well to their first line of platinum-, and taxane-based chemotherapy [5]. Our results confirm, that even after an apparent good response to treatment with NACT, unaffected HGSOC cells are present in the submesothelial peritoneal stroma. In other studies microscopic peritoneal disease is often deemed as scar tissue containing remnants of a peritoneal metastasis after NACT [8,10]. We observed PAX8-positive cells in patients treated with NACT as well as chemotherapy-naïve patients. Furthermore, we did not observe fibrosis or destruction of the architecture of the peritoneum in our macroscopically healthy biopsies. Although it is possible that some of the cells which we observed are remnants of a treated metastasis, it is unlikely that this phenomenon explains our consistent findings in all biopsies. The PAX8-positive cells are mostly attached to submesothelial vascular or lymphatic endothelium, are mostly solitary cells and show no signs of proliferation. This indicates a state of dormancy and could explain why these cells are less vulnerable to chemotherapeutic cell killing. It should be further investigated, whether these cells acquired new characteristics and how this affects their sensitivity to other types of treatment such as poly ADP-ribose polymerase inhibition or immuno-oncology.

In other types of cancers, such as leukemia and glioblastoma, there is evidence that a close relation to endothelial cells in combination with hypoxia creates a niche in which solitary cancer cells can acquire stem-cell properties [22]. These solitary cancer cells can bind to the ECM and remain quiescent using binding molecules such as SDF-1α and its receptor CXCR4 and osteopontin and its receptor CD44. This process occurs in the vicinity of endothelial cells of arterioles. Our finding of PAX8-positive cells in the proximity of endothelium could indicate a similar mechanism in EOC. In EOC, the expression of the same binding molecules, osteopontin and CD44, prevent stress-induced apoptosis in vitro and is associated with poor clinical outcome [30,31]. However, the solitary PAX8-positive cells observed in our study are in direct contact to the endothelium of small blood and lymphatic vessels and not to endothelium of arterioles. The role of the endothelial cells and type of vascular structures that can contribute to an ovarian cancer stem cell niche should be further investigated. In leukemia and glioblastoma therapeutic strategies are currently being developed to overcome the dormancy of cancer cells to sensitize them to chemotherapy and radiotherapy [32,33]. Further research needs to show whether these lines of therapy could also benefit EOC patients in the future.

Another supposed mechanism of recurrent EOC is through disseminated cancer cells in the bone marrow of patients. Bone marrow can serve as a homing organ and can cause hematological spread to the peritoneum in a recurrent active phase. The presence of disseminated cancer cells in bone marrow of HGSOC patients is associated with decreased PFS and OS [34]. It is unknown whether there is a correlation between disseminated cancer stem cells in bone marrow and in the peritoneum. This has not been investigated in mouse models or animal models yet. From our data it is not clear to what degree the cells that we observed in the peritoneum contribute to recurrent disease and the potential prognostic value of this observation.

In conclusion, solitary PAX8-positive cells are present in the submesothelial stroma of macroscopically healthy-looking peritoneum of patients with advanced stage HGSOC. This phenomenon was observed in chemotherapy-naïve patients as well as patients treated with NACT and was observed irrespective of the distance to macroscopically-visible metastases. The majority of these solitary cancer cells are attached to endothelium of either lymphatic vessels or small blood vessels. The solitary character of these cancer cells and the lack of signs of proliferation, suggest a dormant state. Future research should investigate whether these cells have stem cell properties and could contribute to peritoneal recurrences of ovarian cancer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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