

Original Research

# Predicting Tumour Progression of ID-8 Syngeneic Mouse Ovarian Cancer with Blood Biomarkers of CA-125, IL-6 and VEGF: A Prospective Laboratory-Based Study

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Academic Editor: Michael H. Dahan

Submitted: 2 June 2023 Revised: 7 July 2023 Accepted: 27 July 2023 Published: 16 October 2023

## Abstract

**Background:** A preclinical animal model is an imperative tool for uncovering and understanding the tumourigenic hallmarks of human ovarian cancer; the disease is often lethal because it is commonly diagnosed in the advanced stage, where widespread cancer nodules mainly reside within peritoneal regions. Mouse models as a xenograft tumour host or genetic manipulation ovarian cancer-derived mice are widely used for studying specific hypothesis rationale in ovarian cancer. However, limited information associated with disease progression is obtained from such studies; whether it is the best model to study advanced ovarian cancer phenotype or suitable preclinical biomarkers for detecting and monitoring ovarian cancer progression is under study. This study used an ID-8 syngeneic mouse ovarian cancer model with immunocompetence. We monitored cancer growth and development using combination modalities of cancer-specific cancer antigen-125 (CA-125), interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) blood markers, which are well-known for their association with tumour progression in humans. **Methods:** Ten C57/BL6 female mice were intraperitoneally implanted with ID-8 *Trp53* wild-type and monitored the progression of the tumour, until mice developed clinical ascites. Blood was taken at the time of intraperitoneal (IP) implantation (Day 0) and then collected weekly, and levels of biomarkers were analysed with enzyme-linked immunosorbent assay (ELISA). In addition, tumour tissue was collected and proceeded with histological staining. **Results:** We found that blood biomarkers CA-125, IL-6 and VEGF were not readily correlated with tumour progression. However, these biomarkers were markedly elevated in ascitic fluid at the advanced stage of the disease. **Conclusions:** We conclude that blood biomarkers in a syngeneic mouse model are, to some extent, not readily found in the blood as opposed to human ovarian cancer. Model anatomical and physiological differences between rodents and humans might explain this discrepancy.

**Keywords:** ovarian cancer; CA-125; VEGF; syngeneic mouse model; biomarker

## 1. Introduction

Murine models have provided the means to facilitate understanding tumorigenesis's hallmarks and test novel anticancer agents and diagnostic modalities in a preclinical setting for numerous cancers, including ovarian cancer.

In ovarian cancer research, these murine models range from carcinogen-induced tumour models, genetically induced ovarian epithelial tumour models, immunodeficiency mouse models for human ovarian cancer xenograft models, and syngeneic ovarian epithelial tumour models [1–3]. Each ovarian tumour mouse model poses its strengths and weaknesses, but it is an integrative technological tool necessary for the scientific breakthrough of ovarian cancer fields.

Increased mortality in ovarian cancer is due to late diagnosis. *BRCA1/2* germline mutations are widely recognised as significant genetic risk factors for epithelial ovarian cancers, with the highest known impact. These mutations are found in approximately 6–15% of women diagnosed with epithelial ovarian cancer. Understanding a patient's *BRCA1/2* status can provide valuable information for

counselling regarding expected survival. Research suggests that *BRCA1/2* carriers tend to respond more favourably to platinum-based chemotherapies compared to non-carriers. Consequently, *BRCA1/2* carriers may experience improved survival rates, even when the disease is typically diagnosed at a later stage and higher grade [4]. In addition, advanced ovarian cancer is challenging to treat due to its complications [5]. In the study conducted, we focus specifically on epithelial ovarian cancer, which accounts for approximately 90% of all ovarian cancers. Epithelial ovarian cancer encompasses multiple histologic types, each characterised by specific molecular changes, clinical behaviours, and treatment outcomes. The remaining 10% of ovarian cancers are classified as non-epithelial, consisting mainly of germ cell tumors, sex cord-stromal tumors, and a few exceptionally rare tumors such as small cell carcinomas [6,7].

Epithelial high-grade serous ovarian cancer (HGSOC) is considered sensitive to initial chemotherapeutic treatment [8]. However, usually, a chemoresistant phenotype will rapidly develop [9]. Having technologies capable of predicting drug response in chemoresistant ovarian cancer is



crucial. Proteomic technologies, such as mass spectrometry and protein array analysis, have made significant advancements in dissecting molecular signalling events and characterising the proteomic profile of ovarian cancer. By conducting proteomics analysis of ovarian cancer and studying their adaptive responses to therapy, valuable insights can be gained. This can lead to the identification of new therapeutic options, which have the potential to minimise the development of drug resistance and ultimately enhance patient outcomes [10]. Furthermore, many novel cancer treatments modify cancer growth. Poly (ADP-ribose) polymerase (PARP) inhibitors are a prime example of targeted therapies used in ovarian cancer treatment. Mutations in DNA damage repair (DDR) genes, particularly *BRCA* genes, disrupt the homologous recombination (HR) pathway and contribute to aggressive disease. PARP inhibitors leverage synthetic lethality, inducing DNA breaks that cannot be efficiently repaired in HR-deficient or PARP-inhibited cancer cells, leading to their demise. Initially designed for *BRCA*-mutated cancers, PARP inhibitors have extended their reach to non-*BRCA* mutated tumours with similar vulnerabilities [11].

The syngeneic mouse ovarian cancer model is a valuable preclinical tool that mimics the early growth and invasion observed in the peritoneal cavity, closely resembling the advanced stage of ovarian cancer. This model is crucial for studying the role of the microenvironment in the spread of ovarian cancer within the peritoneum. Additionally, the microenvironment of malignant ascites, which includes fully functional immune cells, closely resembles the conditions observed in humans, making it highly relevant for studying ovarian cancer in a translational context [12,13]. To optimise the utilisation of the syngeneic ovarian cancer model, it is essential to have reliable methods for monitoring cancer progression *in vivo*. In this study, we investigate the potential of various tumour marker candidates in a previously under utilised murine ovarian cancer model. By exploring these markers, we aim to identify effective tools for tracking and assessing the development and progression of ovarian cancer in the preclinical setting.

Cancer antigen-125 (CA-125) is a repetitive motif of mucin 16 protein and is the primary blood biomarker utilised at the initial detection stage to determine the risk of ovarian cancer in patients [14,15]. Furthermore, the CA-125, combined with other clinical modalities, including transvaginal sonography (TVS), may aid in disease diagnosis. Monitoring CA-125 in the serum sample is also clinically valuable to follow ovarian cancer response to treatment. However, CA-125 sensitivity and specificity are questionable since elevated serum CA-125 levels can be found in nongynaecological malignancies and non-malignant conditions [16–18]. Therefore, CA-125 could be combined with alternative sera biomarkers to improve its specificity and sensitivity.

Interleukin-6 (IL-6) is a pleiotropic cytokine produced by various cell types, including osteoblasts, monocytes and macrophages, and is typically kept at low levels in normal physiological conditions [19]. However, several physiological factors, including diet, exercise, stress, infection, injury and inflammation, can increase IL-6 levels [20]. In addition, malignant cells can produce IL-6 and allow its stimulation in an autocrine and paracrine manner [21]. IL-6 can be found in serum and ascitic fluids in patients with advanced ovarian cancer, playing a critical role in tumour progression and metastasis [22].

Vascular endothelial growth factor (VEGF) is another cytokine and plays a crucial role in the angiogenic process in normal and pathological conditions. Many cell types produce it. In the advanced stages of ovarian cancer, patients often develop malignant ascites with peritoneal carcinomatosis, and tumour burden and ascites are significantly associated with poor prognosis and survival [14,23–25]. Serum and ascitic fluid from ovarian cancer patients have significantly high levels of VEGF, but its correlation with a poor prognosis is controversial and seems to be VEGF isoforms dependent [26].

This study used the combination of blood biomarkers, CA-125, IL-6 and VEGF, to predict and monitor the tumour progression of a syngeneic mouse ovarian cancer implanted with ID-8 cells. We hypothesised that combining these three biomarkers could be a reliable tool to predict the onset of tumour progression in a syngeneic mouse model.

## 2. Materials and Methods

### 2.1 Tumour Implantation

Ten C57/BL6 female mice at ages 6–8 weeks were intraperitoneally (IP) injected with four million ID-8 cells. Before tumour implantation (Day 0), the blood of each mouse was taken from a saphenous leg vein with a 23 G needle (Thermo Fisher, Auckland, New Zealand). The blood was mixed with a 1:1 ratio of ice-cold 5 mM ethylenediaminetetraacetic acid (EDTA) solution in 1×phosphate buffered saline (PBS) (Thermo Fisher Scientific, Auckland, New Zealand). The blood was taken from mice weekly until mice developed abdominal distention, a sign of clinical ascites. Mice with abdominal distention were then euthanised, and the abdomen was opened to collect ascitic fluid. The blood and ascitic fluid were centrifuged at 2500 rpm to remove red blood and white blood cells. Also, tumour tissue was collected and fixed with 4% paraformaldehyde (Merck, Auckland, New Zealand) until sectioning. Before proceeding with enzyme-linked immunosorbent assay (ELISA), undiluted ascitic fluids and diluted blood samples were stored at –20 °C. Animal ethics was approved by a committee at the University of Otago, Christchurch, New Zealand.

**Table 1. Observational characteristics of ID-8 syngeneic mice tumours, including weights before tumour implantation and prior culling, total weight increased, % weight increased, the volume of ascitic fluid and survival times.**

| Mice ID | Weight of mice before tumour implantation (g) | Weight of mice before culling (g) | Total weight increased (g) | Weight increased (%) | Volume of ascites (mL) | Time of survival (days) |
|---------|---|-----------------------------------|----------------------------|----------------------|------------------------|-------------------------|
| 1       | 18.5  | 27.7                              | 9.2                        | 49.7                 | 7.0                    | 88                      |
| 2       | 18.5  | 23.4                              | 4.9                        | 26.4                 | 3.0                    | 90                      |
| 3       | 20.3  | 24.2                              | 3.9                        | 19.2                 | 8.0                    | 81                      |
| 4       | 19.1  | 27.6                              | 8.5                        | 44.5                 | 5.0                    | 94                      |
| 5       | 18.6  | 25.6                              | 7.0                        | 37.6                 | 5.0                    | 91                      |
| 6       | 15.3  | 20.8                              | 5.5                        | 35.9                 | 3.5                    | 96                      |
| 7       | 15.5  | 21.3                              | 5.8                        | 37.4                 | 2.0                    | 96                      |
| 8       | 18.0  | 23.0                              | 5.0                        | 27.8                 | 4.0                    | 84                      |
| 9       | 17.0  | 21.2                              | 3.7                        | 21.7                 | 3.5                    | 82                      |
| 10      | 17.5  | 19.2                              | 1.7                        | 9.7                  | 0                      | 112                     |

## 2.2 ELISA of CA-125, IL-6 and VEGF

The mouse MUC16/CA-125 ELISA kit was purchased from LifeSpan BioScience (Seattle, WA, USA). Mouse IL-6 DuoSet and mouse VEGF DuoSet were purchased from R&D systems (Minneapolis, MN, USA). A volume of 20  $\mu$ L of serum samples was mixed with 80  $\mu$ L of a sample buffer. For ascitic fluid, 20  $\mu$ L of undiluted ascitic fluid was mixed with 80  $\mu$ L 1 $\times$ PBS. All samples were kept on ice during the procedure. The total volume of 100  $\mu$ L diluted samples was used in all ELISA. For the CA-125 ELISA, we followed the instructions from the manufacturer. For IL-6 and VEGF ELISA, a diluted capture antibody was prepared according to the manufacturer and coated with ELISA plates (Thermo Scientific, Auckland, New Zealand) overnight at room temperature before incubation with the serum and ascitic fluid. After that, all procedures were followed according to the manufacturer.

## 2.3 Tumour Histology

Fixed tumour nodules were embedded in square moulds (Peel-A-Way™, Sigma-Aldrich, Auckland, New Zealand) with an optimal cutting temperature (OCT) embedding medium (Thermo Scientific, Auckland, New Zealand), and samples were kept at  $-80^{\circ}\text{C}$  overnight. Sections of seven-micrometre thickness were cut by Cryostat (Leica CM1860, Leica Biosystems, Germany). Each cut section was transferred to SuperFrost Plus microscope slides (ThermoFisher, Auckland, New Zealand). Then, tumour sections were stained with Haematoxylin & Eosin solution (Merck, Auckland, New Zealand). Dried tissue was then mounted with a mounting solution (DPX, mounting medium, Scharlau). Tumour sections were then imaged by a Zeiss Axioimager Z1 microscope (AxioVision 4.5, Carl Zeiss Oberkochen, Germany).

## 2.4 Immunofluorescence of MUCIN-16

The ID-8 cell line was obtained from Centre for Redox Biology and Medicine, University of Otago, Christchurch, New Zealand. The OV-90 was obtained from Discipline

of Obstetrics and Gynaecology, Robinson Research Institute, Adelaide Medical School, the University of Adelaide, Adelaide, Australia. ID-8 and OV-90 cell lines were tested for the mycoplasma contamination by polymerase chain reaction (PCR) assay using generic primers GPO-3/MGSO (270 bp). The ID-8 cell was authenticated by cell morphology. The OV-90 cell line had been authenticated using STR testing by CellBank (Children's Medical Research Institute, New South Wales, Australia). ID-8 and OV-90 cell lines were fixed with cold acetone: methanol (50%:50%) (Merck, Auckland, New Zealand) solution for 40 min at  $4^{\circ}\text{C}$ . Sections of tumour tissues and cell lines were washed with 1 $\times$ PBS twice and then incubated with 4% bovine serum albumin (BSA) (Meck Ltd, Auckland, New Zealand) for 60 min at room temperature and followed by incubating with 0.1% Triton-X100 (Merck, Auckland, New Zealand) for 20 min. Samples were then washed twice and incubated with anti-mucin-16 (cat# SC99166, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-cytokeratin-18 (cat# SC6259, Santa Cruz Biotechnology, Dallas, TX, USA) at 1/200 dilution overnight at  $4^{\circ}\text{C}$ . After that, samples were washed with 1 $\times$ PBS three times for 10 min on a shaker. Samples were then stained with anti-Mouse-FITC (cat# SC2010, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-Rabbit-Atto594 (Meck Ltd, Auckland, New Zealand) at 1/500 dilution for 60 min and  $37^{\circ}\text{C}$ . Samples were then stained with 20  $\mu\text{g}/\text{mL}$  DAPI (Santa Cruz Biotechnology, Dallas, TX, USA) for 20 min at room temperature. Samples were washed three times for 10 min with a cold 1 $\times$ PBS + 0.5% Tween-20 (Merck, Auckland, New Zealand) solution. The samples were mounted with an anti-fading solution, and fluorescent images were captured by a Zeiss Axioimager Z1 with Apotome software (Carl Zeiss, Oberkochen, Germany).

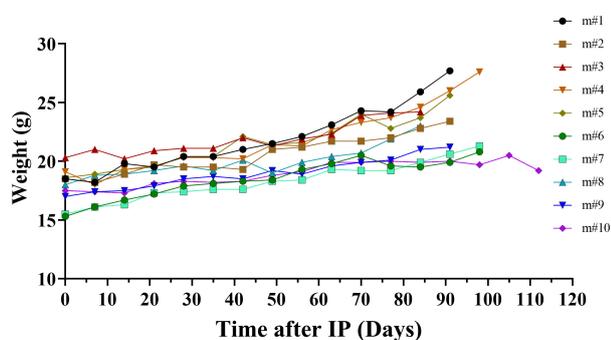
## 3. Results

Each mouse exhibits distinct signs of tumour progression at different time points. However, with the exception of mouse number 10, all other mice show advanced-stage cancer progression characterised by ascites (Table 1).

Mouse number 10, on the other hand, presents small tumour nodules on the mesentery and no signs of abdominal wall growth. Consequently, we conducted a peritoneal wash on mouse number 10 to obtain any remaining peritoneal fluid for further analysis.

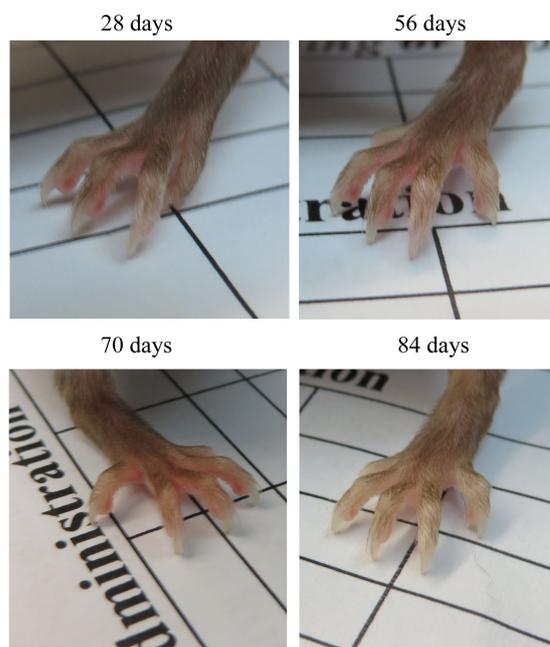
Mice numbered 1 to 9, which developed ascites, experienced shorter survival times and succumbed to the disease between 81 to 96 days. We also analysed the weight increase of mice before tumour implantation and after the development of ascites. While we observed varied weight gains, it is important to note that we cannot definitively attribute these changes solely to the presence of tumours, as we lack control mice without tumours for comparison.

To investigate the potential association between tumour progression and increased total body weight, we conducted weekly weight measurements of each mouse following intraperitoneal implantation of tumour cells. As depicted in Fig. 1, over the course of 60 days, the weight of all mice exhibited fluctuations and a gradual increase. However, starting from day 70, nine out of ten mice (M1–M9: 90%) displayed a consistent and gradual weight gain ranging from 19.2% to 49.7%. In contrast, mouse number 10 exhibited a weight increase of approximately 9.7%. This may suggest that the weight change observed in mouse number 10 may be attributed to a regular growth pattern rather than tumour progression. It is important to note that we did not have control mice without tumour implantation to compare with our study. Therefore, the observed weight increase could potentially be a result of the normal growth pattern in mice.



**Fig. 1. Weights of ten mice after post intraperitoneal injection of ID-8 cells.** Each animal was weighed weekly until a sign of abdominal distention due to ascites was observed. The final weight of each mouse was documented before humane euthanasia. M#1–9 developed ascitic fluid. IP, intraperitoneal.

Also, mice with ascites showed white feet, suggesting low red blood cells in blood circulation (Fig. 2). Ascites was relatively rapid and occurred within 2–3 days without any sign of enlarging abdominal girth to an enlarged abdomen. Mice with abdominal distension were still mobile and maintained their appetite.

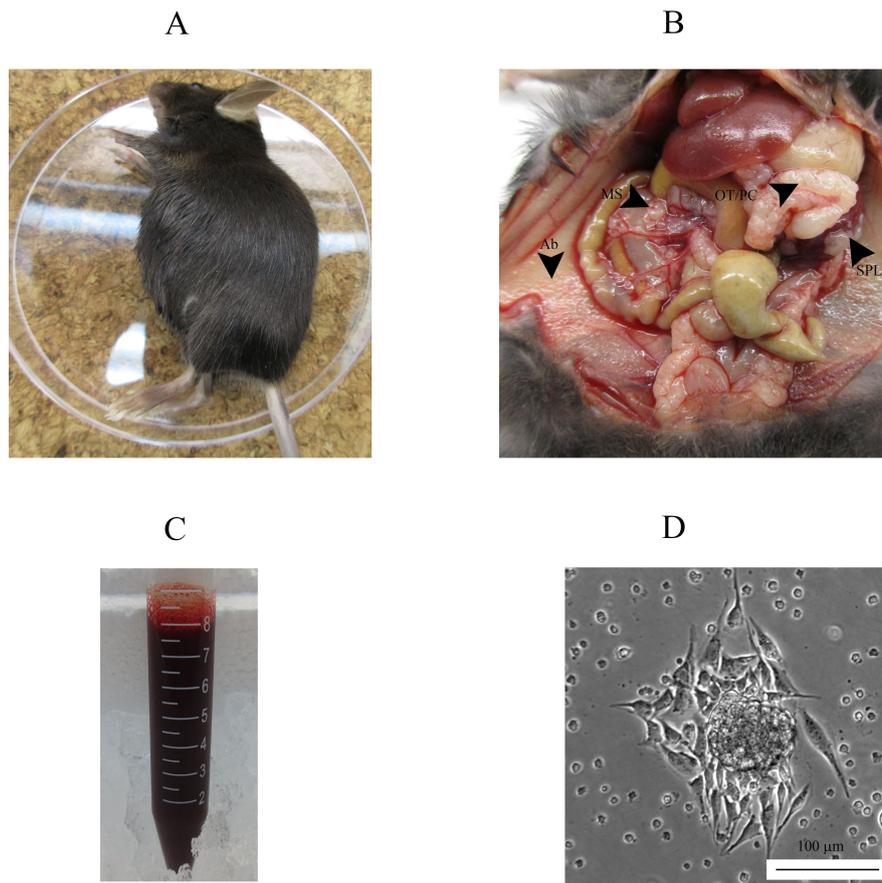


**Fig. 2. Images of the animals' feet were recorded after tumour implantation and before culling the animal.** For example, the anaemic foot of a mouse on day 84 was due to ascites in the abdominal cavity. The selective images are representative of all mice that commonly have white feet associated with ascites.

As shown in Fig. 3, a mouse with ascites has an enlarged abdomen (Fig. 3A). Gross tumour examination showed extensive peritoneal growth consisting of nodules and layers (Fig. 3B). The most common metastatic sites were the surface of the abdominal cavity (Ab), mesentery (MS), omentum/pancreas (OT/PC), diaphragm, and kidneys. Rare but uncommon sites were the surface of the spleen (SPL), colon, liver and ovary. All mice (other than mouse 10) had blood-stained ascitic fluid, and the fluid volume varied among mice (Table 1 and Fig. 3C). Also, clusters of ID-8 cells were present in the ascitic fluid (Fig. 3D).

Tumour nodules and layers were found in various places within the peritoneal cavity, and common places are the surface of the abdominal wall (Fig. 4A), mesentery (Fig. 4B), omentum/pancreas (Fig. 4C) and diaphragm (Fig. 4D).

As shown in Fig. 5, levels of all three sera biomarkers, CA-125 (Fig. 5A), IL-6 (Fig. 5B), and VEGF (Fig. 5C), fluctuated and did not elevate before mice developed ascites. However, these three biomarkers were elevated in ascitic fluid (Fig. 5D). The level of ascitic CA-125, IL-6, and VEGF are high relative to their levels in the blood. Mouse number 10 had small tumour nodules on the mesentery and no ascitic fluid. Therefore, we performed a peritoneal wash on mouse 10 to obtain peritoneal fluid and use it to detect IL-6.



**Fig. 3. The anatomical and pathological appearance of a mouse after developing ascitic fluid.** The image shows abdomen distension due to ascites (A), extensive tumour spread within a peritoneal cavity preferentially to the abdominal surface (Ab), mesentery (MS), and omentum/pancreas (OT/PC) but rarely growing on the surface of the spleen (SPL) (B), ascitic fluid contains red blood cells (C) and clusters of ID-8 cells were present in the ascitic fluid (D). Chosen images in (A–C) are representative of all mice that have abdominal distension due to ascites, tumour burdens and haemorrhagic ascites of advanced ovarian cancer.

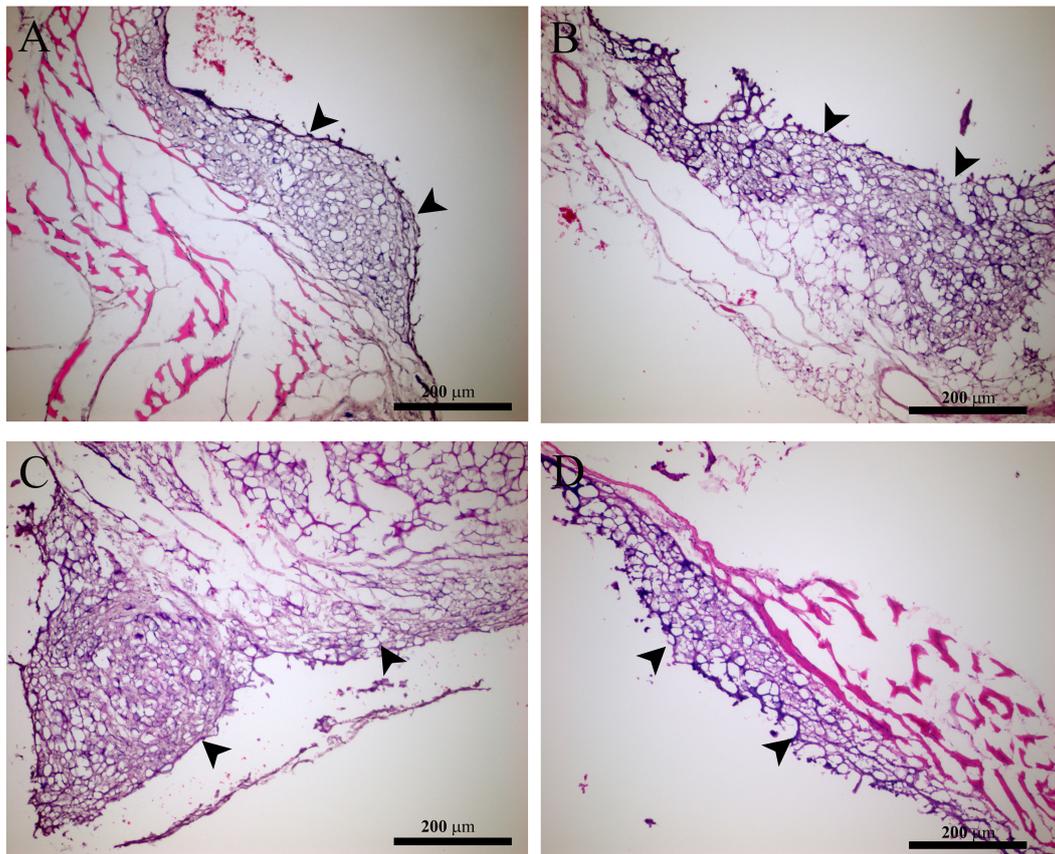
The cellular location of CA-125 is a part of the mucin-16 protein, reflects its biological functions and is an important marker for ovarian cancer. Therefore, we want to determine the presence of mucin-16 in mouse ovarian cancer ID-8 cells compared to a human ovarian cancer cell line, OV-90. OV-90 (Fig. 6A) and ID-8 (Fig. 6B) cell monolayers have mucin-16 in the nucleus and plasma membrane. However, the ID-8 tumour tissues (Fig. 6C,D) showed intense staining of mucin-16 at the cell surface of the plasma membrane but less evidence of the nucleus-associated staining.

#### 4. Discussion

This study demonstrates the feasibility of using the three biomarkers, CA-125, IL-6 and VEGF, to monitor the tumour progression of a syngeneic ovarian ID-8 tumour model. Although these three blood biomarkers are not readily correlated with the ID-8 tumour progression, high levels of these biomarkers are readily detectable in ascitic fluid.

The syngeneic ID-8 tumour model enables the exploration of pathways that potentially contribute to chemoresistance during the advanced stages, mirroring the presence of ascitic fluid, which is commonly observed in patients with advanced ovarian cancer. Ascitic fluid, being a crucial biological sample, plays a pivotal role in tumour progression and significantly influences the efficacy of cytotoxic and targeted agents used in ovarian cancer treatment [27]. Hence, this area of research deserves immediate attention and investment in the field of ovarian cancer.

Detection and monitoring ovarian tumours with peritoneal spread poses immense challenges in preclinical and clinical settings. Unfortunately, routine body examination does not readily recognise silent symptoms associated with ovarian tumour progression. Therefore, the primary clinical practice for detecting tumour progression is to use a blood biomarker, CA-125. The CA-125 is referred to as the N-terminal part of the extracellular domain of Mucin 16 (MUC16) and is cleaved and released into circulation. It

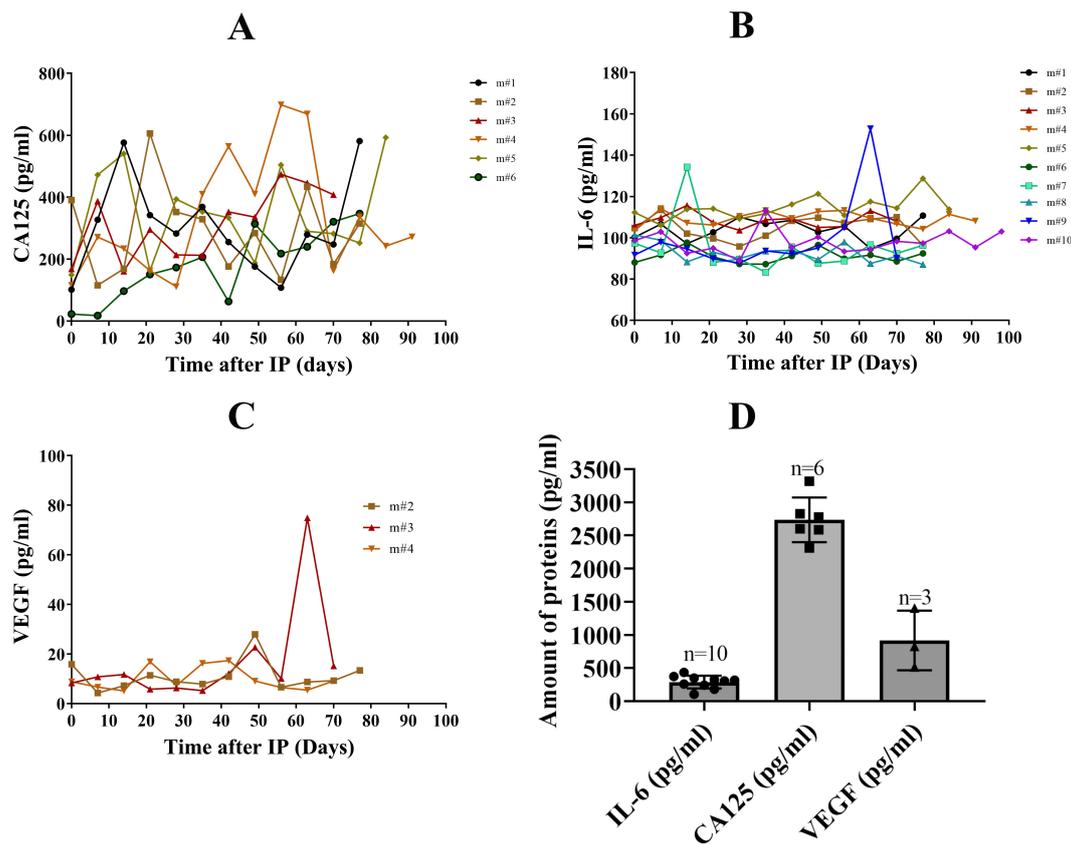


**Fig. 4. Histology of ID-8 tumour in mice that grow on various location of the internal organs.** Haematoxylin and Eosin (H&E) staining of ID-8 tumour nodules and layers on the surface of the abdominal wall (A), mesentery (B), omentum/pancreas (C), and diaphragm (D). The black arrows point at tumour nodules and layers.

was initially believed to be a specific biomarker for ovarian cancer. However, CA-125 is found in several pathological conditions [16,18,28]. Patients with ovarian cancer have significant detectable levels of CA-125 in serum and ascitic fluid. In addition, the level of CA-125 in ovarian cancer patients is higher in ascitic fluid in serum [29]. A high level of CA-125 is strongly associated with advanced-stage disease, suggesting MUC16 might play a key role in tumorigenic progression, which is facilitated by the cytoplasmic domain of MUC16 that have phosphorylation activity. However, little is known about how the MUC16 cytoplasmic domain phosphorylation conveys cell signalling or participates with other cellular proteins. A recent study by Liu *et al.* [17] shows that knocking down the expression of MUC16 mediated via the Janus Kinase (JAK) pathway significantly inhibits the growth and metastasis of colorectal cancer cells *in vitro* and *in vivo*. Interestingly, CA-125 and MUC16 can be found in all ovarian cancer subtypes, suggesting that the underlying tumorigenic phenotype's biological aspects could be molecular subtype-independent, and it might be an excellent cellular target for treatment intervention. A previous study showed that engrafted ovarian tumour cells from ovarian cancer patients into NOD-SCID

IL2R $\gamma$ null (NSG) mice increased levels of CA-125 in both serum and ascitic fluids correlated with the disease progression [30].

IL-6 is a biomarker for ovarian cancer, and patients with high serum IL-6 correlate with a poor prognosis and more chemoresistant tumour phenotype. Interleukins (IL) exhibit both positive and negative effects on endothelial cells and angiogenesis. For instance, IL-6 and IL-8 are associated with increased angiogenesis, while IL-27 and IL-10 are linked to angiogenesis suppression. However, IL-27's antiangiogenic properties are not direct but rather mediated through the downregulation of proangiogenic-related genes such as vascular endothelial growth factor receptor-1 (*VEGFR1/FLT1*), prostaglandin G/H synthase 1 (*PTGS1/COX-1*), and fibroblast growth factor receptor 3 (*FGFR3*), as well as the upregulation of antiangiogenic genes like C-X-C motif chemokine 10 (*CXCL10*) and tissue inhibitor of metalloproteinase 3 (*TIMP3*). Similarly, IL-10 negatively affects proangiogenic cells, such as activated macrophages, by inhibiting proangiogenic matrix metalloproteinase 2 (MMP2) and promoting the overexpression of tissue inhibitor of metalloproteinase 1 (TIMP1) [31]. These mechanisms contribute to the complex interplay between



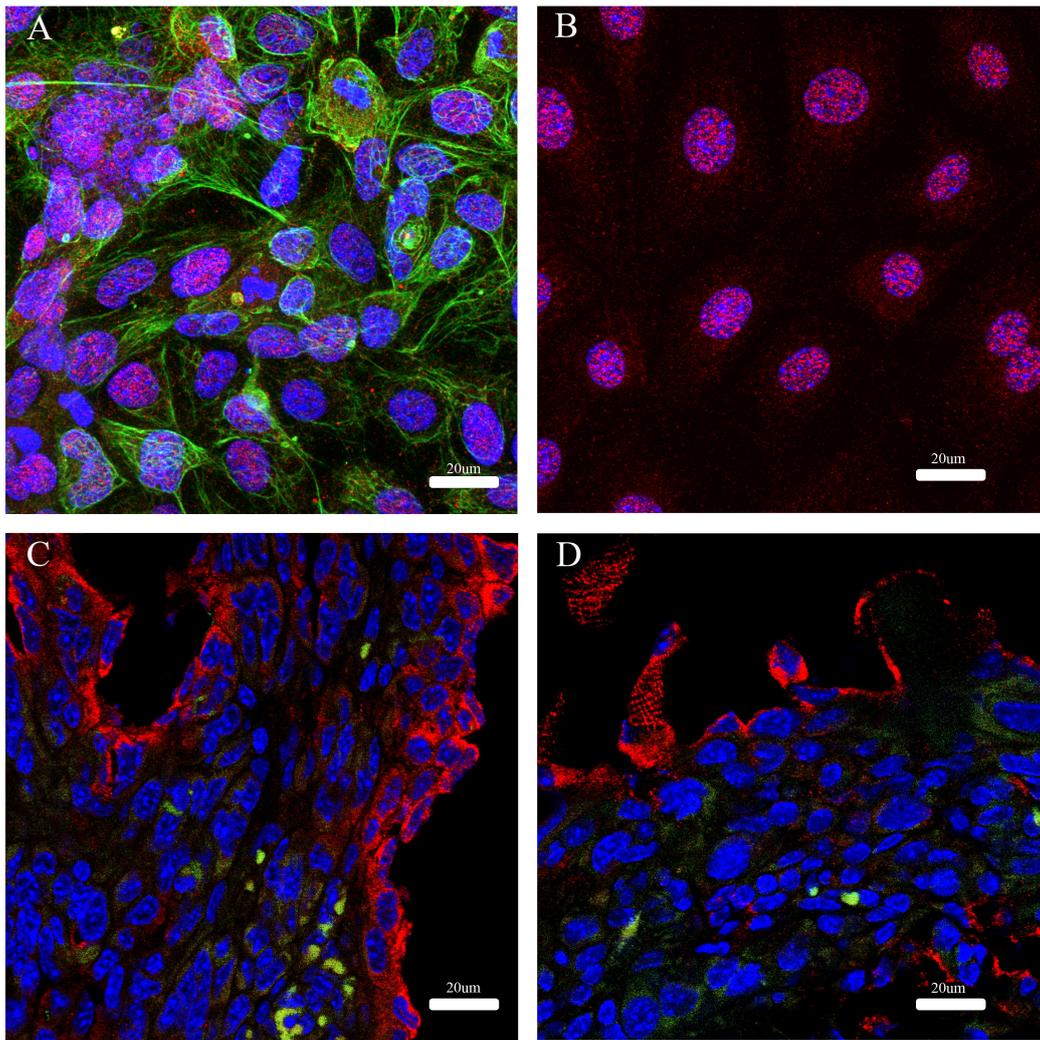
**Fig. 5. Levels of biomarkers in blood and ascitic fluids of mice with tumour.** Levels of CA-125 (A), IL-6 (B), VEGF (C) and ascitic fluid biomarkers (D) of mice implanted with ID-8 cells. Blood and ascitic fluid samples from mice were detected for biomarkers using enzyme-linked immunosorbent assay (ELISA). The levels of three markers were highly detectable in ascitic fluids (D). After culling mice, ascitic fluid from 9 mice (M#1–9) and peritoneal wash of one mouse (M#10) was subjected to IL-6 detection. Ascitic fluid from six mice (M#1–6) was employed for CA-125 detection. Ascitic fluid from three mice (M#2, 3, and 4) was used to detect vascular endothelial growth factor (VEGF). CA125, cancer antigen-125; IL-6, interleukin-6; VEGF, vascular endothelial growth factor.

ILs and angiogenesis regulation. IL-6 levels are significantly high in ascitic fluid and significantly associated with shorter progression-free survival [32]. The level of IL-6 from 18 ovarian cancer patients in ascites is more prevalent than in respective peripheral sera, and the IL-6 is likely a critical factor in the function of effector T cells mediated by tumor necrosis factor receptor 2 (TNFR2) [33]. Furthermore, preclinical and clinical study showed that IL-6 staining in ovarian tumour tissue was significantly associated with poor prognosis, and treatment of ovarian cancer cells with siltuximab inhibited tumour growth in tumour xenograft and patients [34]. Unfortunately, a small single-arm phase 2 clinical trial using monoclonal antibodies to block IL-6 molecules had modest clinical benefits [34].

VEGF is another serum biomarker associated with a poor prognosis in advanced ovarian cancer patients. The VEGF level is higher in serum in advanced-stage ovarian cancer patients compared to patients with benign conditions [35]. Furthermore, a specific isoform of VEGF is significantly associated with tumour lymphatic metastatic pheno-

type [36]. Circulating VEGF is produced from various cell types, including tumour cells and platelets. VEGF binds to its receptors, namely vascular endothelial growth factor receptor (VEGFR1, VEGFR2, VEGFR3) on blood and lymphatic vessel endothelial cells. The binding of VEGF to its receptors triggers a cascade of cell signalling pathways that facilitate a new formation of blood vessels and increase the peritoneal lymphatic and vasculature permeability [37,38]. As a result, intraperitoneal bevacizumab dramatically improves in controlling ascites and the quality of life in patients with advanced ovarian cancer [39]. However, there are contradictory findings regarding the predictive and prognostic value of VEGF levels in ascites that did not predict progression-free survival (PFS) and overall survival (OS) in ovarian cancer [40,41].

A previous study by Cho *et al.* [42] showed characteristics and tumour progression in a syngeneic orthotopic mouse ovarian cancer model of ID-8 cells using non-invasive ultrasound, clinical parameters and tumour biopsies. However, the study did not analyse blood and ascitic



**Fig. 6. Immunofluorescence of Mucin-16 and cytokeratin-18 in cell monolayers and cells in tumour tissue.** Immunofluorescence of Mucin-6 (red) and cytokeratin-18 (green) in a human ovarian cancer cell line, OV-90 (A) and a mouse ovarian cancer cell line, ID-8 (B). Tumour tissue of ID-8 in a mouse model was from the omentum/pancreas (C) and the surface of the abdominal wall (D).

fluid biomarkers that are clinically relevant. Some of our results are similar to this study to some extent. However, we use clinically relevant biomarkers to detect tumour progression and clinical observations. Our study shows that CA-125, IL-6 and VEGF are not elevated in the blood associated with tumour progression. This finding contrasts these three biomarkers in advanced ovarian cancer in humans. In our study, CA-125 is the highest maker in ascites compared to IL-6 and VEGF. Therefore, we would expect to detect CA-125 in blood, but it was not the case. One speculation could be that CA-125 protein fragments in a mouse could be slightly distinct from their human counterpart. One study showed that human MUC16 is present on the cell surface, and its CA-125 soluble fraction is cleaved and shed from the cell membrane [43]. However, murine MUC16 is present intracellularly, and the fraction CA-125 is intracellularly cleaved, shed and secreted out [43]. Our

data in Fig. 6 show cell monolayers of a human ovarian cancer cell line, OV-90 and murine ID-8 cells; the mucin-16 immunological staining is noticeable in the cytoplasm and the nucleus regions. However, MUC16 in ID-8 tumour tissue is starkly different from the *in vitro* cell monolayer, likely having the plasma membrane-cytoplasm associated with MUC16. However, this notion does not explain a lack of elevated IL-6 and VEGF levels in the blood. It might be hypothesised that the physiological contribution of anatomical constraint in a mouse peritoneum might be responsible for this. This hypothesis requires further investigation.

In humans, the peritoneum is well supplied with blood vessels and lymphatics, which generate a dense capillary network. About 70% of the plasma in the arterial capillary end will extravasate by the net balance between the hydrostatic and oncotic pressure to generate the interstitial and lymphatic fluid [44]. As a result, the healthy peritoneal cav-

ity should contain a small volume (20 mL) of serous exudate with various free-floating cells, including macrophages, mesothelial cells, and lymphocytes [45]. A change in the microenvironment within the peritoneum caused by various conditions can tip the balance leading to a pathological state [46]. Ascites occur when tumour cells grow on the surface of the peritoneal cavity, cytokines such as VEGF and IL-8 are released from the tumour, and normal cells interrupt the normal blood and lymphatic vessels, allowing the vessels to become permeable rather than absorptive function [45,47,48]. The presence of protein albumin in ascites is a prominent marker of vascular leakage, a clinical sign of malignant ascites [49,50]. It is uncommon to find red blood cells in ascites in patients with ovarian cancer, but the high amount of red blood cells in mouse ovarian tumour models is prevalent. Furthermore, mice with ascites also associate with white feet incidence, suggesting abnormally low red blood cells in the circulation.

Interestingly, in a mouse model, both syngeneic and immunocompromised mice with intraperitoneal tumour implantations produced bloody ascites [13,51,52].

Alternative imaging techniques use the ID-8 syngeneic tumour model to uncover tumour progression and the immune system's role. However, these imaging modalities pose some hurdles. For instance, the study describes that ascitic fluid can compromise optical imaging for detecting red fluorescent protein-tagged ID-8 cells for metastatic process using the mice harbouring ID-8 tumours [53]. Therefore, in order to optimise the use of this murine cancer model, further research should focus on determining appropriate methods of tumour monitoring.

We acknowledge the limitations of our study. Firstly, we did not include a control group of healthy mice without tumour implantation to use as a comparison for evaluating the association between weight gain and tumour progression. It is possible that both healthy mice and tumour-bearing mice exhibit similar weight gain as an endpoint, despite potential differences in their underlying mechanisms. It is worth noting that healthy mice and tumour-bearing mice may have different appetites for food, with healthy mice following a regular growth pattern, while mice with tumours may experience irregular appetite due to reduced food intake. Consequently, the mechanical pathway of weight gain may differ between the two groups, but ultimately lead to a similar endpoint. Secondly, we acknowledge that the amount of VEGF in serum was obtained from only three mice, which may not be sufficient to draw definitive conclusions. This limitation arises from the challenge of obtaining an adequate amount of blood from the mice for analysis. The limited sample size may introduce potential variability and reduce the accuracy of our findings regarding serum VEGF levels.

## 5. Conclusions

The ID-8 syngeneic mouse model has been widely used in ovarian cancer research, but our study is unique in investigating the association of biomarkers with tumour progression in this model. Our findings suggest that the three biomarkers evaluated in our study are unreliable for the early detection of tumour progression in blood. However, they may be valuable in investigating ascitic fluid as a part of the unique microenvironment of the advanced ovarian cancer model. In order to obtain a clearer understanding of the syngeneic mouse model of ID-8 tumours and its ability to accurately mimic the progression of human ovarian cancer, future studies should consider incorporating control mice without tumours. By including control mice alongside the experimental group, the outcomes of the study can be more effectively evaluated. Additionally, providing information on the number of mice in both the control and experimental arms would contribute to the robustness of the findings. These considerations will enhance our ability to assess the utility of the syngeneic mouse model in predicting the *in vivo* progression of ovarian cancer, thus closely emulating the human disease.

## Abbreviations

CA-125, Cancer antigen 125; IL-6, Interleukine-6; VEGF, Vascular Endothelial Growth Factor; ELISA, Enzyme-Linked Immunosorbent Assay; HGSOc, High-grade serous ovarian cancer; TVS, Transvaginal sonography; IP, intraperitoneal injection; PBS, Phosphate buffer saline; rpm, rate per minute; OCT, Optimal cutting temperature.

## Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

## Author Contributions

KC designed, executed, and analysed the research study. AD performed an animal study. PS designed and wrote the manuscript. KC, AD and PS contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

The study was approved by the Animal Ethics Committee of the University of Otago-Christchurch (approval number: C6/16), and this work and its approval was in accordance with the New Zealand Animal Welfare Act 1999, and Amendment No. 2, 2015.

## Acknowledgment

We like to thank Erin Lally for maintaining animal care.

## Funding

This research received no external funding.

## Conflict of Interest

The authors declare no conflict of interest. Kenny Chitcholtan is serving as one of the Editorial Board members and Guest editors of this journal. We declare that Kenny Chitcholtan had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Michael H. Dahan.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.ceog5010212>.

## References

- [1] Fong MY, Kakar SS. Ovarian cancer mouse models: a summary of current models and their limitations. *Journal of Ovarian Research*. 2009; 2: 1–8.
- [2] Scott C, Becker MA, Haluska P, Samimi G. Patient-derived xenograft models to improve targeted therapy in epithelial ovarian cancer treatment. *Frontiers in Oncology*. 2013; 3: 70425.
- [3] Tudrej P, Kujawa KA, Cortez AJ, Lisowska KM. Characteristics of *in vivo* model systems for ovarian cancer studies. *Diagnostics*. 2019; 9: 120.
- [4] Shah S, Cheung A, Kutka M, Sheriff M, Boussios S. Epithelial ovarian cancer: providing evidence of predisposition genes. *International Journal of Environmental Research and Public Health*. 2022; 19: 8113.
- [5] Playdon MC, Nagle CM, Ibiebele TI, Ferrucci LM, Protani MM, Carter J, *et al*. Pre-diagnosis diet and survival after a diagnosis of ovarian cancer. *British Journal of Cancer*. 2017; 116: 1627–1637.
- [6] Cheung A, Shah S, Parker J, Soor P, Limbu A, Sheriff M, *et al*. Non-epithelial ovarian cancers: how much do we really know?. *International Journal of Environmental Research and Public Health*. 2022; 19: 1106.
- [7] Chitcholtan K. Do we have enough knowledge to win the fight against ovarian cancer? *Edorium Journal of Gynecology and Obstetrics*. 2016; 2: 1–7.
- [8] Neesham D, Richards A, McGauran M. Advances in epithelial ovarian cancer. *Australian Journal of General Practice*. 2020; 49: 665–669.
- [9] Ferrari F, Cisotto F, Gambino A, Ferrari FA, Ardighieri L, Forte S, *et al*. Real World Application of Chemotherapy Response Score in High Grade Serous Cancer of the Ovary. *Clinical and Experimental Obstetrics and Gynecology*. 2022; 49: 147.
- [10] Ghose A, Gullapalli SVN, Chohan N, Bolina A, Moschetta M, Rassy E, *et al*. Applications of proteomics in ovarian cancer: dawn of a new era. *Proteomes*. 2022; 10: 16.
- [11] Shah S, Rachmat R, Enyiomu S, Ghose A, Revythis A, Boussios S. BRCA mutations in prostate cancer: assessment, implications and treatment considerations. *International Journal of Molecular Sciences*. 2021; 22: 12628.
- [12] Wilson AJ, Barham W, Saskowski J, Tikhomirov O, Chen L, Lee H, *et al*. Tracking NF- $\kappa$ B activity in tumor cells during ovarian cancer progression in a syngeneic mouse model. *Journal of Ovarian Research*. 2013; 6: 63.
- [13] Pisano S, Lenna S, Healey GD, Izardi F, Meeks L, Jimenez YS, *et al*. Assessment of the immune landscapes of advanced ovarian cancer in an optimized *in vivo* model. *Clinical and Translational Medicine*. 2021; 11: e551.
- [14] Zang RY, Harter P, Chi DS, Sehoul J, Jiang R, Tropé CG, *et al*. Predictors of survival in patients with recurrent ovarian cancer undergoing secondary cytoreductive surgery based on the pooled analysis of an international collaborative cohort. *British Journal of Cancer*. 2011; 105: 890–896.
- [15] Perez BH, Gipson IK. Focus on Molecules: Human mucin MUC16. *Experimental Eye Research*. 2008; 87: 400–401.
- [16] Epiney M, Bertossa C, Weil A, Campana A, Bischof P. CA125 production by the peritoneum: *in-vitro* and *in-vivo* studies. *Human Reproduction*. 2000; 15: 1261–1265.
- [17] Liu Z, Gu Y, Li X, Zhou L, Cheng X, Jiang H, *et al*. Mucin 16 Promotes Colorectal Cancer Development and Progression through Activation of Janus Kinase 2. *Digestive Diseases and Sciences*. 2022; 67: 2195–2208.
- [18] Streppel MM, Vincent A, Mukherjee R, Campbell NR, Chen S, Konstantopoulos K, *et al*. Mucin 16 (cancer antigen 125) expression in human tissues and cell lines and correlation with clinical outcome in adenocarcinomas of the pancreas, esophagus, stomach, and colon. *Human Pathology*. 2012; 43: 1755–1763.
- [19] Woolery KT, Kruk PA. Ovarian Epithelial-Stromal Interactions: Role of Interleukins 1 and 6. *Obstetrics and Gynecology International*. 2011; 2011: 1–9.
- [20] Ara T, DeClerck YA. Interleukin-6 in bone metastasis and cancer progression. *European Journal of Cancer*. 2010; 46: 1223–1231.
- [21] Jones SA, Scheller J, Rose-John S. Therapeutic strategies for the clinical blockade of IL-6/gp130 signaling. *Journal of Clinical Investigation*. 2011; 121: 3375–3383.
- [22] Matte I, Lane D, Laplante C, Rancourt C, Piché A. Profiling of cytokines in human epithelial ovarian cancer ascites. *American Journal of Cancer Research*. 2012; 2: 566.
- [23] Roszkowski P, Wronkowski Z, Szamborski J, Romejko M. Evaluation of selected prognostic factors in ovarian cancer. *European Journal of Gynaecological Oncology*. 1993; 14: 140–145.
- [24] Huang H, Li YJ, Lan CY, Huang QD, Feng YL, Huang YW, *et al*. Clinical significance of ascites in epithelial ovarian cancer. *Neoplasma*. 2013; 60: 546–552.
- [25] Krugmann J, Schwarz CL, Melcher B, Sterlacci W, Ozalinskaite A, Lermann J, *et al*. Malignant ascites occurs most often in patients with high-grade serous papillary ovarian cancer at initial diagnosis: a retrospective analysis of 191 women treated at Bayreuth Hospital, 2006–2015. *Archives of Gynecology and Obstetrics*. 2019; 299: 515–523.
- [26] Sopo M, Anttila M, Hämäläinen K, Kivelä A, Ylä-Herttuala S, Kosma V, *et al*. Expression profiles of VEGF-a, VEGF-D and VEGFR1 are higher in distant metastases than in matched primary high grade epithelial ovarian cancer. *BMC Cancer*. 2019; 19: 584.
- [27] Hassan W, Chitcholtan K, Sykes P, Garrill A. Ascitic fluid from advanced ovarian cancer patients compromises the activity of receptor tyrosine kinase inhibitors in 3D cell clusters of ovarian cancer cells. *Cancer Letters*. 2018; 420: 168–181.
- [28] Szubert M, Suzin J, Wierzbowski T, Kowalczyk-Amico K. CA-125 concentration in serum and peritoneal fluid in patients with endometriosis—preliminary results. *Archives of Medical Science*. 2012; 3: 504–508.
- [29] Vergote IB, Onsrud M, Børner OP, Sert BM, Moen M. CA125 in peritoneal fluid of ovarian cancer patients. *Gynecologic Oncology*. 1992; 44: 161–165.
- [30] Bankert RB, Balu-Iyer SV, Odunsi K, Shultz LD, Kelleher Jr

- RJ, Barnas JL, *et al.* Humanized mouse model of ovarian cancer recapitulates patient solid tumor progression, ascites formation, and metastasis. *PLoS ONE*. 2011; 6: e24420.
- [31] Ioannidou E, Moschetta M, Shah S, Parker JS, Ozturk MA, Pappas-Gogos G, *et al.* Angiogenesis and anti-angiogenic treatment in prostate cancer: mechanisms of action and molecular targets. *International Journal of Molecular Sciences*. 2021; 22: 9926.
- [32] Dalal V, Kumar R, Kumar S, Sharma A, Kumar L, Sharma JB, *et al.* Biomarker potential of IL-6 and VEGF-a in ascitic fluid of epithelial ovarian cancer patients. *Clinica Chimica Acta*. 2018; 482: 27–32.
- [33] Kampan NC, Madondo MT, McNally OM, Stephens AN, Quinn MA, Plebanski M. Interleukin 6 present in inflammatory ascites from advanced epithelial ovarian cancer patients promotes tumor necrosis factor receptor 2-expressing regulatory T cells. *Frontiers in Immunology*. 2017; 8: 1482.
- [34] Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Lester DA, *et al.* Interleukin-6 as a Therapeutic Target in Human Ovarian Cancer. *Clinical Cancer Research*. 2011; 17: 6083–6096.
- [35] Wang J, Zhu M, Zhou X, Wang T, Zhang J. Changes in tumor markers, coagulation function and serum VEGF in patients with ovarian cancer and benign ovarian disease. *Journal of B.U.ON*. 2020; 25: 2287–2292.
- [36] Kuerti S, Oliveira-Ferrer L, Milde-Langosch K, Schmalfeldt B, Legler K, Woelber L, *et al.* VEGF-C expression attributes the risk for lymphatic metastases to ovarian cancer patients. *Oncotarget*. 2017; 8: 43218–43227.
- [37] Masoumi Moghaddam S, Amini A, Morris DL, Pourgholami MH. Significance of vascular endothelial growth factor in growth and peritoneal dissemination of ovarian cancer. *Cancer and Metastasis Reviews*. 2012; 31: 143–162.
- [38] Adham SAI, Coomber BL. Glucose is a key regulator of VEGFR2/KDR in human epithelial ovarian carcinoma cells. *Biochemical and Biophysical Research Communications*. 2009; 390: 130–135.
- [39] Hamilton CA, Maxwell GL, Chernofsky MR, Bernstein SA, Farley JH, Rose GS. Intraperitoneal bevacizumab for the palliation of malignant ascites in refractory ovarian cancer. *Gynecologic Oncology*. 2008; 111: 530–532.
- [40] Trachana S P, Pilalis E, Gavalas NG, Tzannis K, Papadodima O, Lontos M, *et al.* The development of an angiogenic protein “signature” in ovarian cancer ascites as a tool for biologic and prognostic profiling. *PLoS ONE*. 2016; 11: e0156403.
- [41] Ford CE, Werner B, Hacker NF, Warton K. The untapped potential of ascites in ovarian cancer research and treatment. *British Journal of Cancer*. 2020; 123: 9–16.
- [42] Cho S, Sun Y, Soisson AP, Dodson MK, Peterson CM, Jarboe EA, *et al.* Characterization and evaluation of pre-clinical suitability of a syngeneic orthotopic mouse ovarian cancer model. *Anticancer Research*. 2013; 33: 1317–1324.
- [43] Goodell CA, Belisle JA, Gubbels JA, Migneault M, Rancourt C, Connor J, *et al.* Characterization of the tumor marker muc16 (ca125) expressed by murine ovarian tumor cell lines and identification of a panel of cross-reactive monoclonal antibodies. *Journal of Ovarian Research*. 2009; 2: 8.
- [44] Zawieja DC, Thangaswamy S, Wang W, Furtado R, Clement CC, Papadopoulos Z, *et al.* Lymphatic Cannulation for Lymph Sampling and Molecular Delivery. *The Journal of Immunology*. 2019; 203: 2339–2350.
- [45] Ritch S J, Telleria CM. The transcoelomic ecosystem and epithelial ovarian cancer dissemination. *Frontiers in Endocrinology*. 2022; 13: 886533.
- [46] Yu F, Chen J, Wang X, Cai Q, Luo J, Wang L, *et al.* Establishment of a novel mouse peritoneal dialysis-associated peritoneal injury model. *Clinical and Experimental Nephrology*. 2022; 26: 649–658.
- [47] Ahmed N, Stenvers K. Getting to know ovarian cancer ascites: opportunities for targeted therapy-based translational research. *Frontiers in Oncology*. 2013; 3: 63576.
- [48] Penet MF, Krishnamachary B, Wildes FB, Mironchik Y, Hung CF, Wu TC, *et al.* Ascites volumes and the ovarian cancer microenvironment. *Frontiers in Oncology*. 2018; 8: 595.
- [49] Stachowska-Pietka J, Waniewski J, Flessner MF, Lindholm B. Distributed model of peritoneal fluid absorption. *American Journal of Physiology-Heart and Circulatory Physiology*. 2006; 291: H1862–H1874.
- [50] Vorgias G, Iavazzo C, Mavromatis J, Leontara J, Katsoulis M, Kalinoglou N, *et al.* Determination of the Necessary Total Protein Substitution Requirements in Patients with Advanced Stage Ovarian Cancer and Ascites, Undergoing Debulking Surgery. Correlation with Plasma Proteins. *Annals of Surgical Oncology*. 2007; 14: 1919–1923.
- [51] Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE. Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. *Cancer Cell*. 2002; 1: 53–62.
- [52] Walton J, Blagih J, Ennis D, Leung E, Dowson S, Farquharson M, *et al.* CRISPR/Cas9-mediated *Trp53* and *Brca2* knockout to generate improved murine models of ovarian high-grade serous carcinoma. *Cancer Research*. 2016; 76: 6118–6129.
- [53] Lewellen KA, Metzinger MN, Liu Y, Stack MS. Quantitation of intra-peritoneal ovarian cancer metastasis. *Journal of Visualized Experiments*. 2016; 113: e53316.