The Potential Role of Platelets in the Progression of Ovarian Cancer

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Thesis for the degree of Bachelor of Science
University of Iceland
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Abstract

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Background: Epithelial ovarian cancer (EOC) generally presents with non-specific symptoms and is diagnosed at an advanced stage. Malignant cells spread from the ovaries, along the peritoneal cavity, and formation of ascites in patients is common. Paraneoplastic thrombocytosis is associated with adverse prognosis in various solid tumors, and in EOC it is associated with advanced disease and shortened survival. If platelets are present and active in the ascites of patients with EOC, it is possible that they play a role in the progression of the disease.

Research goal: Assess evidence of platelet migration and activation in the ascites of patients with EOC.

Materials and methods: Patient samples consisted of plasma (n=47) and ascites (n=26) samples. Plasma samples from healthy donors (n=54) were used as controls. Hemoglobin levels were measured in ascites samples to determine which samples had blood contamination, as platelets in those samples would not represent migration only. Western blots were performed to acquire a qualitative view (full length and cleaved form) of GPIb, a platelet specific membrane protein, in patient samples. In order to get a quantitative view of GPIb in ascites samples, and thus an estimate of platelet mass, a method for GPIb ELISA was put into development. Platelet activation was determined by measuring PF4 levels with an ELISA assay, as PF4 is a cytokine specific for platelets and is released from alpha granules following activation.

Results: The median hemoglobin level in ascites samples was 0.4% of the median hemoglobin level in plasma, suggesting negligible blood contamination in most samples. Western blot showed GPIb protein present in plasma and ascites of EOC patients. Development of an ELISA method for GPIb is still in progress. PF4 was present in plasma and ascites of EOC patients as well as healthy control plasmas.

Discussion: The presence of GPIb indicates that platelets are present in malignant ascites of EOC patients. Only ascites samples that had negligible hemoglobin levels were used for analysis which suggests that the presence of platelets was due to migration into the ascites, rather than blood contamination, although further confirmation is needed. PF4 levels also suggest that platelets are activated in malignant ascites of EOC patients. These results support our hypothesis that platelets are present and active in the tumor microenvironment of EOC, the peritoneum. Based upon evidence of platelet interaction with cancer cells, platelets may influence the progression of EOC in the peritoneum. To what extent is a question that still needs to be answered. Our hope is that further research into the contribution of platelets on the progression of EOC might lead to new therapeutic targets against mechanistic EOC pathways that involve platelets and platelet activation, or in fact against platelets themselves.
Ágrip

Hlutverk blóðflögna í útbreiðslu eggjastokkakrabbeins

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Inngangur: Einkenni eggjastokkakrabbeins af þekjuuppruna (Epithelial Ovarian Cancer, EOC) eru ósértæk og greinist sjúkdómurinn því oft á háu stigi. Meinið dreifir sér innan kviðarholsins og er uppsófnun kviðarholsvökvak algeng meðal kvenna með langt genginn sjúkdóm. Blóðflögumergð (paraneoplastic thrombocytosis) er algengur fylgivilli ýmissa krabbameina og í EOC hefur verið synt fram á tengsl blóðflögumergðar við verri sjúkdómsmynd og stytttri lífin. Finnist blóðflögur í kviðarholsvökv kvenna með EOC er mögulegt að þær gegni hlutverki í framgangi sjúkdómsins.

Markmið: Meta hvort blóðflögur ferðist sértaækt yfir í kviðarholsvökv kvenna með EOC og virkist þar.

Efni og aðferðir: Úrtak rannsóknarinnar samanstöð af blóðvökvavísun frá 47 konum með EOC, þar af 26 með kviðarholsvökv, og 54 heilbrig um konum til samanburðar. Hemóglóbín magn í blóðvökv og kviðarholsvökv var mælt til þess að meta hvaða kviðarholsvökvavísni innihéldu blóðflögur vegna blæðingar í kviðarholin en ekki vegna sértaæks flutnings. GPIb er himnubundi glykóprótein sértaækt fyrir blóðflögur. Western blot aðferðir var notuð til þess að meta hvort GPIb (í fullri lengd eða klofið) væri til staðar í kviðarholsvökv kvenna með EOC. Til þess að meta magn GPIb í sýnum og út frá því áætla magn blóðflagna í þeim þurfti að þróa ELISA aðferðir fyrir GPIb. Að því gefnu að blóðflögur væru sértaækt til staðar í kviðarholsvökv, vildum við meta hvort þær væru virkjaðar. PF4 er cýtokín sértaækt fyrir blóðflögur sem þær losa við virkjun. Því var PF4 mælt með ELISA aðferð í blóð- og kviðarholsvökv kvenna með EOC og borð saman við PF4 í samanburðarhópi.

Niðurstöður: Miðgildi hemóglóbíns í kviðarholsvökvavísun var 0,4% af miðgildi hemóglóbíns í blóðvökv sem bendir til óverulegar blóðmengunar í flestum sínum. Western Blot sýndi að GPIb prótein val til staðar í blóð- og kviðarholsvökv kvenna með EOC. ELISA aðferðir fyrir GPIb próteini er enn í þróun. PF4 var til staðar í blóð- og kviðarholsvökv EOC kvenna sem og í blóðvökv samanburðarhóps.

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Abbreviations

ADP: Adenosine diphosphate
BSA: Bovine serum albumin
DVT: Deep venous thrombosis
ELISA: Enzyme-linked immunosorbent assay
EOC: Epithelial ovarian cancer
EMT: Epithelial-Mesenchymal Transition
FIGO: Federation of Gynecology and Obstetrics
HRP: Horseradish peroxidase
IL-6: Interleukin-6
LMP: Low malignant potential
NK cells: Natural killer cells
PBS: Phosphate-buffered saline
PE: Pulmonary embolism
PF4: Platelet factor 4
SDS: Sodium dodecyl sulfate
SNARE’s: Soluble NSF attachment protein receptors
TF: Tissue factor
TGF- β: Transforming growth factor β
TNM: Tumor-node-metastasis
TPO: Thrombopoietin
TP53: Tumor protein p53
VEGFA: Vascular endothelial growth factor A
VTE: Venous thromboembolism
VWF: Von Willebrand factor
1 Background

Cancer is a complex disease that is a product of multiple changes within the tumor cell of origin and within the microenvironment that fuels disease progression and dissemination.\(^1\) Epithelial ovarian cancer (EOC) commonly presents with non-specific symptoms and is diagnosed at an advanced stage.\(^2,3\) Paraneoplastic thrombocytosis and increased rates of venous thromboembolism are associated with clinically aggressive EOC and reduced survival rates.\(^4,5\) Although the procoagulant state has been associated with worse clinical outcomes,\(^6\) there has been little study on how the hemostatic system might influence intra-peritoneal EOC progression. In this project, we investigated platelet presence, activity and role in EOC.

1.1 The ovaries

The ovaries are paired female reproductive organs located on either side of the uterus and below the fallopian tubes. Although they vary in size depending on a woman’s age and hormonal status, the ovaries are generally small and almond-shaped. The exterior is smooth in a woman’s early reproductive years but with time becomes scarred and convoluted. The ovaries are divided into three histological sections; the outer cortex, the inner medulla and the hilus. The outer cortex is lined with epithelial cells.\(^7\) Ovaries are both endocrine glands and gonads. They secrete cholesterol derived steroid hormones; estrogens, progestogens and androgens. They also produce and release the female gamete, the oocyte.\(^8\)

1.2 Ovarian cancer

Although a relatively rare malignancy, causing 1.3% of all new cancer cases in the U.S.,\(^9\) ovarian cancer is the second most common gynecologic malignancy. In the US, new cases were estimated to be 11.7 per 100,000 women between the years 2010-2014 and in Iceland, 6.8 per 100,000 women between the years 2006-2010.\(^9,10\) Ovarian cancer is also the most common cause of death from gynecologic cancer worldwide and is the fifth most common cause of cancer mortality in women.\(^11\) Five year survival rate is 92.1% for localized cases but 28.8% for cases where the cancer has spread.\(^9\)

The normal ovary has three cell types: surface epithelium, germ cells and sex cord stromal cells.\(^12\) These cell types give rise to different growths in the diverse group of ovarian tumors (Figure 1).\(^13\) The most common malignancy arises from the epithelium, accounting for approximately 80% of all ovarian cancers.\(^3\) While benign neoplasms are usually cystic, malignant neoplasms may be cystic (cystadenocarcinoma) or solid (carcinoma).\(^12\) Interestingly, recent evidence suggests that some ovarian cancers originate not from the ovary itself but from dysplastic regions in the distal fallopian tube. One of the current theories is that malignant tubal epithelial cells spill onto the ovarian surface and create the appearance of ovarian origin.\(^14\)
1.2.1 Classification

Epithelial ovarian tumors are classified based on cell type into serous, mucinous, endometrioid, clear cell, transitional and squamous cell tumors. These tumors are further categorized into benign, intermediate and malignant. Malignant epithelial tumors, carcinoma, have been categorized based on histopathology, immunohistochemistry and molecular genetics into five main types; high grade serous (70%), endometrioid (10%), clear cell (10%), mucinous (3%) and low grade serous carcinomas (<5%). These five types differ in response to chemotherapy, risk factors and mortality.\(^\text{15}\)

Another way to categorize malignant ovarian cancers is to divide them by grade into type I cancers composed of low-grade and borderline tumors of low malignant potential (LMP) and type II cancers composed of high-grade carcinomas. Borderline tumors are so named as they show the epithelial proliferation associated with malignancy yet without obvious invasion of the underlying tissue. About 10% of borderline cancers can metastasize and prove lethal to the patient.\(^\text{16}\) In general, patients with type I cancers are diagnosed at a younger age, have higher resistance to conventional cytotoxic therapy and yet have a better prognosis. Type II cancers are most common in postmenopausal women, are very sensitive to conventional chemotherapy at first but patients develop chemoresistance and only have a median survival time of 30 months.\(^\text{2}\)

1.2.2 Staging

The International Federation of Gynecology and Obstetrics (FIGO) Committee on Gynecologic Oncology sets the criteria for staging of ovarian cancer. Changes were last made to the staging system in 2012 (Table 1). At stage I the tumor is confined to ovaries or fallopian tube(s). Stage II, the tumor involves ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer. By stage III the tumor has spread to the peritoneum outside the pelvis and/or metastasized to retroperitoneal lymph nodes. At stage IV, distant metastases have formed excluding peritoneal metastases.\(^\text{17}\) Most patients (75%) present with advanced-stage disease with metastases within the peritoneal cavity at time of diagnosis.\(^\text{2}\)
The tumor-node-metastasis (TNM) staging can also be applied to ovarian cancer but the FIGO system is most commonly used world-wide.\textsuperscript{18}

<table>
<thead>
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<th>FIGO staging of ovarian cancer</th>
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<tr>
<td>STAGE I: Tumor confined to ovaries or fallopian tubes</td>
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<tr>
<td>STAGE II: Tumor involves one or both ovaries or fallopian tubes with pelvic extension or primary peritoneal cancer</td>
</tr>
<tr>
<td>STAGE III: Tumor involves one or both ovaries or fallopian tubes, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes</td>
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<td>STAGE IV: Distant metastasis excluding peritoneal metastasis</td>
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Table 1: FIGO staging classification of ovarian cancer (From Prat J, 2015)\textsuperscript{15}

1.2.3 Etiology and Risk Factors

It has been theorized that continuous ovulation is a risk factor for epithelial ovarian cancer. Epithelial cells of the ovary generally have a low proliferative rate but rupture of mature follicles induces proliferation of the epithelium in order to repair the ovarian surface. Spontaneous mutations can occur during cell proliferation that can lead to a cell turning malignant. One of the most frequent genetic abnormalities in ovarian cancer is the loss of function of the \textit{TP53} tumor suppressor gene, present in 60-80\% of both sporadic and familial cases.\textsuperscript{16} Rupture of follicles and the following repair can also lead to the entrapment of epithelial cells within the ovary forming an inclusion cyst. These cysts can develop neoplastic properties and result in epithelial malignancies.\textsuperscript{19}

Factors that decrease the number of ovulatory cycles, such as use of oral contraceptives, multiple pregnancies and breast feeding, decrease the risk of ovarian cancer while factors that increase ovulation, such as late menopause and nulliparity, increase the risk of ovarian cancer in later life.\textsuperscript{16} Family history and genetic factors, especially \textit{BRCA1} and \textit{BRCA2} mutations and Lynch syndrome, also increase the risk of ovarian carcinoma.\textsuperscript{3} Type I tumors more frequently have mutations in \textit{KRAS} and \textit{BRAF} oncogenes and microsatellite instability while mutations in the tumor suppressor genes \textit{BRCA1} and \textit{BRCA2} are more common in high-grade, type II, tumors.\textsuperscript{16}

1.2.4 Natural history of epithelial ovarian cancer

Cancer metastasis involves a series of complex interactions between cancer cells and the host. The main steps of metastasis include:

1. Loss of cell-to-cell adhesion and detachment of malignant cells from the primary mass
2. Migration of malignant cells into the local microenvironment
3. Intravasation of malignant cells into vascular and/or lymphatic vessels
4. Transportation along circulation systems
5. Extravasation from vascular and/or lymphatic vessels
6. Generation of metastatic foci in distal tissues

7. Growth into clinically detectable metastatic cancer mass

Epithelial ovarian cancer most commonly metastasizes along the peritoneal cavity but can also spread to the lungs, skin, lymph nodes, bones and brain. The spread to the peritoneum is often early and rapid and is known as transcoelomic dissemination. Development of malignant ascites, i.e. peritoneal fluid containing cancer cells, is frequently seen in EOC. More than one in three women with ovarian cancer present with malignant ascites at diagnosis. Lymphatic obstruction, activation of peritoneal mesothelial cells and increased vascular permeability due to high levels of tumor-derived vascular endothelial growth factor A (VEGFA) contribute to the accumulation of ascites. Unlike many other malignancies, ovarian cancer cells rarely disseminate through the vascular system. Ascitic fluid facilitates dissemination independent of angiogenesis to the peritoneum, the omentum and viscera in the peritoneal cavity. The tumor cells can survive as single cells or form multicellular aggregates in ascites. Within the ascites the EOC cells can interact with other cells in the microenvironment such as immune cells, fibroblasts and platelets; adhere to pelvic organs; and become secondary lesions. Therefore, it has become a widespread belief that malignant ascites plays a key role in formation of metastases to peritoneal organs. The presence of ascites is also associated with chemoresistance, higher risk of recurrence and poorer prognosis.

1.2.5 Diagnosis

Screening for ovarian cancer has not yet been proven to reduce mortality in the general population and is currently not recommended by most professional societies. Among the screening methods that have been employed are pelvic examinations, transvaginal ultrasounds and CA125 assays. The best strategy now seems to be to screen only among those in increased risk groups.

Ovarian carcinoma rarely produces specific symptoms. In early stages it may present itself in lower abdominal pressure or pain and in later stages it could present with symptoms such as constipation, nausea, anorexia and ascites formation. The presence of a pelvic mass is highly suggestive of an ovarian malignancy and often leads to an exploratory laparotomy. Because of this non-specific presentation, more than 50% of patients diagnosed with ovarian cancer have already developed metastases beyond the ovaries. Only 14.8% of ovarian cancer cases are diagnosed at the localized stage.

1.2.6 Treatment

For early stage ovarian cancer, FIGO stages I-II, the main recommendation is surgical treatment. For advanced cases, FIGO stages III-IV, a combination of surgery and chemotherapy is most often employed. Ovarian cancer is among the few malignancies in which cytoreductive surgery is performed to reduce tumor bulk, even when complete resection is impossible. Prognosis is correlated with the extent to which the initial surgery reduces tumor mass. Adjuvant chemotherapy with a platinum-paclitaxel combination has been shown to improve survival of women with advanced cancer. Initially, over 70% of patients respond to chemotherapy, but the majority relapses.
Chemoresistance is also problematic in the treatment of ovarian cancer, especially in advanced cases. Drug-resistant cells can persist and remain dormant in the peritoneal cavity for many months before rousing and growing progressively, leading to recurrent disease. Approximately 80% of women with advanced ovarian cancer will have tumor progression or a recurrence. Relapse of the disease usually leads to the death of the patient despite aggressive treatment.

1.2.7 Ovarian cancer and thrombosis

Hypercoagulability and increased incidence of venous thromboembolism (VTE) in cancer patients was first described by Trousseau in the 19th century. Thromboembolic disease can often be the first clinically recognizable manifestation of cancer. According to Virchow’s triad, three factors can contribute to thrombosis: endothelial injury, stasis and hypercoagulability. Endothelial injury can occur due to chemotherapy or intravasation of cancer cells, and stasis can be a consequence of a large tumor burden or ascites formation which compresses veins. Tumor cells and their products enhance coagulation by influencing thrombin- and plasmin-generation systems, as well as interacting with leukocytes, endothelial cells and platelets.

Although many cancers induce hypercoagulable states, deep venous thromboembolisms (DVT) and pulmonary embolisms (PE) seem to be more common in ovarian cancers than in any other solid tumor. Levitan et al. demonstrated a rate of 120 DVT/PE per 10,000 patients, compared to rates of 44 in uterus cancer and 22 in breast cancer. In EOC, there is an increased rate of VTE, association of VTE with clinically aggressive variants and an adverse effect on overall survival, suggesting that systemic hypercoagulability is an innate part of ovarian cancer biology. Thromboembolism is the second leading cause of death in cancer patients overall, with progression of cancer being the first, and DVT in patients with ovarian cancer has been shown to increase risk of death significantly.

While the mechanisms behind hypercoagulability in cancer patients are not fully known, it is likely that important factors taking part in those mechanisms are tissue factor (TF), inflammatory cytokines and, the focus of this thesis, platelets.

1.3 Platelets

Platelets are circulating anucleate cells that are essential for hemostasis and thrombosis. They also play part in other physiological processes such as inflammation, angiogenesis and wound healing.

1.3.1 Production

The mechanism by which platelets are produced is not yet completely understood. Platelets are thought to be produced by pro-platelet budding from megakaryocytes located primarily in the bone marrow. However, a recent murine study suggests that megakaryocytes in the lungs could also contribute to thrombopoiesis, accounting for up to 50% of the total platelet production in the mice of that study.
Thrombopoietin, a hormone produced mainly in the liver, is the main stimulator of platelet production. Inflammatory mediators such as interleukin-6 (IL-6) can increase hepatic production of thrombopoietin, thus boosting platelet count (Figure 2).\textsuperscript{38} IL-6 is overexpressed in most ovarian cancers, leading to autocrine stimulation of the IL-6 receptor and activation of the JAK-STAT signaling pathway.\textsuperscript{16} This signaling upregulates thrombopoietin (TPO) mRNA expression and expression of other genes that not only increase thrombopoiesis but also stimulate angiogenesis, cell proliferation and inhibition of apoptosis.\textsuperscript{39-41}

Figure 2: The proposed paracrine signaling pathway leading to increased thrombopoiesis in ovarian cancer and potential therapeutic targets. Modified from Stone et al., 2012, from Blood comment section.\textsuperscript{42}

1.3.2 Activation of platelets

Inactivated platelets are kept in a discoid and nonadherent form by endothelial cells, which produce inhibitory substances such as prostaglandin $I_2$ and nitric oxide and inactivate platelet agonists such as ADP and thrombin.\textsuperscript{43}

Platelet activation does not normally occur in an intact blood vessel. Activation occurs upon vessel wall injury causing platelets to change in shape, increase in adhesiveness and develop a prohemostatic surface.\textsuperscript{44} An early step is platelet adhesion, which represents the initial binding of the platelet to an injury site, mediated by interaction between the platelet surface GPIb-V-IX receptor and a conformationally active form of von Willebrand factor (VWF) bound to exposed collagen surface.\textsuperscript{43} The GPIb-V-IX complex consists of the glycoprotein subunits GPIba, GPIbβ, GPV and GPIX and is exclusively found on platelets and megakaryocytes.\textsuperscript{45} Binding of VWF and the glycoprotein complex leads to so-called “inside-out” signaling in the platelet, which increases the affinity and avidity of the platelet integrin $\alpha_{Ib}\beta_3$ (GPIIb/IIIa) for ligands such as fibrinogen.\textsuperscript{46} Binding of fibrinogen results in linkage and aggregation of the activated platelets, enabling the formation of a platelet plug.\textsuperscript{44} It also initiates intracellular signaling cascades known as “outside-in” signaling, which leads to stable adhesion and granule secretion.\textsuperscript{47}
Platelets contain different types of storage granules, including dense granules and alpha granules (Figure 3). Dense granules contain small molecules such as calcium, polyphosphates, ADP and serotonin. Alpha granules contain many proteins, including hemostatic factors such as fibrinogen and factor V, angiogenic factors such as VEGF, anti-angiogenic factors such as PF4, growth factors, proteases and other cytokines. As mentioned above, the contents of these granules are released upon activation. Involved in this exocytosis is both the fusion between granules and between the granule membrane and cell membrane. This fusion is mediated by soluble NSF attachment protein receptors (SNAREs). The SNARE proteins are regulated by protein kinase C (PKC)-dependent phosphorylation that is controlled by GTPases such as RAB27.

Platelets also release two membrane derived vesicles during activation, microvesicles from the cell surface and exosomes of endosomal origin. Microvesicles contain several platelet surface glycoproteins including GP Ib.

Platelet factor 4 (PF4), or CXCL4, is a chemokine expressed exclusively in megakaryocytes and in mature platelets. In the platelet, it is a component of the alpha granule and is released upon platelet activation. Normal levels of PF4 in plasma are approximately 2-10 ng/mL. Released PF4 has a procoagulant function and affects coagulation in many ways, for example by regulating thrombin/thrombomodulin complexes and promoting stable thrombus formation through the binding of fibrin.

1.3.3 Thrombocytosis
Normal values for platelet count varies between individuals, from 150,000 to 450,000 per microliter of blood. Thrombocytosis is defined as a platelet count of over 450,000 per microliter and is divided into primary and secondary forms. Primary thrombocytosis is associated with myeloproliferative syndromes while secondary thrombocytosis, also known as reactive thrombocytosis, can be observed in inflammatory states such as infections, trauma and malignancy. Thrombocytosis occurs in 10-50% of patients with solid malignancies. Thrombocytosis associated with cancer is thought to be related to the increased cytokine production by tumor and host tissues, such as the production of IL-6, which drives thrombopoiesis.

1.3.4 Platelets and cancer
The concept that platelets play a role in cancer growth and metastasis was recognized early on. One of the first studies on this relationship published its findings in 1964, showing that thrombocytopenic mice were protected against cancer metastasis.
Studies have since demonstrated that thrombocytosis is an adverse prognostic factor in various solid tumors such as breast, lung, colon and ovarian cancers.\textsuperscript{55} A vicious cycle forms where tumor cells increase platelet production and activation and in turn platelets drive cancer growth, angiogenesis and metastasis (Figure 4).\textsuperscript{55}

Cancer cells can induce platelet activation through various mechanisms, including direct cell-cell contact and release of platelet activating mediators such as ADP and thrombin. Various cancers also disrupt hemostasis, producing a hypercoagulable state in the patient and leading to further platelet activation.\textsuperscript{20}

Activated platelets influence cancer progression by stimulating angiogenesis, cell mobility, adhesion, proliferation, chemoresistance and immune system evasion.\textsuperscript{56} As previously mentioned, platelets contain granules with an abundance of proteins, such as the angiogenic mediator VEGF.\textsuperscript{49} Upon activation, platelets release these factors which enhance tumorous angiogenesis, an important factor in growth and metastasis.\textsuperscript{20} Platelets have been shown to stimulate cell proliferation and resistance to apoptosis through release of granule contents.\textsuperscript{1} Upon exposure to platelet derived TGF-\(\beta\)1, cancer cells reduce E-Cadherin expression and increase expression of epithelial to mesenchymal transition (EMT) markers, leading to increased cell mobility.\textsuperscript{56} Platelets can enhance invasive qualities of cancer cells through release of TGF-\(\beta\) and facilitate extravasation of cancer cells via release of serotonin and histamine.\textsuperscript{20,57}

Studies suggest that yet another way platelets facilitate metastasis is by protecting tumor cells in circulation from shear stress and detection of the immune system by forming a layer cloaking the malignant cells.\textsuperscript{58} TGF-\(\beta\), released from activated platelets, suppresses NK-mediated cytolytic activity by interfering with mobilization and interferon-\(\gamma\) secretion.\textsuperscript{59} and recent studies have demonstrated that tumor cells can acquire platelet major histocompatibility complex, thus preventing recognition by NK cells.\textsuperscript{60}

Antiplatelet therapy has been shown to inhibit metastasis in animal models, raising hope that platelets might be utilized as a therapeutic target in humans.\textsuperscript{20}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Overview of known and proposed interactions between malignant cells and platelets. From Buergy et al., 2012\textsuperscript{55}}
\end{figure}
1.3.4.1 Platelets and ovarian cancer

One in three women diagnosed with ovarian cancer have a platelet count exceeding 450,000 per microliter, fulfilling the diagnostic criteria of thrombocytosis. A recent study showed that thrombocytosis at time of diagnosis of stage III and IV ovarian cancer had prognostic value regarding overall survival and progression free survival. Another study published data demonstrating that overall survival among ovarian cancer patients with thrombocytosis was 2.62 years compared with 4.65 years among those with normal platelet counts.

Platelets in the microenvironment of malignancies create a growth factor rich setting, increasing the growth and spread of neoplastic cells. As ovarian cancer primarily spreads within the peritoneal cavity, the microenvironment of advanced ovarian cancer is usually comprised of the peritoneum and ascites within, making ascites samples a good model within which to study the EOC microenvironment.

Platelets have been shown to extravasate into the tumor bed and ascites in mice with ovarian cancer. The platelets were detected primarily in a perivascular location in the tumor bed but free-floating and adherent to tumor cells in ascites. The same study demonstrated that platelet count was not elevated in the normal peritoneum or in the peritoneum after induction of peritonitis.

In humans, a recent study demonstrated that, in vitro, platelets were activated upon contact with ovarian cancer cell medium and enhanced ovarian cancer cell migration. It also detected platelets in peritoneal fluid extracted from ovarian cancer patients, both in contact with cancer cells and free platelets. It did not, however, explain how the platelets came to be in the ascites, whether they migrated there independent of whole blood or if their presence was a consequence of a ruptured vessel. Therefore, it has not yet been ascertained whether platelets migrate into ascites and undergo activation there, a process that could significantly drive disease progression.

As the importance of platelets in the progression of cancer is becoming increasingly evident, the research question driving this study is: Do platelets preferentially migrate into and undergo activation in the ascites of human patients with advanced ovarian cancer?

1.4 Aim of this study

The primary aim of this study was to investigate specific evidence for platelet migration and activation in the tumor microenvironment of EOC. In order to answer the research question, the following studies were performed:

1. Hemoglobin levels were measured in the tested ascites samples in order to determine if any samples had been contaminated by spontaneous bleeding into the peritoneum or during surgical debulking. Samples with high levels of hemoglobin were considered to be contaminated with blood and hence, platelets. Platelets present in those samples would therefore not represent specific migration.

2. Presence of the platelet membrane protein GPIb in EOC samples was determined with the western blotting method. As GPIb is specific for platelets, it was used as a marker for their presence in the study samples. Western blotting provides a qualitative or semi-quantitative view of GPIb and the different forms of the protein present in each sample.
3. For a quantitative analysis of GPIb in ascites, and thus an estimate of platelet mass, an enzyme-linked immunosorbent assay (ELISA) was chosen. As there was no defined, published method for GPIb ELISA available and the few commercially available were prohibitively expensive, questionably reliable or ill-defined (i.e. no information on epitope recognition or antibody specificity), the study involved development of a GPIb ELISA.

4. Platelet activation was determined using PF4 levels in samples, as this cytokine is specific for platelets and is released from their alpha granules following activation. PF4 levels were determined using a commercially available, quality tested and reliable ELISA assay.
2. Material and methods

Refer to appendix for recipes of solutions and more detailed description of material and methods.

2.1 Study population and ethics approval

The study population consisted of women diagnosed with EOC from 2013-2016, who were referred to the University of Wisconsin Hospital and Clinics for surgical debulking of their tumor. The study protocol was approved by the University of Wisconsin-Madison’s Institutional Review Board (IRB) and all participants signed informed consent. Pre-operative plasma (n=47) and ascites (n=26) samples were collected from patients with EOC and plasma samples (n=54) were also collected from healthy women recruited to be sex-matched non-EOC controls (Table 2). Patients were excluded if they had been therapeutically anticoagulated within 2 weeks prior to study entry.

2.2 Blood and ascites sample processing

Blood samples were collected into 3.2% sodium citrate tubes and 50µg/mL corn trypsin inhibitor (CTI) added prior to plasma isolation. Samples were centrifuged at 200xg for 10 minutes to separate RBC from plasma. Plasma was transferred to fresh tubes and centrifuged at 13000xg for 10 minutes. Supernatant, platelet poor plasma, was transferred into fresh tubes and portions either further processed to obtain microparticles or aliquoted, snap frozen and stored at -80°C; the platelet pellet was lysed using IP lysis buffer with proteinase inhibitor cocktail. Ascites were collected intraoperatively into 3.2% sodium citrate tubes.

2.3 Hemoglobin quantification

Hemoglobin levels in ascites samples were determined using Drabkin’s assay from Sigma-Aldrich®. Samples were diluted with water to lyse cells under hypotonic conditions before adding Drabkin Reagent and letting hemoglobin react. Samples were transferred to a 96 well plate with standards and water for zero point. Plate was read in a UV spectrophotometer and absorbance at 540nm and 600nm

Table 2: Overview of study population. Age and BMI values presented in mean and standard deviation. *Menopause status missing for n=9
measured. Each sample was measured in triple biological replicates (n=3) and the hemoglobin content ascertained via the standard curve.

Hemoglobin levels in plasma samples were determined by the hospital clinical lab, along with other values in the blood panel.

2.4 Western Blotting

Due to its specific expression in platelets and megakaryocytes, western blotting was performed to determine the presence of GPIbα (CD42b), a subunit of GPIb, in ascites samples as a marker for platelet content. Samples and molecular weight marker were loaded onto gels and then run at 190V for 50 minutes at room temperature. Gels were then transferred to Immobilon-FL® membranes using the wet transfer method with Towbin buffer. Membranes were blocked with blocking solution containing 5% dry milk in TBST for at least 60 minutes. Membranes were washed with TBST and then incubated overnight with a 1:3000 dilution of the primary antibody, 1mg/mL rabbit anti-human CD42b polyclonal antibody. Membranes were washed with TBST, incubated with a 1:5000 dilution of the secondary antibody, 1mg/mL HRP conjugated goat anti-rabbit IgG, for 45 minutes at room temperature, and then washed again. Chemiluminescence substrate was added and blots imaged on a Bio-Rad ChemiDoc Imaging System.

The main goal of performing western blots for GPIb was to detect what forms of the protein were present in the ascites samples (Figure 5). Variations in forms of protein, most likely due to different protein cleavage and post-translational modification, result in differences in molecular weight and therefore separate bands. Detecting these different bands helped with deciding which antibodies to use for the GPIb ELISA as antibodies were needed that could recognize epitopes that represented all forms and polymorphisms of GPIb.

![GPIb alpha subunit](image)

Figure 5: GPIb alpha subunit. The chromosomal location, splicing variants and preliminary protein product. Various forms of protein fragments after modification or cleavage are not fully known.
2.5 ELISA

2.5.1 GPIb ELISA

ELISA’s were intended to test GPIb quantitatively in ascites samples of patients with ovarian cancer. As an adequate protocol for GPIb ELISA was not found in any references, we developed a sandwich ELISA method (Figure 6). For capture antibody, we tested combinations of two antibodies, the polyclonal GPIbα from ThermoFisher and the monoclonal GPIbα from Santa Cruz. GPIbα is a protein with a length of 652 amino acids. The polyclonal antibody recognizes epitopes corresponding to a region within amino acids 92-266 of human GPIbα and the monoclonal antibody (SZ2 clone) recognizes a region within amino acids 276-282. We wanted antibodies that could recognize all the different forms of GPIb present in the samples but would not be conformation-sensitive for the N41H mutation of CD42b. For detection we tested three different antibodies, the polyclonal GPIbα antibody from ThermoFisher, the monoclonal GPIbα antibody from Santa Cruz and a monoclonal biotinylated GPIbα antibody from Novus Biologicals.

For the capture antibodies, we tried different working concentrations, from 0.5µg/mL up to 10µg/mL. For coating buffer, we first used phosphate-buffered saline (PBS) with a pH of 7.4 and then changed the buffer to 0.2M sodium bicarbonate with a pH of 9.4.

For blocking buffer, we tried both 1% bovine serum albumin (BSA) in PBS and 5% skim milk in PBS with 0.05% TWEEN 20.

For the detection antibodies, we used working concentrations of 0.5µg/mL. When using a non-biotinylated detection antibody, we added a secondary horseradish peroxidase (HRP) conjugated antibody in a working concentration of 200ng/mL. The secondary antibodies were anti-rabbit, for the polyclonal GPIbα, and anti-mouse, for the monoclonal GPIbα. When using the biotinylated GPIbα antibody for detection, we added Streptavidin-HRP in a 1:200 dilution.

The wash buffer used was PBS with 0.05% TWEEN 20. For reagent diluent we tested 2% skim milk in PBS with 0.02% TWEEN 20 and 1% BSA in PBS.

Wells of an EIA microtiter plate were coated with 100µL of capture antibody and incubated overnight at 4°C. The following day, wells were washed with wash buffer and blocked for 2 hours at room temperature. Standard dilutions of human GPIb recombinant protein (960, 640, 320, 160, 80, 40, 20, 10, 5 and 0 pg/mL as well as 40pg/mL and 0pg/mL control standards with a 1:100 dilution of 10.5mmolar 3.2% sodium citrate added) were made with reagent diluent. Ascites samples were prepared by diluting them 1:100 in reagent diluent. Wells were washed before adding 100µL of standard or sample, then left to incubate for 2 hours at room temperature. After incubation, wells were washed and 100µL of detection antibody added. The plate was incubated for 2 hours at room temperature and washed before adding secondary HRP-conjugated antibody or streptavidin-HRP and incubated again for 45 minutes out of contact with direct light. Wells were washed, then 100µL of substrate solution was then added to each well and the plate was incubated again for 20 minutes at room temperature protected from light. Finally, 50µL of stop solution were added to each well and the optical density determined using a microplate reader set to 450nm with corrections at 540nm.
2.5.2 PF4 ELISA

ELISA’s were performed to detect PF4 levels in plasma and ascites samples of EOC patients as well as in plasma of healthy donors for comparison. A similar protocol as in the GPIb ELISA was followed, using human PF4 standards, capture and detection antibodies from R&D Systems™. As the detection antibody was biotinylated, streptavidin conjugated to HRP was able to bind to the antibody. Substrate solution, a 1:1 mixture of color reagent A and B from R&D Systems™, was cleaved by the HRP to produce a “free” chromophore, and a stop solution halted the reaction. The optical density of each well was determined using a microplate reader set to 450nm with corrections at 540nm.

2.6 Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.00 by GraphPad Software, Microsoft Excel 2016 and SPSS 23. D’Agostino & Pearson omnibus normality test was performed to determine whether data had a normal/Gaussian distribution or not. Grubb’s test was performed to identify outliers. For comparisons between groups with non-normally distributed continuous data, Mann-Whitney U tests were used for analysis. For comparisons between non-continuous data, Fisher’s Exact tests were used. A p-value of <0.05 was considered statistically significant. Bonferroni corrections were performed for multiple comparisons.
3. Results

3.1 Hemoglobin quantification

Hemoglobin levels in ascites samples were measured and compared to hemoglobin levels in plasma (Table 3).

<table>
<thead>
<tr>
<th></th>
<th>Median Hb g/dL (IQR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOC plasma (n=46)</td>
<td>13.2 (12.6-13.9)</td>
</tr>
<tr>
<td>EOC ascites (n=26)</td>
<td>0.05 (0.02-0.2)</td>
</tr>
</tbody>
</table>

Table 3: Hemoglobin (Hb) levels in EOC patient samples (IQR, interquartile range)

Based upon these results, a rough estimate of blood contamination was devised in order to exclude contaminated samples as “biased”. Blood contamination was considered to be present if hemoglobin in the ascites sample was more than 1% of hemoglobin in the plasma sample from the same patient. If hemoglobin in the ascites sample was less than 1% that of plasma, blood contamination was considered to be negligible, i.e. the amount of blood present, if any, would theoretically not have enough platelets to be biologically relevant. Samples with non-detectable levels of hemoglobin, i.e. with levels below the detectable range of the assay, were considered to have no contamination. We chose 1% based upon observations of the amount of blood or platelet number per blood volume that was theoretically negligible for platelet activity/physiologic effects, absorbance measurements and standard curve. Thus, blood contamination was calculated for each sample and 7/26 or 27% of the ascites samples were considered to have blood contamination.

3.2 Western blotting of GPIb

Platelet lysate samples derived from plasma (#-Plt) and raw ascites (#-Asc) samples from six patients with EOC were blotted, along with platelet lysate from the plasma of a healthy donor for comparison (C Plt). Different sets of bands could be seen for platelet lysate and ascites, which is most likely due to differential protein cleavage and post-translational modification. For the platelet lysate from plasma, bands could be seen at approximately 190 kDa, 170 kDa and 110 kDa. For the ascites samples, bands were detectable at 170 kDa, 130 kDa, 80 kDa, 60 kDa and 34 kDa. (Figure 7)
Hemoglobin levels in the ascites samples were measured and 4 of 6 samples run in this assay had negligible hemoglobin contamination, less than 1% of hemoglobin in plasma. Samples #55 and #63 had what we determined to be blood contamination, with hemoglobin levels higher than 1% of that in plasma.

The molecular weight of GPIb is reported to range from 168kDa to 153kDa, due to heterogeneity of the protein. The molecular weights of its subunits have been determined as approximately 143kDa and 22kDa for GPIbα and GPIbβ, respectively. The cleaved form of GPIb, glycocalicin, has the molecular weight of 130kDa.68 The bands seen on the western blot performed (Figure 7) are not completely consistent with the reported molecular weights of the proteins. Factors that might explain this are post-translational modifications that add to the molecular weight69 and the possibility that GPIbα was still bonded with GPIbβ through their disulfide bond68, as no reducing agent was used in the assay and only a denaturing agent, SDS, was used. As the antibody used was a GPIbα polyclonal antibody, bought pre-tested from a trusted manufacturer, we were confident that we were detecting GPIbα and not some other protein through nonspecific binding. The true test for specificity would have been to run GPIb deficient plasma on the blot, but this was unavailable.

Having determined that GPIb was present in 6 of the 26 ascites samples randomly selected we moved on to developing the ELISA assay to quantify GPIb protein in the samples.

3.3 GPIb ELISA

As we could not find a method for GPIb ELISA in the literature, we decided to develop our own as listed above. Twelve trials were performed over a three-week period, without success. No increase was seen in absorbance with increasing protein concentration or with increasing antibody concentration, suggesting lack of binding of or to the antibodies. We do not fully know what was
lacking in the protocol and what needed to be altered for the assay to work. Many factors need to be considered, but those that we find most relevant are the following:

a) Capture antibody: The capture antibody can stick to the plate in a way that its binding region is not accessible to the antigen or there might be steric hindrance from neighboring antibodies. The capture antibody worked well in the western blotting but there the protein antigen is denatured so the epitopes open up and are more accessible. In ELISA’s the secondary structure of the protein is intact and so the epitope the antibody recognizes might have been unaccessible.

b) Coating buffer: When choosing a coating buffer the main issue is the pH as an environment with higher pH is more charged which helps the capture antibody bind to the plate. However, too high of a pH could lead to degradation of protein, including the antibody. At first, we used PBS with a pH 7.4 as a coating buffer, which did not give good results, so we changed the buffer to a 0.2M sodium bicarbonate buffer with a pH 9.4 and that yielded better results. PBS is sometimes preferred for use in ELISA kits as it has a long shelf life and is less sensitive to exposure to air than sodium bicarbonate, however it has a lower pro-binding ability.

c) Blocking solution: Blocking the high protein binding plate is very important in performing ELISA’s as you do not want the detection antibody to bind to non-specific antigen that is stuck to the plate directly instead of the capture antibody. We tried two different methods of blocking: we used 5% dry milk but that coated the plate “too well” resulting in no signal at all and so we switched to 1% BSA in PBS which worked but had some nonspecific binding evident from a high background signal. Milk can have a tendency to deposit a film onto the plate and captured antibody, thus blocking binding. Although we use non-fat milk, this does not necessarily mean the milk only contains proteins. However, BSA can also be problematic as antibodies can bind non-specifically to serum proteins or Fc receptors. Using heat denatured serum albumin or serum from other species would be the next step.

d) Detection antibody: The detection antibody can have problems as well. It can only recognize a specific epitope that it may not have access to if the antigen has bound to the capture antibody in a certain way and it can have nonspecific binding to the BSA used to block the plate.

In short, we were not able to develop an ELISA protocol for GPIb in this study and were thus not able to quantify GPIb.
3.4 PF4 data

Results from the PF4 ELISA assay are presented in Table 4.

<table>
<thead>
<tr>
<th>PF4</th>
<th>Average (±SD)</th>
<th>Min</th>
<th>25th percentile</th>
<th>Median</th>
<th>75th percentile</th>
<th>Max</th>
<th>Number of samples (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy plasma</td>
<td>30.76 (±34.82)</td>
<td>4.13</td>
<td>9.52</td>
<td>20.21</td>
<td>36.68</td>
<td>177.37</td>
<td>42</td>
</tr>
<tr>
<td>Cancer plasma</td>
<td>64.07 (±46.15)</td>
<td>12.53</td>
<td>27.86</td>
<td>51.79</td>
<td>79.9</td>
<td>211.56</td>
<td>45</td>
</tr>
<tr>
<td>Cancer ascites</td>
<td>41.16 (±71.43)</td>
<td>0.46</td>
<td>1.90</td>
<td>6.05</td>
<td>42.33</td>
<td>284.02</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 4: PF4 levels in cancer patients and healthy donors

As the PF4 level data was not normally distributed according to the D'Agostino & Pearson omnibus normality test we used the Mann Whitney statistical test and determined that median PF4 levels were significantly higher in the plasma of cancer patients compared to the median PF4 levels in plasma of healthy individuals, (51.79 v 20.21, p < 0.0001). These results might indicate that EOC patients have more platelet activity compared to healthy individuals. PF4 levels in ascites had a wide range, from almost non-detectable to very high levels. Outliers were determined, using Grubb’s test, and are depicted as colored points on the graph below (Figure 8). Interestingly, outliers in the ascites samples, are the samples that were contaminated with blood. As seen on the graph, PF4 levels in plasma of EOC patients were higher than in plasma of healthy controls, as reported above.

Figure 8: PF4 levels in plasma of healthy donors, plasma of EOC patients and ascites of EOC patients. The graph shows median PF4 levels with interquartile ranges of each subgroup. Colored points represent statistical outliers.
3.5 Comparison of disease markers by FIGO stage

To investigate whether the EOC disease markers of interest to this study differed by disease stage, the patient group (as described in Table 2) was divided into two groups by severity of disease; Patients with less advanced disease (FIGO I/II) were compared to those with more advanced disease (FIGO III/IV). The results are presented in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>FIGO stage I/II</th>
<th>FIGO stage III/IV</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=16</td>
<td>N=31</td>
<td></td>
</tr>
<tr>
<td>Platelets (CBC)</td>
<td>275.5 (135-385)</td>
<td>360.0 (215-668)</td>
<td>0.001</td>
</tr>
<tr>
<td>Thrombocytosis, n (%)</td>
<td>0 (0%)</td>
<td>6 (18%)</td>
<td>0.082 f</td>
</tr>
<tr>
<td>PF4 blood (ng/ml)</td>
<td>33.8 (12.5-160.7)</td>
<td>68.7 (16.6-211.6)</td>
<td>0.041</td>
</tr>
<tr>
<td>Ascites, n (%)</td>
<td>4 (25%)</td>
<td>22 (71%)</td>
<td>0.005 f</td>
</tr>
</tbody>
</table>

Table 5: Results of comparison of disease markers between EOC disease stages. Mann-Whitney and Fisher’s Exact test were used to compare the groups. Continuous values are presented in median with range.

When these two groups were compared there was a significant difference in platelet count between less advanced disease and more advanced disease, 275.5 vs 360.0 p=0.001, which is consistent with other studies. However, there was not a significant difference in prevalence of thrombocytosis, which might be due to the small number of cases with thrombocytosis (6/47, but all in the more advanced disease group). PF4 levels and prevalence of ascites were significantly higher in more advanced disease compared to less advanced disease. Although 3 of the quantitative comparisons have p<0.05, only platelet count and ascites prevalence survive bonferroni correction for multiple testing (p<0.05/4=0.013).
4. Discussion

Epithelial ovarian cancer is a difficult disease in that it presents with non-specific symptoms commonly leading to diagnosis at an advanced stage. Although the disease is sensitive to initial therapy, relapse occurs in up to 80% of patients, often with development of resistance to conventional therapeutic approaches.³

This project was part of a larger ongoing study on the role of the hemostatic system in epithelial ovarian cancer progression, led by Pamela R. Westmark, Ph.D., and John P. Sheehan, MD. As hypercoagulability is a highly significant clinical problem, the goal of this larger study is to shed light on the mechanisms behind the pro-coagulant phenotype. Epithelial ovarian cancer patients represent a good model of the hypercoagulable state in cancer as venous thromboembolism is an integral part of the disease³⁴ and as malignant ascites samples provide a window into sampling the tumor microenvironment directly.

In this substudy, we wanted to determine whether platelets were present in the ascites of patients with EOC, and if so whether they were activated. We were able to find evidence of platelets in the malignant ascites based upon our western blotting of GPIb protein in ascites samples. As all but two of the ascites samples we ran had negligible hemoglobin levels, the western blot results support our hypothesis that platelets migrate into ascites specifically. The ELISA development was not successful as previously stated and hence, we do not have data on GPIb presence in more than the 6 samples randomly selected for western blot testing. However, the results of the PF4 measurements in all 26 ascites samples indicate that platelets are indeed present and undergo activation in the ascitic fluid. Interestingly, the highest PF4 levels were present in the samples that contained blood contamination. As the blood contamination is likely a consequence of a ruptured vessel in the peritoneum or bleeding from the paracentesis, a higher level of platelets might have been present and activated during this minor trauma. But can we be confident that PF4 present in the ascitic fluid is a result of local activation or could this small cytokine be leaking into the peritoneum from the systemic circulation? Although we cannot be certain, we think the PF4 levels are due to local activation. Our reasoning for this is that the half-life of PF4 is short, T₁/₂<20 minutes in rabbits⁷⁰ and so rapid in humans that it’s T₁/₂ could not be estimated,⁷¹ and so little PF4 from the systemic circulation would be able to leak into the peritoneum before being metabolized. Cervi et al. even hypothesize that molecules such as PF4 are exchanged locally at sites of platelet aggregation and remain bound to glycosaminoglycans such as heparan sulphate in tissues.⁷² Interestingly, the same research group found evidence that PF4 could predict microscopic human tumors in mice and showed that it might be a potential tumor biomarker.⁷² This raises the question of whether PF4 could be utilized as a method for screening.

Our findings of platelets being present in the ascitic fluid are also consistent with a study performed by Orellana et al.,⁵⁶ who used GPIb and flow cytometry to show that platelets were present with tumor cells in peritoneal ascites from patients with ovarian cancer. We could not find any previous studies on PF4 or other markers for platelet activity in ascites for comparison. Based upon our results of PF4 in plasma of EOC patients compared to in plasma of healthy controls we hypothesized that platelets might be hyperreactive in ovarian cancer patients. However, a study by Feng et al.,
concluded that platelets from ovarian cancer patients were not hyperreactive, so the few studies available remain inconsistent and further research is required.

Stone et al. showed that ovarian cancer patients with thrombocytosis were significantly more likely to have advanced-stage disease and thromboembolic complications. Analysis of our study population showed that women with advanced stage disease (FIGO III/IV) had significantly higher platelet counts than those with less advanced disease (FIGO I/II), consistent with these findings. In the same study by Stone et al., those with thrombocytosis had a significantly shorter median time to disease progression and shorter overall survival compared to those with normal platelet counts.

Unfortunately, we currently have no data on clinical outcome for our study group.

The results of this study suggest that platelets are indeed present and active in the tumor microenvironment of ovarian cancer. Based on evidence of platelet interaction with cancer cells, it is likely that they are contributing to the progression of ovarian cancer in the peritoneum. To what extent is a question that still needs to be answered.

Strengths of the study are that both plasma and ascites samples were obtained from the same EOC patients and that blood contamination was considered and adjusted for.

Limitations of the study are that we were not successful in developing an ELISA method for GPIb. The development of an ELISA protocol is a complex and time-consuming process. Although we were not successful within the time limits of this B.Sc. study, the development of the ELISA will continue in the lab and can build upon the steps taken and the experience already gained. While the unsuccessful development of the GPIb ELISA was a disappointing outcome of my study, this process taught me perhaps the most important lesson of all those I learned during this project. That science is trial and error, it is hard work and requires determination and patience. In light of the fact that we could not quantify GPIb with an ELISA assay, it is also a limitation that we did not confirm the presence of GPIb, and therefore platelets, by western blot in all the ascites samples. Although we have the results of PF4 measurements in all ascites samples suggesting the activation of platelets in ascites, it would have strengthened our results to see GPIb present on western blotting in all samples. The study is also limited by not having information on the clinical outcome of EOC patients. It would have been interesting to compare PF4 and GPIb levels in ascites with clinical outcome, prevalence of VTE and disease progression. That will be a topic for future study.

Other future steps should be developing a working ELISA for GPIb in order to get a quantitative view of platelet mass in ascites and researching further platelet releasate in ascites, especially TGF-β and VEGFA, as they have been shown to increase tumor cell proliferation. Future studies should also research methods of inhibiting platelet function and activation in the malignant ascites and its effect on tumor cells present in that environment.

Platelet inhibitors and other anti-platelet drugs have been regarded with interest as a potential therapeutic option in the treatment of various tumors, including ovarian cancer. Aspirin, a platelet inhibitor, has been shown to reduce long-term incidence and mortality due to colorectal cancer in human patients. Stone et al. showed that reducing platelet counts decreased the size and number of tumor growths in a murine model of orthotopic ovarian cancer. We found no evidence of platelet
inhibitors tested as treatment in human patients with ovarian cancer but in light of these studies and our suggestive results, such a trial would be of great interest.

In conclusion, while the role of platelets in cancer progression is a relatively new field of study, our results indicate that platelets are likely to have a role in EOC progression that requires clarification. Further knowledge on the mechanisms by which platelets influence EOC progression may lead to the possibility of testing new methods of screening for EOC and new therapeutic targets against platelets in the battle against this difficult disease.
References

63. Genetics Home Reference N. Chromosomal location GPIBa. NCBI: NCBI.
64. Ensembl. Glycoprotein Ib platelet alpha subunit. Ensemble release 882017.
Appendix

1. List of reagents and materials

<table>
<thead>
<tr>
<th>Reagent/material:</th>
<th>Catalog nr:</th>
<th>Manufacturer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.2% Sodium citrate blood collection tubes</td>
<td></td>
<td>BD Vacutainer Systems</td>
</tr>
<tr>
<td>10% NextGel solution (acrylamide)</td>
<td></td>
<td>Amresco</td>
</tr>
<tr>
<td>Costar® EIA microtiter plate</td>
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<td>Corning</td>
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<td>Biotek Synergy2 plate reader</td>
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<td>Biotek Instruments</td>
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<td>CD42b mouse monoclonal antibody</td>
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<td>Santa Cruz Biotechnology</td>
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<td>CD42b mouse monoclonal biotinylated antibody</td>
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<td>CD42b rabbit polyclonal antibody</td>
<td>PA5-29664</td>
<td>ThermoFisher Scientific</td>
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<td>4067-GP-050</td>
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<td>DuoSet® Human PF4 ELISA system</td>
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<td>R&amp;D Systems</td>
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<tr>
<td>Immobilon-FL membrane</td>
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<td>EMD Millipore</td>
</tr>
<tr>
<td>Other general chemicals</td>
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<td>Fisher</td>
</tr>
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</table>

2. Recipes:

2.1 IP Lysis Buffer

- 500 µL of 1M Tris
- 750 µL of 2M NaCl
- 20 µL of 0.5M EDTA
- 100 µL of 10% SDS
- 2 mL of 10% Triton-X
- 1 mL of 10% NP-40
- 1 mL of 10x Protease cocktail inhibitor (1x contains: 500µM AEBSF, 150nM Aprotinin, 1µM E-64, 0.5mM EDTA, 1µM Leupeptin)

Add purified H₂O up to 10mL total volume

2.2 Towbin Buffer

- 12.12g Tris base
- 56.16g Glycine
- 400mL Methanol (5%)

Add purified H₂O up to 4L total volume
Store at 4°C
2.3 TBS-T for western blots
6.68g Tris base
32g NaCl
4mL Tween-20
Add HCl to pH 7.6
Add purified H₂O up to 4L total volume

2.4 SDS loading dye
3.8mL purified H₂O
1.0mL 0.5M Tris
0.8mL Glycerol
1.6mL 10% SDS
0.4mL β-mercaptoethanol
0.4mL 0.05% bromophenol blue in water
Add HCl to pH 6.8

2.5 Gels for western blots
48mL 10% Nextgel®
288µL 10% APS
96µL TEMED

2.6 Blocking solution for western blots
2.5g non-fat dry milk
Add TBS-T up to total volume of 50mL

2.7 Platelet wash buffer
50mL ACD-A
5.95g HEPES
4.36g NaCl
0.45g dextrose
NaOH to pH 7.4
Add purified H₂O up to 500mL total volume
3. Protocols

3.1 Protocol for blood sample processing

1. Blood was collected into two 3.2% sodium citrate tubes and kept at 4°C until processed, normally within 5 minutes, but no longer than 30-45 minutes from collection

2. Platelet rich plasma
   a. 50µg/mL of CTI was added to citrate tubes and centrifuged at 200xg for 10 minutes at 4°C. Transfer supernatant of platelet rich plasma (PRP) to fresh tubes and discard red blood cells

3. Platelet free plasma
   a. Centrifuge PRP at 13000xg for 10 minutes at 4°C. Transfer supernatant of platelet free plasma (PFP) to fresh tubes and save platelet pellets for further processing

4. Platelet pellet preparation
   a. Wash platelet pellets with HEPES platelet wash buffer (see recipe above), 400µL per tube and recentrifuge at 13000xg for 10 minutes at 4°C
   b. Lyse platelet pellet with IP lysis buffer (see recipe above), 200µL per tube

5. Plasma microparticles
   a. Centrifuge PFP at 20800xg for 10 minutes at 4°C
   b. Remove supernatant of MP-depleted PFP and transfer to fresh tubes
   c. Resuspend MP pellets in 100µL HBS Wash Buffer (140mM NaCl, 10mM Hepes, no calcium) and recentrifuge at 20800xg for 10 minutes at 4°C
   d. Remove supernatant and discard. Resuspend MP pellets in 250µL of HBS Wash Buffer

3.2 Protocol for western blotting

1. Pour 1.5mm gels (see recipe above)
2. Add Buffer B1 to sample up to 25µL total volume
3. Add 8µL of loading dye
4. Heat samples at 95°C for 5 minutes
5. Load total volume in tube into well, one tube per well
   a. Add 3µL of Fisher BioReagents™ EZ-Run™ Prestained Rec Protein Ladder into first well as marker
6. Run gels at 190V for 50 minutes using running buffer (see recipe above)
7. Transfer gels to Immobilon-FL membrane® using wet transfer method with Towbin buffer (see recipe above) and run at 100V for 75 minutes in 4°C
8. Block membrane with blocking solution (see recipe above) for 60 minutes in 4°C
9. Wash membrane in TBS-T 3x5minutes
10. Prepare primary antibody by adding 7µL of 1mg/mL rabbit anti-CD42b from ThermoFisher™ into 21mL of TBS-T, making up a 1:3000 dilution. Incubate membrane overnight in 4°C
11. Wash membrane in TBS-T 3x5minutes
12. Prepare secondary antibody by adding 4µL of 1mg/mL HRP conjugated goat anti-rabbit antibody from GE Healthcare Life Sciences™ into 20mL of TBS-T, making up a 1:5000 dilution. Incubate membrane for 45 minutes in room temperature.

13. Wash membrane in TBS-T 3x5minutes


3.3 Protocol for GPIb ELISA

1. Bring all reagents to room temperature.
   a. PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.2-7.4, 0.2 µm filtered.
   b. Reagent Diluent – 1%BSA in PBS, pH 7.2-7.4, 0.2 µm filtered
   c. Wash Buffer - 0.05% Tween® 20 in PBS, pH 7.2-7.4
   d. Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine). Protect from light.
   e. Human CD42b Standard – (R&D Systems™ Cat# 4067G050) Reconstitute the standard (50 mg) with 0.5mL PBS for a 100 mg/mL stock. Mix the standard to ensure complete reconstitution and allow the standard to sit on ice for 10 min with gentle agitation prior to making dilutions. Use the stock solution to produce a dilution series. Mix each tube thoroughly before the next transfer.

2. Dilute the capture antibody to the appropriate working concentration in coating buffer and coat the wells of a EIA microtiter plate with 100 µL of the antigen dilution per well. Cover plate with an adhesive plastic and incubate overnight at 4°C. Seal plate to keep from drying out.

3. Remove the antigen coating solution and wash the plate four times by filling the wells with wash buffer. The solutions or washes are removed by flicking the plate over a sink and remaining drops are removed by patting the plate on a paper towel.

4. Block the remaining protein-binding sites in the coated wells by adding 300 µl Reagent Diluent per well. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

5. Wash the plate four times with wash buffer.

6. Make standard dilutions (960, 640, 320, 160, 80, 40, 20, 10, 5, 0 pg/mL as well as 40pg/mL and 0pg/mL standards with a 1:100 dilution of 10.5mmolar 3.2% sodium citrate added). Make sample dilutions in Reagent Diluent. Add 100 µl of each dilution to an antigen-coated well in duplicate. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

7. Wash the plate four times with wash buffer.

8. Add 100 µl of detection antibody (unconjugated or biotin conjugated), diluted to a working concentration of 0.5µg/mL in Reagent Diluent. Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.

9. Wash the plate four times with wash buffer.

10. If unconjugated antibody, add 100 µL of 200ng/mL secondary Ab-HRP conjugated and incubate for 45 min at room temperature. Else add 100 µL of the working dilution of Streptavidin-HRP to
each well. Cover the plate and incubate for 20 minutes at RT. Avoid placing the plate in direct light.

11. Wash the plate four times with wash buffer.

12. Add 200 µL of substrate solution to each well. Incubate for 20 minutes at RT. Avoid placing the plate in direct light.

13. Add 50 µL of Stop Solution (2 N H₂SO₄) to each well. Gently tap the plate to ensure thorough mixing.

14. Determine the optical density of each well immediately, using a microplate reader set to 450 nm with wavelength correction set to 540 nm.

3.4 Protocol for PF4 ELISA

Human PF4/CXCL4 antibodies, standard and streptavidin-HRP are from a DuoSet ELISA development system from R&D Systems™, catalog number: DY795.

1. Bring all reagents to room temperature.
   a. PBS - 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.2-7.4, 0.2 µm filtered.
   b. Reagent Diluent – 1%BSA in PBS, pH 7.2-7.4, 0.2 µm filtered
   c. Human PF4 Standard – Reconstitute the Human PF4 Standard from R&D Systems™ with 0.5 mL Reagent Diluent for a 240 ng/mL stock. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Use the stock solution to produce a dilution series (250, 200, 125, 75, 50, 35, 25, 15, 10 and 0 pg/mL). Mix each tube thoroughly before the next transfer.
   d. Wash Buffer - Prepare 500 mL of Wash Buffer. (0.05% Tween® 20 in PBS, pH 7.2-7.4)
   e. Substrate Solution - Color Reagents A and B from R&D Systems™ should be mixed together in equal volumes within 15 minutes of use. 1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine). Protect from light.

2. Dilute the Capture Antibody, mouse anti-human PF4 from R&D Systems™, to a working concentration of 2 µg/mL in PBS. Immediately coat a 96-well microplate with 100 µL per well of the diluted Capture Antibody. Seal the plate and incubate overnight at 4°C.

3. Aspirate each well and wash with Wash Buffer for a total of three washes. Wash by filling each well using a squirt bottle. After the last wash, remove any remaining Wash Buffer by inverting the plate and blotting it against clean paper towels.

4. Block plates by adding 300 µL Reagent Diluent to each well. Incubate at RT for a minimum of 1 hour.

5. Repeat wash as in step 3.

6. Add 100 µL of sample or standards in Reagent Diluent per well. Cover with an adhesive strip and incubate 2 hours at RT.

7. Repeat wash as in step 3.
8. Add 100 µL of the Detection Antibody, biotinylated goat anti-human PF4 from R&D Systems™, diluted to a working concentration of 0.2 µg/mL in Reagent Diluent, to each well. Cover with a new adhesive strip and incubate 2 hours at RT.

9. Repeat wash as in step 3.

10. Add 100 µL of Streptavidin-HRP from R&D Systems™ to each well. Cover the plate and incubate for 20 minutes at RT. Avoid placing the plate in direct light.

11. Repeat wash as in step 3.

12. Add 200 µL of Substrate Solution to each well. Incubate for 20 minutes at RT. Avoid placing the plate in direct light.

13. Add 50 µL of Stop Solution (2 N H₂SO₄) to each well. Gently tap the plate to ensure thorough mixing.

14. Determine the optical density of each well immediately, using a microplate reader set to 450 nm with wavelength correction set to 540 nm.

3.5 Protocol for hemoglobin quantification of ascites samples

1. Set plate reader wavelength to 540nm (dye) and 650nm (turbidity)

2. Use 96 well plates

3. Prepare Drabkin’s solution
   a. Reconstitute one vial of Drabkin’s Reagent with 200mL of water for 5x solution
   b. Add 0.5mL of 30% Brij 35 Solution per 200mL of 5x reconstituted solution
   c. Store at room temperature and protect from light

4. Make up standards by diluting 100mg/mL Hb Stock Solution
   a. Make 0.1mL each of 25, 20, 15, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.025 mg/mL dilutions

5. Dilute 25µL of ascites sample or standard to 120µL with water in order to lyse cells under hypotonic conditions. Let sit for 5 minutes at 37°C

6. Add 30µL of 5x Drabkin Reagent, shake vigorously for 1 minute and let sit for 15 minutes in room temperature until all hemoglobin has reacted

7. Centrifuge at 13000xg for 5 minutes

8. Transfer 125µL of standard of sample to plate. Add water to first well for zero point

9. Measure samples in plate reader