Exosomes for the Early Detection of Ovarian Cancer

By

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Abstract

Epithelial ovarian cancer (EOC) is a silent killer that strikes with few, if any, symptoms. By the time a woman is diagnosed with EOC, it is often at an advanced stage where the outlook is grim. However, if caught early, the prognosis is excellent as it can be cured in up to 90% of patients. Therefore, developing a highly specific blood-based test is extremely appealing for presymptomatic screening and early detection of EOC. However, all blood biomarkers to date lack the necessary sensitivity-specificity for early detection of this disease. A fundamental challenge in biomarker discovery is the extremely low concentrations released from developing tumors into the circulation at pre-clinical stages, which can be $10^4$-fold lower than the clinically detectable levels. For this reason there is a pressing need to uncover novel biomarkers, and apply new strategies to propel the advancement of EOC diagnostics. We have focused our efforts on extracellular vesicles (EVs), primarily exosomes derived from the endosomal pathway that play important roles in intercellular communication, immune responses and cancer pathogenesis via transfer of a selective repertoire of biomolecules. A proteomic analysis was performed on plasma and EV-depleted plasma obtained from patients diagnosed with stage III/IV serous ovarian cancer (n=14). Bead-based Luminex flow cytometry assays were performed on the complete or EV/exosome-free plasma samples to examine circulating or EV-associated levels of 23 growth factors, cytokines, and other cancer related molecules. In addition, similar experiments were completed using immune-purified CA125$^+$ (EOC associated marker) subpopulation of cell culture derived exosomes. Immunoprecipitation was also utilized to isolate three subtypes (CA125$^+$, EpCAM$^+$, and FAPα$^+$) of extracellular vesicles, primarily exosomes, from plasma. Our proteomic analysis indicated that the levels of several circulating biomarkers decrease upon the removal of EVs from total plasma (TRAIL, leptin, and OPN), likely indicating
a direct association between these analytes and EVs. Three of the 23 analytes were specifically detectable in immunoisolated CA125⁺ cell culture derived exosomes (OPN, HE4, CYFRA-21) and undetectable in the unfractionated exosome population. Of the 23 analytes investigated 21 were detectable at varying levels in the three subtypes of EVs immuno-captured from plasma. When the growth factors, cytokines, and other cancer related molecules were examined in the subpopulations of EVs derived from cases and controls we found that using CA125 or EpCAM as the capture agents, and subsequently measuring CA125 and CYFRA21-1, respectively, may offer improved specificity and sensitivity for ovarian cancer. This study is the first to report free versus microvesicle-associated proteins in EOC plasma samples, and further characterize a limited number of intravesicular proteins in subtypes of EOC EVs. Our work presents the framework for developing an ovarian cancer specific assay to capture and evaluate protein signatures in tumor-derived EVs, primarily exosomes, with the goal of significantly improving the detection of this deadly disease at a stage when curable.
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Chapter 1. Introduction
1.1 Ovarian Cancer

Ovarian cancer only accounts for 3% of cancers in women; however, it is the fifth most common cause of cancer death in women and the leading cause of death of all gynecological cancers (1). In 2016 it is estimated that over 22,000 women will receive a new diagnosis, and over 14,000 will succumb to the disease. The five-year survival rate for this disease ranges from 22% to 92%, depending on the stage at diagnosis. The average five-year survival is only 44%. When a patient is diagnosed with localized disease the five-year survival is over 90% while distant and unstaged disease is roughly a quarter of that. Strikingly only 15% of cases are diagnosed when the disease is localized and survival is promising, while an astounding 60% of cases are diagnosed when the disease is distant and has a dismal five-year survival rate (2). Over the years the five-year survival rate for ovarian cancer has improved slightly due to improvement in treatment options; however, the overall rate of cure has not increased, which is impart due to the late stage at diagnosis (3). When compared to other cancers, the death rate per 100,000 females has remained relatively unchanged for ovarian cancer (7.5 per 100,000) while most cancers have decreased during the past 40 years (from 1975 to 2013) (2).

While the lifetime risk of ovarian cancer is relatively low (1 in 72) compared to other forms of cancers (1 in 8 for breast), there are a number of risk factors which increase a woman’s chance of developing ovarian cancer (3, 4). Half of all cancers, including ovarian, show an association with modifiable risk factors such as: obesity, lack of physical activity and smoking (5-7). One risk factor specific to ovarian cancer is hormone replacement therapy. Women who underwent hormone replacement therapy were 37% more likely to develop ovarian cancer as opposed to women who never received hormone replacement therapy both estrogen-only and estrogen-progesterone formulations (5).
There is also an increased risk of developing ovarian cancer associated with an increase in ovulatory cycles as described by the incessant ovulation hypothesis. The incessant ovulation hypothesis correlates tumor formation as a result of an accumulation of mutations from the repetitive wounding and recurring cell proliferation associated with postovulatory repair of the ovarian surface epithelium (8). Support for this hypothesis comes from the majority of women diagnosed with ovarian cancer being post-menopausal and in the sixth and seventh decade of life, when they have reached their maximum number of ovulatory cycles (8, 9). Further support for the incessant ovulation hypothesis is shown through factors capable of suppressing or interrupting ovulation thus protecting against ovarian cancer (10-13). Ovulation cycles can be interrupted and suppressed through the use of oral contraceptives, polyparity, breast feeding and tubal ligation, all of which reduce the chance of developing ovarian cancer (9, 10, 14).

Endometriosis is another means by which a woman’s risk for ovarian cancer can be increased, specifically clear cell ovarian cancer. In a genomic study conducted by Prowse et al. a total of 63 loss of heterozygosity (LOH) events were detected in carcinomas, 22 of which were detected in corresponding endometriosis samples. When comparing genomic profiles from endometriosis samples with that of carcinomas that later developed, in each case the same allele was lost in the endometriosis and carcinoma samples; however, no markers showed LOH in the endometriosis samples alone (15). This data is supportive for the hypothesis that endometriosis is a precursor to ovarian cancer, and shows that women with endometriosis are at a higher risk of developing ovarian cancer.

To date the most significant factors influencing the development of ovarian cancer come from family history and genetics. The risk of developing ovarian cancer is most elevated in woman
with a family history of ovarian and/or breast cancer, as well as by germline mutations in \textit{BRCA1} and \textit{BRCA2} (8). Women in the general population have a risk of \(<2\%\) of developing ovarian cancer; however, inheriting mutations in \textit{BRCA1} or \textit{BRCA2} increase the lifetime risk to approximately \(40\%\) and \(20\%\), respectively (9, 10, 16, 17). Factors that are known to influence and suppress ovulatory cycles have also been shown to decrease the risk of ovarian cancer in the population of women with an inherited susceptibility (10). While these epidemiological facts and statistics are used to describe ovarian cancer as a whole it is also vital to note the importance of the histological origin of the disease and the role it plays in progression and overall survival.

Ovarian cancer can be classified based on the tissue of origin (surface epithelial, stromal endocrine cells, and germ cells). Carcinomas of the epithelial origin account for \(>90\%\) of ovarian cancer cases (18). Transformed cells can also develop into serous, mucinous, clear cell and endometrioid histotypes. Serous ovarian cancer can exist as low and high grade subtypes. Low grade or type I carcinomas are more often identified in the early stages and progress more slowly than high grade or type II carcinomas. High grade carcinomas tend to present at the more advanced stages and are more aggressive (3). For instance 86\% of high grade serous ovarian cancer cases present at late stage as compared to only 3\% of low grade serous ovarian cancer cases (19). A majority of all ovarian cancer cases as well as deaths are attributed to advanced stage, high grade epithelial serous ovarian cancer (3, 20). For this reason, it is desirable to find a means of detecting these high grade cases before they have advanced.

Advanced cases are associated with the FIGO stage at which the carcinoma is detected as determined by a gynecologic oncologist. Staging is an assessment of how far the disease has spread and typically performed when samples are collected for the pathologist’s analysis.
Staging has been standardized by the International Federation of Gynecology and Obstetrics (FIGO) and is typically the most important predictor of overall survival. Ovarian cancer can present anywhere from stage I to stage IV with increasing severity respectively (21).

Stage I disease can be broken up into three subcategories IA, IB, and IC. To be classified as stage I the disease must be confined to the ovaries. Detecting ovarian cancer at stage I is when the disease is the most curable and has the greatest overall survival rate. The disease has progressed to stage II when the tumor extends into other pelvic structures and can also be identified based on three sub-categories: IIA, IIB, and IIC. Progression to stage III has occurred when the tumor has spread beyond the pelvis into the lining of the abdomen and/or to the regional lymph nodes and can also be subdivided into IIIA, IIIB, and IIIC. Greater than 50% of all cases are diagnosed at stage III. Descriptions of the subcategories for stage I, stage II, and stage III are shown in Table 1. Stage IV disease is characterized by metastasis to distant locations beyond the peritoneal cavity, such as the liver and lungs (21). Unfortunately, these late stages, where the disease has spread beyond the pelvis and/or metastasized, are where a majority of cases as diagnosed and treatment options are limited.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Confined to Ovaries</th>
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<tbody>
<tr>
<td>IIA</td>
<td>Uterus/Tubes</td>
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<tr>
<td>IIB</td>
<td>Other pelvic organs</td>
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<tr>
<td>IIC</td>
<td>A/B with malignant ascites</td>
</tr>
<tr>
<td>IIA</td>
<td>Microscopic peritoneal metastases</td>
</tr>
<tr>
<td>IIB</td>
<td>Macroscopic (&lt;2cm) peritoneal metastases</td>
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<tr>
<td>IIC</td>
<td>Macroscopic (&gt;2cm) peritoneal metastases or regional lymph node metastases</td>
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Symptomology, or lack thereof, also plays a significant role in the late stage at diagnosis seen with ovarian cancer. The symptoms associated with ovarian cancer include bloating, pelvic or abdominal pain or pressure, abdominal swelling, dyspepsia, and early satiety (9, 22, 23). These vague abdominal symptoms coupled with the age at onset, are often the reason ovarian cancer is often misdiagnosed as early menopause or gastrointestinal problems. Only after the symptoms persist does a woman go on to receive additional screening such as transvaginal ultrasonography (TVUS) and monitoring of cancer antigen 125 (CA125). While TVUS and CA125 are the most reliable screening methods to date, challenges associated with distinguishing between diseased and disease free women still exist.

The only 100% specific and sensitive test for ovarian cancer is histological examination of biopsy samples; however, histological examination cannot be implemented as a screening technique and is typically only utilized after other screening methods has suggested further tests be completed. When a woman has symptoms of ovarian cancer and/or a suspicious pelvic mass, a serum CA125 test and an abdominal and vaginal ultrasound is requested (24). Based on the patient’s menopausal status, ultrasound findings, and CA125 level the risk of malignancy index
(RMI) is calculated (24). RMI, is a simple scoring system used for evaluating the risk of ovarian cancer in women and is typically utilized because it gives much better results than using a single parameter to calculate risk (25). Even still the diagnosis of ovarian cancer is often prolonged as the only one and true test involves an invasive biopsy followed by histological examination. Coupled with the symptoms associated with ovarian cancer being vague and nonspecific a tumor specific biomarker for diagnostic and screening purposes is greatly sought-after.

Ideally a tumor marker would be able to detect subclinical disease, monitor the response to treatment and identify early recurrence. To date the best tumor marker for ovarian cancer is CA125. CA125 is a large, 200 kd, glycoprotein expressed in coelomic epithelium during embryonic development (25). In the early 1980’s Canney et al., performed some of the initial studies using CA125 for detection and monitoring of ovarian cancer. In initial studies a cutoff value of >35 U/mL was set and the radioimmunoassay provided an overall sensitivity of 83% for all ovarian cancer histological subtypes with a specificity of 58%. In this initial study, CA125 levels positively correlated with tumor burden (25). The discovery of this antigen offered an alternative and less invasive means to monitoring disease progression, as opposed to the laparotomies previously utilized. A quarter of a century later and CA125 is still the superior biomarker for ovarian cancer detection and monitoring, making it the only marker approved for clinical use. While the discovery of CA125 was groundbreaking in the ovarian cancer field, limitations that were present at the time of initial discovery are still present today, including specificity, sensitivity, and ability to detect the disease in the early stages.

In a phase I study CA125 was elevated in 82% of women with epithelial ovarian cancer and only 1% of healthy donors. However, CA125 is not specific to ovarian cancer and can be elevated in
various other forms of cancer such as lung, breast, pancreas, and colorectal (25). In addition, elevated levels of CA125 can be attributed to numerous benign gynecologic and non-gynecologic diseases (24). For these reasons CA125 is not a reliable tumor biomarker as elevated levels are not always a result of ovarian cancer.

In addition to the problems associated with low specificity, the sensitivity of CA125 is lower than ideal. There is a subpopulation of women diagnosed with ovarian cancer, approximately 20%, who will not have elevated serum levels of CA125, making disease undetectable with this antigen (24). In addition, the sensitivity of this marker is low for detecting early stage disease due to CA125 only being elevated in 25% to 50% of patients with stage I ovarian cancer (9, 24, 26, 27). Not all tumors cells express the antigen and the lack of sufficient antigen released by small tumors, contribute to CA125 levels not being elevated and detection via CA125 almost impossible, especially at the pre-clinical stages (25). Thus the need still exists to develop a diagnostic assay or screening procedure capable of detecting ovarian cancer at the earliest possible time point.

To improve upon the sensitivity of CA125 and the ability to detect early stage disease, transvaginal ultrasounds are being examined as a supplemental tool or alternative to CA125. Three large studies have been conducted examining transvaginal ultrasound as a screening method; however, in each of these studies TVUS did not offer the desired positive predictive value of 10%. Based on the prevalence of the disease the annual cost would be too significant to implement TVUS as a primary screening tool (28-31). However, TVUS may prove to be an excellent supplement to CA125 screening. It has been reported that when the two methods are combined a specificity of 99.9% was achievable (31, 32). Two separate screening studies were
conducted examining different modalities for screening and the effects on mortality due to ovarian cancer. The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial screened women of the general population with CA125 and transvaginal ultrasound. The second study was one of the largest to date and conducted by the UK Collaborative Trial of Ovarian Screening (UKCTOCS) examining three screening procedures.

The PLCO screening trial randomly assigned over 78,000 women to two groups, annual screening or usual care. The screening cohort received CA125 screening for 6 years in addition to screening with transvaginal ultrasound for 4 years, while the control group received neither screening method only usual medical care. Women in this study were followed for 13 years to assess the mortality associated with ovarian cancer. The results from this study showed that among the general population simultaneous screening with CA125 and transvaginal ultrasound did not reduce the mortality associated with ovarian cancer (33). Even though combining TVUS with CA125 has shown to improve specificity at diagnosis the combination does not prove to be of much utility for screening purposes.

Similar to the PLCO Screening Trial, the UKCTOCS conducted a study examining the effects of screening on mortality in a much larger cohort, with over 200,000 women. These women were randomly allocated to three screening groups: annual multimodal screening (MMS) with CA125 and the risk of ovarian cancer algorithm (ROCA), annual transvaginal ultrasound screening (USS), or no screening (34). In the MMS group ROCA was used to monitor serum CA125 levels over time and identify any significant rises. Women were triaged via ROCA based on their risk as normal, intermediate, or elevated. For women identified as having an elevated risk TVUS was used as a second-line test following the CA125 screening. In an interim report
Menon and colleagues reported that the sensitivity for early stage disease increased from a historical baseline of 20% under standard care to almost 50% (35). Additional analyses by the UKCTOCS found that for each ovarian and peritoneal cancer detected by screening, an additional two women in the MMS group and ten women in the USS group had false-positive surgery and furthermore the decrease in mortality as a result of screening was not significantly (33, 34). This study conducted by UKCTOCS is the largest study to date and the results show that for the general population screening using MMS based on circulating CA125 and ROCA does not reduce ovarian cancer mortality, and there is no significant benefit associated with multimodal screening or transvaginal ultrasound screening (33, 34). Since, the sensitivity with ROCA, for early stage disease is limited to 50%; other plasma protein biomarkers such as HE4, CA72.4, and MMP7 have been studied to evaluate whether they complement serum CA125 and thus increase sensitivity for pre-diagnostic early stage disease while maintaining specificity at 98% (36). These studies support the need for a novel screening method if we truly hope to combat the challenges associated with ovarian cancer mortality.

The results of the UKCTOCS trial further support the U.S. Preventive Services Task Force (USPSTF) recommendation against screening for ovarian cancer (37). The USPSTF found that as a result of the low prevalence rate of ovarian cancer the positive predictive value of screening is low and a majority of positive tests are actually false-positives (37). Another major consideration in this recommendation comes from the balance of benefits and harms associated with screening. Screening can lead to major surgical interventions in women without cancer; therefore, harms associated with screening outweigh the benefits. For instance, based on false-positive screening alone twenty-one major complications occurred per one hundred procedures
performed (37). If the number of false positives could be limited, or even eliminated, screening could more easily be implemented in the general population.

An alternative to screening the general population would be screening only woman with an elevated risk of ovarian cancer. In a study conducted by Terada and colleagues, data from the Prostate, Lung, Colorectal, and Ovarian Cancer (PLCO) Screening Trial, previously mentioned, was used to analyze the effects of screening on a subcategory of women with an increased risk of ovarian cancer. The study focused on menopausal women who reported a first degree relative with breast or ovarian cancer and examined overall mortality and disease specific mortality in the screening versus usual care groups. Although there was a slight shift in the stage at detection as well as survival, there was no significant difference in overall mortality or disease specific mortality between the screening group and the control group (38). This further supports the limitations associated with the markers currently available for screening.

For these reason, there has been a significant amount of research focused on trying to find alternatives or supplements to CA125 to create an efficient mode of screening. Various studies have been conducted with the aim of finding a single marker to replace CA125, or panels that could be used to replace or be used in combination with CA125 (36, 39, 40). All of these studies use CA125 as the standard and the performance of these candidate biomarkers are only studied in cases at diagnosis when the disease is already detectable with current procedures (36). In addition, various studies have been conducted implementing varying techniques for screening purposes; however, still there exists no data supporting a beneficial, reasonable and affordable method for screening (40-49). Even with all of the research aimed at finding a novel screening technique we are still solely reliant on a marker discovered in the early 1980’s.
CA125 has also been used to monitor disease recurrence as well as response to treatment. Once an advanced-stage diagnosis has been confirmed a patient will undergo a cytoreductive debulking surgery to reduce tumor to the point where there is no grossly visible tumor (50, 51). Following debulking first-line chemotherapy is administered in the form of intravenous paclitaxel. Paclitaxel is often times combined with carboplatin in both advanced-stage cases as well as those cases with early stage disease (50). Despite the course of treatment ovarian cancer has the tendency to recur. Of the women who present with advanced-stage disease two-thirds will recur, for which a cure remains elusive (50). Following neoadjuvant chemotherapy, CA125 is typically monitored as a means of evaluating response to treatment, specifically monitoring for recurrence (52-55). In one study elevated levels were able to detect recurrent disease in 70% of patients (3). In another study CA125 was monitored every three months for women in complete clinical remission following front-line therapy. When a woman’s CA125 level exceeded twice the upper limit of normal she either remained blinded until symptomatic recurrence or became un-blinded and underwent second-line treatment. Women who were unblended started second-line treatment five months earlier than women who remained blinded until symptomatic recurrence. However, this earlier treatment did not contribute to an overall improvement in survival (50, 56). While CA125 is clinically used and approved by the FDA for monitoring patients following treatment the same limitations exist that exist when using CA125 as a screening biomarker, therefore there is a pressing need to identify new diagnostic and disease monitoring biomarkers.

If we hope to decrease the mortality associated with ovarian cancer the most promising tactic will be to implement screening as a means of detecting early-stage disease, when patients have an increased survival advantage. To date the benefits associated with screening have been less
than ideal. The lack of success associated with screening thus far is likely due to a majority of trials utilizing CA125, even though CA125 has been shown to lack sensitivity or specificity as a primary test in detection. As a result, the race to find a new diagnostic tool continues.

1.2 Extracellular Vesicles and Exosomes

The term “extracellular vesicle” (EV) is one that has been coined in recent years to refer to a range of small lipid-bounded vesicles secreted into the extracellular space. There are three subcategories of EVs recognized today, 1. exosomes 2. microvesicles 3. apoptotic bodies (57-59). Distinguishing between the populations is often based on size, density, subcellular origin, function and molecular cargo (59, 60). The secretion of extracellular vesicles is an evolutionarily conserved process, suggesting biological significance to the vesicles. In recent years research in the field has led to the belief that cancer cells rely extensively on EVs to invade tissues and propagate oncogenic signal at distances (58, 59, 61). While the term EV encompasses a range of vesicles a significant amount of research in the field has focused primarily on exosomes and their role in cancer development and communication.

Exosomes are membrane bound microvesicles secreted by most cells in vitro and in vivo. First discovered in the 1980’s exosomes were thought to be a waste removal system for the cell, specifically maturing reticulocytes, removing unneeded membrane proteins. Following the initial discovery of exosomes, four decades of intensive research has gone into understanding their biological significance revealing functions far beyond “waste removal systems”; however, there still lies an overabundance of questions regarding their biological function. Exosomes are commonly described as nucleus-free, mitochondria free, membrane bound microvesicles ranging in size from 40-100 nm. Vesicles are characterized as exosomes based on their structure
(bilipidic layer), size, density (1.13 g ml$^{-1}$ to 1.19 g ml$^{-1}$), and overall protein content. Exosomes are representative of their cell of origin, as depicted by their protein content. There are also a number of proteins which are found in a vast majority of exosome preparations and are known as exosomal markers. These include members of the tetraspanin family such as CD9, CD63, and CD81, members of the Endosomal Sorting Complexes Required for Transport (ESCRT) such as ALIX and TSG101 and heat shock proteins such as Hsp70 and Hsp90 (62-66).

The term ‘exosomes’ was initially used by Trams and colleagues in the early 1980s to describe exfoliation of membrane bound ecto-enzymes, with 5’ nucleotidase activity (64, 67, 68). Half a decade later Johnstone and colleagues observed vesicles released during the maturation of reticulocytes to erythrocytes, these vesicles would later come to be known as exosomes and refers to exosomes as we know them today. Not only was the report of these extracellular vesicles a significant discovery, Johnstone was able to link the biogenesis of exosomes to the formation of multivesicular bodies (MVB) through tracking of the transferrin receptor. Johnstone was not the first to track the transferrin receptor and the formation of MVBs; however, she was the first to link the process to the biogenesis of exosomes (69-72). During that time the transferrin receptor had been tracked during the maturation of reticulocytes and found to be internalized into large (1-1.5 µm in diameter) MVBs which harbored round bodies ~50 nm in diameter. These 50 nm vesicles contained the transferrin receptor at the external surfaces, and were released into the extracellular milieu upon the fusion of MVB with the plasma membrane, a key component of the biogenesis process as we know it today (69-72).

While there are still many questions regarding the biogenesis of exosomes, the process has been well characterized by the inward budding of the plasma membrane followed by the formation of
early endosomes, which mature to late endosomes ultimately forming the MVB that will later fuse with the plasma membrane to release microvesicles (exosomes); as shown in **Figure 1.1**. Inward budding of the plasma membrane occurs, leading to the formation of endocytic vesicles, through clathrin-mediated or non-clathrin-mediated endocytosis, these vesicles are then transported to the early endosome (63, 73). The early endosome undergoes inward budding of the limiting membrane to form intraluminal vesicles (ILV). There are various components of the endosomal sorting complex required for transport (ESCRT) associated with this process; ESCRT-0, I, II & III, TSG101, and accessory proteins as well such as VPS4. During this time the early endosome undergoes acidification and changes in protein content, fully maturing into late a endosome (63, 73). ESCRT complexes and accessory proteins are also involved in this phase of biogenesis by assisting in ubiquitin-mediated cargo trafficking, and scission of ILVs (62, 63, 74, 75). The late endosome undergoes reversed budding leading to the formation of MVBs, which have two fates. Once the MVB has been formed it can fuse to the lysosome resulting in degradation or fuse with the plasma membrane releasing microvesicle cargo, known as exosomes. Members of the RAB GTPase family are responsible for this trafficking and fusion of the MVB to the plasma membrane (76, 77). Many cell types release exosomes in this fashion: hematopoietic cells, reticulocytes, B- and T-lymphocytes, dendritic cells, mast cells, platelets, intestinal epithelial cells, astrocytes, neurons and tumor cells (63, 71, 78-85). Once released from their cells of origin exosomes can travel locally and systemically to interact with local and distant cells.

Exosomes can be characterized by the enrichment of certain proteins known as exosomal markers as compared to cell lysates. These exosomal markers have a range of function and include: polypeptides associated with antigen presentation, adhesion molecules, membrane
transport and fusion, heat-shock proteins, cytoskeletal proteins, and raft-associated proteins and glycolipids (62, 63, 84, 86-89). One of the first observations of these markers occurred when the protein composition of exosomes derived from murine DCs, human intestinal epithelial cell (IECs), and EBV-transformed human B lymphocytes all showed common proteins. These included but were not limited to CD86, CD63, CD9, CD 37, CD53, CD81, CD82, HSC70, HSP84/90, Annexins, Rab 7, Rab 2, ALIX and TSG101 (62). A vast majority of these are now known as exosomal makers and used to characterize exosomes. While there is a significant amount of similarities between exosomes secreted from the various cell types there are also remarkable differences, which is seen when the protein composition of exosomes secreted from reticulocyte, lymphocytes and dendritic cells are compared. Red cell exosomes contain the transferrin receptor, a major component of reticulocyte plasma membrane; however, lymphocyte and dendritic cell derived exosomes show little to no transferrin receptor. In contrast these exosomes are rich in MHC Class I and II complexes, undoubtedly reflecting their cell of origin (64, 67, 75, 79). With the protein composition of all exosomes being unique it is important to understand the fate of these extracellular vesicles once released.

Typically, exosomes will travel systemically to a distant cell where they will partake in one of three forms of communication with the recipient cell. The three mechanisms by which exosomes are believed to interact with recipient cells are shown in **Figure 1.2**. The first two mechanisms, internalization and membrane fusion, result in the transfer of exosomal membrane and cytosolic material to the recipient cell; this could include oncogenic proteins, mRNAs and miRNAs (57, 64, 75). The third mechanism by which exosomes interact with distant cells is through ligand/receptor interactions. Through this mechanism, exosomes are able to interact with the distant cell in a similar fashion to two cells communicating with one another; however, without
the need for direct cell-cell contact. Upon binding of the receptor and ligand the exosome activates a signaling pathway in the recipient cell leading to changes in gene expression and phenotype (57, 64, 75). Because of their content, ability to travel systemically, and different modes of communicating with distant cells exosomes can be seen as packaged delivery systems, capable of delivering material from the cell of origin; such as microRNA, nucleic acids, and proteins; to distant cells in the body.

Due to their communication capabilities exosomes, specifically tumor-derived exosomes, have shown to play an important role in tumorigenicity. Tumor-derived exosomes are capable of inducing phenotypic changes in non-neoplastic cells promoting an environment for further tumor development. In particular tumor-derived exosomes have been shown to play roles in: enhancing metastasis by preparing new tumor niches, inducing neoplastic transformation, increasing angiogenesis, inducing anti-tumor immune evasion, educating and recruiting macrophages and fibroblast and lastly promoting drug resistance (57, 90, 91). For these reasons tumor-derived exosomes are thought to have a vital role in the overall development and progress of cancer.

In addition to the important roles exosomes play in cancer development, the biogenesis and output of exosomes has been shown to be elevated in cancer cells and often times correlates with the clinical status of the patient (92-94). Coupled with the fact exosomes are extremely stable compared to circulating proteins and nucleic acids (95) and can be easily isolated from a variety of biological fluids such as blood, ascites, urine, saliva and malignant effusions exosomes are ideal targets for biomarker discovery (96-102). For example, in one study exosomes derived from patients with melanoma were assessed as diagnostic and prognostic biomarkers. Melanoma biomarkers, MIA and S100B, were detectable in the exosomes of patients and the exosomal
concentrations correlated with serum concentrations of the biomarkers. Following analysis the exosomal associated MIA and S100B proved to have as much diagnostic and prognostic utility as the serum markers currently used (90). In another study, conducted by Melo and colleagues, exosomes expressing Glypican-1 were able to detect early pancreatic cancer with 100% specificity and sensitivity (103, 104). In both of these studies exosomes secreted as a byproduct of tumors were interrogated and utilized for novel diagnostic purposes.

Of increased interest, exosomes have also been evaluated as potential biomarkers for ovarian cancer. In one study exosomes were captured from plasma of ovarian cancer patients, patients with benign disease and healthy controls. The total exosome number and protein content was significantly greater for exosomes captured from patients with disease as opposed to benign disease and healthy controls (105, 106). Utilizing exosomes as biomarkers for ovarian cancer has also been performed through the examination of miRNA. In one such study performed by Taylor and colleagues the levels of miRNAs in exosomes was examined for ovarian cancer patients, patients with benign disease and healthy controls. The miRNA expression was significantly higher for patients with the disease as opposed to benign disease and undetectable in healthy controls. Of the seven miRNAs examined the expression of two, miR-200c and miR-214, showed correlation with staging of the tumor (107). Overall this data and others support the utility of exosomes as a source of biomarkers for a range of malignant conditions. Because of the many unique advantages exosomes harbor, they could play a vital role in the advancement of blood based assays or liquid biopsies.

According to Roche and the National Institute of Cancer at the National Institute of Health a liquid biopsy is a simple and non-invasive blood test to examine tumor-circulating byproducts, as
a means gaining molecular information regarding the tumor. Most commonly liquid biopsies are used to analyze circulating tumor DNA (ctDNA), circulating tumor cells (CTC), tumor-relevant protein molecules and miRNAs (108-115). Genotyping of a tumor is the best strategy in determining the most beneficial treatment option for a patient. However, tissue samples collected during biopsies typically only provide a snapshot of the tumor environment and are not representative of the intratumor heterogeneity (116-118). Noninvasive blood-based liquid biopsies can provide a means of monitoring therapy-related markers and molecular changes throughout tumor progression, providing crucial information for patient management (116).

Novel research in the field of exosomes has been aimed at implementing exosomes as a surrogate for tumor liquid biopsies (105, 118-122). This is largely a result of tumors continually secreting exosomes, as well as exosomes being strongly representing of their cell of origin (118). Changes in the tumor environment will more rapidly be reflected in exosomes than through biomarkers that are typically measured in circulation. For this reason, liquid biopsies using exosomes is a novel method to provide accurate information regarding response to treatment and progression of the tumor environment over time.

With this growing interest in utilizing exosomes for liquid biopsies microfluidic devices are being developed to automate and expedite the isolation of exosomes and ultimately analysis. One such device was developed in our lab integrating immunomagnetic isolation and enrichment, chemical lysis, and immune-sandwich chemifluorescence probing in one sequential process. This microfluidic device or “lab-on-a-chip” was developed to capture non-small cell lung cancer (NSCLC) and ovarian cancer-associated exosomes and showed that exosomal protein markers could accurately distinguish cancer cases from healthy individuals (123). Of most importance, the complete analysis was completed in ~2 hrs (0.5 hrs off-chip incubation and
~1.5 hrs on-chip assay) with as low as 30 µL of plasma samples, compared to up to ten hours required for traditional isolation and 1 mL of plasma required. There are many other types of devices that exist which utilize different platforms and technology to capture exosomes including size-exclusion chromatography, surface plasmon resonance, membrane filters and magnetic sensing, as well as antibodies and affinity agents (123-127). The future of early detection as well as disease monitoring is contingent on developing devices such as the one developed by He and colleagues which focus on isolating and analyzing tumor-derived exosomes as biomarkers. Not only do these assays have the potential to offer high specificity and sensitivity they are non-invasive, relatively inexpensive, and offer timely results.
**Figure 1.1 Biogenesis of exosomes.** The plasma membrane undergoes endocytosis to form the early endosome. Inward budding and a series of maturing steps occur to form the late endosome from the early. Reversed budding of the late endosome leads to the formation of the multivesicular body (MVB). The MVB can be trafficked to the lysosome for degradation or to the plasma membrane where it can be fused and exosomes released into the extracellular milieu.
Figure 1.2 Different methods for exosome communication with recipient cells. 1. Fusion of the exosome membrane to the plasma membrane of the recipient cell, exchanging membrane and cytosolic material. 2. Endocytosis of the exosome resulting in exchange of membrane and cytosolic material. 3. Ligand/Receptor interaction between the exosome and recipient cell leading to activation of a signaling cascade in the recipient cell.
Chapter 2. Methods and Materials
Depleting Plasma of microvesicles and exosomes. Prior to removal of extracellular vesicles (EVs), 50 µl of plasma was reserved for downstream proteomic studies. Three 150 µL aliquots of plasma from 14 patients diagnosed with stage III/IV ovarian carcinoma were centrifuged at 100,000 x g for one hour to pellet all EVs, including exosomes, using a Beckman Airfuge ultracentrifuge. EVs and exosomes pelleted were pooled for each patient.

Proteomic Studies. Milliplex® Human Circulating Cancer Biomarker Panel 1 Immunoassay (Millipore cat#HCCBP1MAG-58K) containing 23 cancer related biomarkers (Table 2) was used to measure the concentrations of analytes in: plasma of EOC patients, plasma of EOC patients depleted of EVs, cell cultured derived EVs, primarily exosomes, cell culture derived subpopulations of EVs, primarily exosomes (CA125⁺), as well as patient derived immune-purified extracellular vesicles including exosomes (CA125⁺, EpCAM⁺, and FAPα⁺). From the immunoassay, Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method was used to calculate analyte concentrations as per manufacturers’ guidelines.

Immunomagnetic bead and antibody coupling. Streptavidin-coated magnetic microbeads (Dynabeads MyOne, 1 µm in diameter) were purchased from ThermoFisher Scientific. Antibodies against CA125, EpCAM, and FAP-α were coupled to the Dynabeads through biotin-streptavidin linkage, per manufacturer’s instructions. Linkage of CA125, EpCAM, and FAP-α to Dynabeads was performed utilizing a 30-minute incubation time at room temperature, with 20 µg of biotinylated antibody per 1 mg of beads. 20 µL of antibody-coated beads (1 mg/mL) was mixed with 150 µL human plasma or 150 µL of purified cell culture derived EVs, primarily exosomes. Dynabeads coupled to antibodies against CA125, EpCAM, and FAP-α, were
incubated with the samples for 30 minutes at room temperature. Captured EVs and exosomes were released from the Dynabeads through incubation at 65°C for 5 minutes to break the biotin-streptavidin bond. EVs and exosomes were lysed by 5% Triton X-100 with agitation for 5 minutes.

**Biotinylating Antibodies for Immunoprecipitation.** EpCAM and FAPα antibodies purchased from R&D Systems (Catalog Number: MAB960 and MAB3715-500) were biotinylated using Thermo Scientific EZ-Link Sulfo-NHS-Biotin following manufacturer’s protocol for biotinylating proteins in solution. Following steps to label the antibodies Zeba Spin Desalting Columns 7KMWCO were used to remove excess biotin. An aliquot of purified labeled antibody was taken for protein assay.

**Patient plasma samples.** Human blood samples were collected from healthy donors and ovarian cancer patients under protocol HSC #5929 (Biospecimen Repository Core Facility, Director, Andrew Godwin). De-identified samples were obtained from the University of Kansas Cancer Center’s BRCF after approval from the internal Human Subjects Committee.

**Cell Culture.** We utilized the human ovarian cancer cells A1847, and OVCAR3 as representative epithelial ovarian cancer cell lines (128). Ovarian cancer cell lines have been authenticated by using multiplex short tandem repeat (STR) testing and compared to historical reference DNA preserved in the lab for these cell lines. Testing was performed by the Clinical Molecular Oncology Lab at KUMC, a CLIA/CAP-accredited molecular diagnostics laboratory using the Promega PowerPlex 16 System used for human identity testing run on an Applied Biosystems instrument. Both cell lines were cultured in RPMI-1640 (Gibco, Thermo Fisher) media supplemented with 10% (v/v) exosome-depleted FBS, 2 mm L-glutamine, 0.2 units/mL
human insulin, and 100 units per mL penicillin-streptomycin at 37°C with 5% CO₂. Exosome-depleted FBS was obtained by centrifuging FBS for 18 hours at 100,000 x g followed by filtration through a 0.22 µm filter.

**Exosome Isolation.** Cell lines were cultured to 70-80% confluency in complete media. Conditioned media was collected after 24-48 hours and pooled. Exosomes were isolated by differential centrifugation as previously reported (64). Briefly, media was spun for 10 minutes at 2000 x g to isolate cell debris, 45 minutes at 10,000 x g to pellet large vesicles, and twice at 100,000 x g to pellet and wash exosomes. Exosome pellets were resuspended in 50-100 µL of cold PBS.

**CA125 Enzyme-Linked Immunosorbent Assay (ELISA).** RayBio® Human CA125 ELISA Kit (RayBiotech cat#ELH-CA125) was used to quantify circulating CA125 levels in plasma of EOC patients and controls, as well as plasma of EOC patients and controls depleted of EVs including exosomes, following manufacturer’s protocol.

**Nanoparticle Tracking Analysis.** Purified exosomes from cell culture as well as exosomes and microvesicles isolated from plasma samples using immunoprecipitation were analyzed using a NanoSight LM10 instrument (NanoSight, Salisbury, United Kingdom). Analysis was performed by applying a monochromatic 404 nm laser to diluted samples and measuring the Brownian movements of each particle. The Nanoparticle Tracking Analysis software version 2.3 was used to analyze five 60 second videos from different fields of view were collected for each sample and averaged to give mean, mode, and median vesicle size as well as concentration of particles.
Statistical Analysis. Statistical analysis was performed using a two tailed Student’s t-test on Graph Pad Prism Program. To reduce the probability of making a Type I error Bonferroni correction was applied reducing the significance level to 0.0025.
Chapter 3. Results
Cancer related biomarkers detected in plasma are in free circulation as well as associated with extracellular vesicles.

Exosomes are known to carry a wide range of proteins representative of their cell of origin making them excellent biomarker candidates (64, 67, 75, 79, 118). In one study, exosomes containing the biomarker Glypican-1 were capable of detecting pancreatic cancer from benign disease with absolute specificity and sensitivity (103, 104). With tumors generating a significant amount of exosomes and extracellular vesicles as compared to normal cells is it possible that cancer biomarkers typically measured in plasma are actually associated with tumor derived extracellular vesicles in circulation as opposed to in free circulation? To investigate this further a Luminex multiplex panel of 23 cancer related biomarkers, shown in Table 2, was utilized to analyze whole blood plasma as well as plasma depleted of all EVs, from patients diagnosed with advanced stage (III/IV), high grade serous ovarian cancer. Through the comparison of the analyte concentrations in plasma to the plasma depleted of all extracellular vesicles (PDEV) we gain an understanding of where these cancer related biomarkers can be located, in free circulation or in direct association with EVs.
Table 2: List of analytes examined in the Milliplex® Human Circulating Cancer Biomarker Panel 1 Immunoassay

<table>
<thead>
<tr>
<th>Human Circulating Cancer Biomarker Panel 1</th>
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<tbody>
<tr>
<td>AFP</td>
</tr>
<tr>
<td>CA 15-3</td>
</tr>
<tr>
<td>CEA</td>
</tr>
<tr>
<td>sFas</td>
</tr>
<tr>
<td>FGF-2</td>
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<td>HE4</td>
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<td>IL-6</td>
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<td>Leptin</td>
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<td>OPN</td>
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<td>SCF</td>
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<td>TNFα</td>
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<td>VEGF</td>
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Plasma samples were depleted of EVs including exosomes through ultracentrifugation using the Airfuge ultracentrifuge. Both whole plasma and PDEV were analyzed with the Luminex multiplex panel using a 1:6 dilution. The concentration of each analyte in the plasma as well as PDEV is shown in Figure 3.1. It is important to note that while 23 analytes were investigated, signal was detectable for 20 markers. For the other three analytes (SCF, CYFRA21-1, and β-HCG), the concentration was outside of the lower detection limit (SCF: 2.0 pg/mL, CYFRA21-1: 59.3 pg/mL, β-HCG: 0.029 mU/mL). TRAIL, Leptin, and OPN show a small, but statistically significant decrease in the PDEV as opposed to the whole plasma based on a paired t-test (TRAIL and Leptin: P<0.05, OPN: P<0.001). However, when the statistical analysis is corrected for using the Bonferroni correction, only OPN shows a statistical decrease in the PDEV, Figure 3.2. This decrease in biomarker concentration suggests that when the EVs including exosomes are removed from the plasma, a portion of the circulating analyte is depleted; representing a potential association between the analyte and the EVs. In contrast, the concentration of the
remaining analytes does not show a statistically significant decrease following the removal of extracellular vesicles and exosomes.

Current screening methods for ovarian cancer include monitoring CA125 levels. The CA125 used for diagnostic purposes showed to be mostly free circulated as opposed to microvesicle-associated. This is shown in Figure 3.1 and Figure 3.2 with data from the Luminex panel as well as with data from a CA125 specific ELISA (Figure 3.4.A). There is not a statistically significant difference in the concentration of CA125 measured in the plasma as compared to the PDEV. Taken together this data suggest that a majority of the cancer biomarkers studied in this panel are relatively low or potentially absent in extracellular vesicles and that the signal detected is attributed primarily to analytes in free circulation.

**Cell line derived exosomes contain subpopulations with varying levels of cancer related biomarkers, implying there is heterogeneity within the exosome populations.**

Exosomes were isolated from conditioned media of A1847 and OVCAR3 ovarian cancer cell lines through ultracentrifugation (64). Immunoprecipitation was used to select for exosomes expressing CA125 on the surface. Using MyOne streptavidin coated beads coupled with a biotinylated antibody against CA125, exosomes expressing CA125 were selected for from the whole exosome population isolated. The CA125+ exosomes as well as the whole exosome population were lysed and analyzed using the same Luminex multiplex panel of 23 cancer related biomarkers. The concentration of each analyte for the two populations is shown in Figure 3.3, for both cell lines. In both A1847 and OVCAR3 derived extracellular vesicles, the analyte concentration for the CA125+ population does not match that of the whole exosome population, suggesting cargo of these subpopulations differ and there is heterogeneity among the
cell culture derived exosomes. In exosomes from A1847 there are some analytes such as MIF, IL-8, HGF, sFas, CA 19-9, and β-HCG where the concentrations were lower in the CA125⁺ exosomes as compared to the whole exosome population. This is attributed to the CA125⁺ population only contributing to a fraction of the observed signal detected in the whole exosome population. In addition, there are other analytes such as prolactin, SCF, CYFRA 21-1, OPN, FGF-2, and HE4 in A1847 and MIF, Leptin, IL-6, sFasL, HGF, sFas, prolactin, SCF, CYFRA 21-1, OPN, FGF-2, HE4, and VEGF in OVCAR3 where the concentration is higher in the subpopulation as compared to the entire population. When this subpopulation is enriched for, the signal for these biomarkers is also enriched for, which can result in > 300-fold increase in the analyte concentration.

While these exosomes were both derived from ovarian cancer cell lines there are striking differences between the protein levels of the exosomes. This is likely a result of the heterogeneity of the disease itself. For instance, when the CA125⁺ population is selected for in OVCAR3 exosomes the concentration of MIF, HGF, and CA 19-9 is enriched for, as depicted by an increase in concentration from the whole exosome population. However, in the CA125⁺ population of the A1847 derived exosomes these signals are not enriched for. In contrast there are some analytes that are enriched for in the CA125⁺ population of both cell line derived exosomes, such as Leptin, CYFRA 21-1, HE4 and OPN.

At least three subtypes of extracellular vesicles can be isolated from patients with stage III and IV ovarian cancer, of which heterogeneity can be observed in the levels of 23 biomarkers studied.
The subpopulations of extracellular vesicles observed in the A1847 and OVCAR3 cell lines are also observed in vesicles derived from clinical plasma samples of women with stage III/IV high grade serous ovarian cancer. Using the same amount of whole plasma (150 μL), immunoprecipitation was used to select for exosomes and extracellular vesicles expressing CA125, EpCAM, or FAP-α on the surface. Epithelial cell adhesion (EpCAM) is a 30- to 40-kDa membrane protein expressed by a variety of human epithelial cells, but is overexpressed and homogenously expressed on the surface of cancer cells (144). EpCAM, while it is not specific for ovarian cancer, has been shown to be expressed on the surface of ovarian cancer derived exosomes (145). While little research has been done with FAP-α and extracellular vesicles, it is understood that expression is restricted to pathologic sites such as cancer (146). For the cell culture related data, the starting material used was ovarian cell line derived exosomes isolated via the widely accepted method of differential centrifugation. In contrast, these extracellular vesicles (CA-125⁺, EpCAM⁺, and FAP-α⁺) were isolated directly from plasma using immune-capture, which contains a diverse population of vesicles within the circulation as opposed to solely exosomes. For this reason, they are representative of a wider population of extracellular vesicles. When the Luminex multiplex panel of 23 cancer related biomarkers is used to compare the three subpopulations (CA-125⁺, EpCAM⁺, and FAP-α⁺) of extracellular vesicles there are differing concentrations for each analyte within the subtypes. Some analytes such as AFP, MIF, Leptin, and HGF levels are relatively consistent between the three subtypes while other analytes such as CEA, sFas, prolactin, CYFRA 21-1, OPN, HE4, and CA125 vary between the three populations (Figure 3.4). This observation is most likely attributed to the subtypes being secreted from different cell types resulting in differences in the cargo and the levels of protein in the immuno-captured vesicles.
Enrichment of CA125+ EVs results in increased limit of detection between EOC patients and healthy controls.

When a woman presents with symptoms of ovarian cancer that persist for more than two weeks her physician would likely request a CA125 blood test and/or a TVUS. As discussed in Chapter 1, the problem with both of these screening methods is the lack of specificity and sensitivity, which was seen when we measured circulating CA125 in cases and controls (Figure 3.5.A). Any screening strategy for ovarian cancer must have an exceptionally low false positive rate in order to achieve a low and acceptable number of unnecessary operations per screen-detected case. Given the relatively low prevalence in the general population (1 in 2,500), an effective screening strategy must not only have a high sensitivity for early-stage disease (>75%), but also a very high specificity (99.6%) to prompt less than ten operations for each ovarian cancer case diagnosed (a minimum positive predictive value of 10%), which represents a generally accepted limit for balancing risk with benefit among practitioners and advocates (37, 147-149). Therefore, we began to explore if EVs could serve as new biomarkers and/or complement existing screening approaches.

EVs were immuno-captured with antibodies against CA125 and EpCAM from 150 µL of plasma of EOC cases and controls. Following the immuno-capture the EVs were lysed and evaluated using the Luminex multiplex panel (Figure 3.6). Importantly, even in this very limited samples set; we observe up to a 13-fold elevation in CA125 for patients with ovarian cancer compared to controls (Figures 3.5.B and 3.6). Although it is not possible to establish sensitivity or specificity from this limited sample set, our results show a clear separation between cases and controls when we enrich for the CA125+ subpopulation of EVs and then measure the total CA125 concentrations. This is most likely a result of the concentration of CA125 within each
microvesicle being greater, as a similar amount of vesicles were isolated from both cases and controls (Figure 3.6). This pilot study suggests that we are likely measuring a different form of CA125 than what is traditionally measured with an ELISA. The typical ELISA for CA125 measures a free circulating form of the protein while the immunoprecipitation is isolating vesicles with the membrane associated form of the protein, essentially acting as a different biomarker than the cleaved form (Figure 3.5C). While the CA125 ELISA is capable of detecting the membrane associated form, it is important to note that the concentration detected in plasma of the cleaved circulating form is significantly greater than that of the EV associated form, thereby masking the differences observed between cases and controls due to the membrane associated form. Thus, selecting for only the membrane associated form of CA125, we might be eliminating any background associated the cleaved form, which can be elevated above the clinical level that elicits concern (≥35U/ml) in benign and non-gynecologic malignancies. One caveat to our detection approach is that since CA125 is cleaved and secreted by ovarian tumor cells, it may result in decreased levels of CA125 available on the surface of the tumor cell, potentially impacting the amount of tumor associated vesicles immuno-captured. However, as shown in Figures 3.4 and 3.5 we are still able to efficiently immuno-capture and interrogate CA125 levels in ovarian cancer patients.

This pilot study also included examining EpCAM+ EVs and comparing the relative analyte concentrations in the same cases and controls (Figure 3.5). Interestingly, we observe lower levels of a few analytes in cases versus controls in EpCAM+ EVs. For instance HE4, the second most common circulating marker used in ovarian screening studies (150), was higher in EpCAM+ EVs from control samples versus cases. This was also observed for a number of other
growth factors and cytokines, including β-HCG, CEA, CYFRA21-1, IL8, Leptin, SCF, sFasL, TNF-α, and TRAIL.

In our experiments, both the CA125⁺ and EpCAM⁺ vesicles were selected directly from plasma, thus the cell of origin for these vesicles is unknown and potentially different for the two subtypes, attributing to the differences observed in the concentration of sFasL, as well as other analytes. Interestingly, even though these two subtypes may be a result of differing cells of origin there are trends between cases and controls that can be observed in both the EpCAM⁺ and CA125⁺ vesicles. This is seen with CYFRA21-1 which is elevated in controls compared to the cases for both of these subtypes, indicating the decreases in EV associated CYFRA21-1 is potentially associated with disease state (Figure 3.6). Plasma levels of CYFRA21-1 are monitored in cases of non-small-cell lung cancer (151, 152), indicating in diseased states the protein in secreted. Therefore, we can speculate that when the protein becomes secreted, the intracellular levels are decreased and thus fewer proteins are available to be packaged into EVs. Taken together our studies demonstrate that subpopulations of EVs can offer specificity for certain biomarkers.
**Figure 3.1 Analytes detected from the Milliplex® Human Circulating Cancer Biomarker**

**Panel 1.** A majority of analytes detected appear to be attributed to a free circulating form as opposed to directly associated with extracellular vesicles including exosomes. The concentrations for each biomarker in plasma (red) as well as plasma depleted of EVs (blue) is shown. *P<0.05, ***P<0.0001
Figure 3.2 Analytes detected from the Milliplex® Human Circulating Cancer Biomarker

Panel 1. A majority of analytes detected appear to be attributed to a free circulating form as opposed to directly associated with extracellular vesicles including exosomes. The concentrations for each biomarker in plasma (red) as well as plasma depleted of EVs (blue) is shown. ***P<0.0001
Figure 3.3 Subpopulations of exosomes exist in cell culture derived EVs for which analytes can be detected at varying concentrations. A,B. Analyte concentrations for whole EV population (orange) and the CA125\(^+\) subpopulation of EVs (green). These EVs, primarily exosomes, were derived from the (A) OVCAR3 cell line and (B) A1847 cell line. Concentrations for CA19-9, CA125, and β-HCG are shown in U/mL with the remaining 20 analytes shown in pg/mL.
Figure 3.4 EV cytokine, growth factor and cancer molecule concentrations vary for three subtypes of EVs derived from clinical plasma samples. Immunoprecipitation was performed using antibodies against CA125, EpCAM, and FAP-α, to isolate EVs from plasma (150 µL) of three patients presenting with stage III and IV ovarian cancer. Three subtypes of EVs were isolated CA125⁺ (orange), EpCAM⁺ (green), and FAP-α⁺ (purple). Protein quantity for the three populations were analyzed using the Milliplex® Human Circulating Cancer Biomarker Panel 1. Concentrations from the three individual patients were averaged for each biomarker and the standard deviation is represented by the error bars. Concentrations for CA 19-9, CA-125, and β-HCG are shown in U/mL with the remaining 20 analytes shown in pg/mL.
Figure 3.5 CA125 measured in CA125\(^+\) exosomes offers greater distinction between cases and controls than CA125 measured in plasma. A. CA125 levels measured through standard ELISA in plasma (red) and plasma depleted of microvesicles (blue) for healthy controls (n=5) and patients (n=14) presenting with stage III/IV ovarian cancer. B. CA125 levels measured in CA125\(^+\) immuno-captured exosomes with from patients presenting with stage III/IV ovarian cancer (red) and healthy controls (blue). C. Transmembrane and cleaved forms of CA125. *P<0.05
Figure 3.6 EpCAM⁺ and CA125⁺ subtypes of EVs and exosomes present with differing levels of various analytes in both clinical samples and healthy controls. Concentrations for each biomarker from the Luminex Milliplex® panel is shown for both the EpCAM⁺ and CA125⁺ exosomes and microvesicles of cases (red) and controls (blue).
Figure 3.7 Size and concentration of immuno-captured vesicles is relatively consistent regardless of antibody used for capture. A. Concentration and B. size of vesicles immuno-captured with antibodies against CA125, EpCAM, and FAP-α for high grade serous cases (red) and controls (blue)
Chapter 4. Discussion
The role TRAIL, leptin, and OPN play in cancer development can offer a potential explanation for the observed decrease in concentration upon the removal of extracellular vesicles. TRAIL or TNF-related apoptosis-inducing ligand is part of the TNF superfamily, capable of inducing apoptosis. TRAIL selectively induces apoptosis in cancer cells. Induction of apoptosis is through the extrinsic pathway involving formation of the Death-inducing signaling complex (Disc), a pathway independent of p53 (129, 130). For this reason TRAIL-receptor agonists (TRAs) have been developed and are showing robust anticancer activity in preclinical studies (130). Once apoptosis has been initiated and the cell has undergone condensing and fragmentation, apoptotic bodies, vesicles ranging in size from 100-400 nm are formed. These apoptotic bodies, because of their size, were removed during the depletion of the plasma. As TRAIL is associated with the cell undergoing apoptosis it will become part of and removed with the apoptotic bodies, resulting in the observed decrease in concentration.

Leptin, which also showed a decrease in concentration with the removal of extracellular vesicles including exosomes, has been shown to play a significant role in cancer progression. Leptin is known to assist in cell differentiation, proliferation and survival, through endocrine, autocrine, and paracrine signaling (131). Increased leptin signaling has been shown in various forms of cancer to activate a multitude oncogenic pathways enhancing proliferation and decreasing apoptosis (131-133). In addition, leptin signaling has shown to assist in acquisition of the mesenchymal phenotype, important for enhanced invasion and angiogenic properties of tumor cells (131, 133). For these reasons it is crucial to the progression of tumor development for leptin to be secreted by cancer cells and bind to receptors on neighboring cancer cells and cells of the tumor microenvironment. To date there have been no studies which solely focus on the association between leptin and extracellular vesicles including exosomes. However, the role
EVs play in cell to cell communication may assist in some of this essential leptin signaling, which is supported by data shown in Figure 3.1 and 3.2. The slight decrease in Leptin in the PDEV is suggestive that a portion of the leptin signaling that occurs in cancer cells is a result of extracellular vesicle or exosomes associated signaling. Similar to leptin, osteopontin (OPN) has been shown to promote tumorigenicity and metastasis so it is possible EVs including exosomes play a similar role with OPN as they do with leptin (134, 135). In a study conducted by Monika and colleagues OPN was significantly elevated in peritoneal fluid of patients with ovarian cancer compared with patients with other gynecological conditions (135). Interestingly, Atay and colleagues (unpublished data from the Godwin laboratory) have determined that the circulating exosome levels are increased by 12-fold in the plasma of EOC patients (0.711 x10\text{11} \pm 0.03 particle/mL of initial plasma (n=17)) versus age-matched healthy donors (control) [0.06 x10\text{11} \pm 0.01 particle/mL of initial plasma (n=11 samples)], and by 56-fold in ascites fluids of EOC patients [3.35 x10\text{11} \pm 12.7 particle/mL of initial plasma (n=14)] when compared to controls. This and other findings support the idea that increased levels of OPN measured in plasma from EOC patients may be in part associated with circulating EVs. Overall, we found that when EVs were removed from plasma there was a decrease in TRAIL, leptin, and OPN concentrations suggesting an association between the analytes and vesicles. Further, it may also suggest a role for these EVs and analytes in EOC development and progression.

While the isolation of exosome subpopulations in cell culture derived exosomes was initially unexpected, others have reported on these subpopulations, and their ability to be distinguished and isolated from one another using a sucrose density gradient, as these subpopulations often have different densities. Suggesting this heterogeneity observed in these exosome population is not unique to these two cell lines (136-139). In one study conducted by Willms and colleagues it
was reported that cells release distinct exosome subpopulations, and that these subpopulations have unique composition. In this study it was also reported that these subpopulations had the ability to elicit differential effects on recipient cells (136). Furthermore, Bobrie et al. have suggested that common protocols for exosome isolation co-purifies vesicles from the endosomal origin as well as other origins. As this protocol was used for our studies, it may be possible that the “exosomes” isolated from OVCAR3 and A1847 cells actually represent a diverse extracellular vesicle population. The different extracellular vesicle populations observed in these studies, as well as our own, can be closely linked to the biogenesis of exosomes. During biogenesis different MVBs are formed within the cell and the ILVs that form the MVBs are all different in morphology and composition, therefore variation in exosome populations can be expected (140-143).

This heterogeneity observed in the cell culture derived exosomes is supports the idea of utilizing exosomes as biomarkers. For example, selecting exosomes expressing CA125 on the surface and measuring another cancer related marker such as HE4, CYFRA21-1 or OPN, all markers where the signal is enhanced in this CA125^+ subpopulation, may prove to be a better biomarker than simple analyzing a single analyte in circulation. Previous studies attempting to use multimarker screening in ovarian cancer have coupled CA125, and HE4 which is supported by the HE4 signal being enriched for when vesicles expressing CA125 on the surface are selected for (36). Based on this and previous studies, the combination of markers to select for a subtype of vesicles and evaluate specific proteins within this population may prove to be a valuable technique in discrimination between ovarian cancer and other benign gynecological conditions.
The particles that have been selected for from plasma using immunoprecipitation represent vesicles in circulation and can be derived from any given cell in the body, not just the cancer cells. Because the cargo in exosomes and extracellular vesicles is highly representative of their cell of origin, if these vesicles are derived from different cell types it would explain the differences observed in the proteomic profile. Regardless of their source these vesicles can be isolated using immunoprecipitation and interrogated for various cancer markers, expressed at varying levels for the three subpopulations studied.

When these subpopulations of EVs were analyzed in clinical samples and controls, we noted an unexpected elevation in cancer related analytes for the controls compared to cases in EpCAM$^+$ EVs. We hypothesize that this may be the result of the biogenesis of these immune-captured molecules. Typically, growth factors are secreted and bind to cell surface receptors and trigger intracellular signaling cascades, resulting in cell proliferation and/or differentiation. Cytokines are a subtype of growth factors that are produced by hematopoietic and immune cell types, and include interferons and interleukins. They are able to inhibit, as well as stimulate, cell proliferation and differentiation. Overproduction of growth factors and dysregulation of cytokine signaling are common features of malignancies. Our results might suggest that in ovarian cancer and other malignancies, these factors are rapidly secreted from cells independent of EVs and are therefore at lower levels in the tumor cells and are not as efficiently shuttled to/captured in EVs.

Finally, it is important to note that the level for some markers (i.e., β-HCG, CEA, CYFRA21-1, sFas, and sFasL) are increased in EVs immuno-captured with EpCAM as compared to CA125. This could potentially be associated with the expression level of CA125 and EpCAM within the
tumor cell. In one study by Kloudová and colleagues the mRNA expression of 21 tumor associated antigens was examined in 41 patients with stage III/IV high grade serous epithelial ovarian cancer. This study showed that the mRNA expression of EpCAM was elevated compared to CA125 in primary tumor cells. Therefore, it is possible the vesicles isolated using EpCAM contain more tumor associated EVs which is reflected by elevated levels of multiple cancer biomarkers compared to the CA125 population. This is also seen when the levels of sFasL are compared for CA125+ and EpCAM+ EVs. sFasL levels are elevated in EpCAM+ EVs compared to CA125+ EVs, for both cases and controls. Based on this observation it appears that sFasL is more strongly associated with vesicles that express EpCAM on the surface, which could be a result of the origin, i.e., primarily EpCAM+ tumor cells, of these EVs. Although the number of vesicles isolated with EpCAM is similar to that of CA125 (Figure 3.6), it is possible that the vesicles isolated using EpCAM are more heterogeneous in their origin than CA125+ EVs.

Overall, while the concentration of vesicles isolated based on their surface phenotype does not differ widely, the content within these vesicles is immensely different between EOC cases and healthy controls. These differences observed in the subpopulations is likely a result of the diseased state as well as the origin of the vesicles. For these reasons, we believe that examining subpopulations of EVs offers a unique strategy for evaluating novel and existing biomarker, with future potential in detecting ovarian cancer at the early stages when the 5-year survival is promising.
Chapter 5. Conclusion
The lack of precise early warning signs is one of the factors that contribute to the high mortality rate of ovarian cancer, with only 15% of ovarian tumors identified at stage I, when the disease can be cured in up to 90% of cases (1-3). Current screening methods include serum cancer antigen 125 (CA125), which has proven to be inadequate for early detection due to its low sensitivity and specificity (153-155). The focus of this study was to evaluate and examine extracellular vesicles, primarily exosomes, as potential sources of new biomarkers for early detection of ovarian cancer.

This is the first study of our knowledge to measure and compare growth factors and cytokines in total plasma and plasma-depleted of extracellular vesicles. An association was shown between three cancer related markers (TRAIL, Leptin, and OPN) EVs, as upon the removal of EVs the concentrations of these three markers decreased. Each of these markers is shown to play an important role in the development of cancer; therefore it would be of interest to investigate to what extant EV associated communication plays a role in their tumorigenic signaling. In addition, based on our study it appears that the form of CA125 measured clinically with a blood test is distinct from the protein measured in and on EVs from circulation. It would be of interest to investigate further the similarities and differences between the two forms of CA125 in an effort to gain a better understanding of the roles played in ovarian cancer.

The presence of subtypes of EVs was shown in vesicles isolated from conditioned media of ovarian cancer cell lines. These subtypes were also observed in plasma samples of patients with stage III/IV ovarian cancer as well as healthy controls. The three subtypes detected in clinical samples (EpCAM⁺, CA125⁺, and FAP-α⁺) all presented with varying levels of the 23 cancer related markers studied. This is a proof-of-principle study to begin to better understand the
content of EOC-derived EVs and their utility as biomarkers that can be evaluated using a microfluidic device, similar to the one developed by He and colleagues (123). Based on the cytokines, growth factors, and cancer molecules studied; measuring CA125 in the CA125+ subpopulation of EVs offers potential in distinguishing cases from controls. Considering these findings were in a pilot study of only three cases and three controls it will be essential in future studies to expand to larger sample sets. If these findings are consistent they could fairly easily be translated over for use with the microfluidic device, using CA125 as the capture agent to enumerate particle numbers and in turn measure CA125 in the captured and lysed vesicles.

It is important to note that this study was limited to three subpopulations of EVs and the potential number of subpopulations is endless. Studies are proposed in the Godwin laboratory to use these three subpopulations for deep global mass spectrometric (MS) analysis of the exosome preps using a novel Simple Nano-Proteomic Platform (SNaPP) specifically tailored for improving proteomic coverage and quantitative reproducibility in characterization of small-sized biological samples in order to define the proteome of ovarian cancer-associated exosomes. These studies have developed the necessary techniques to allow for Phase I protein discovery experiments. Although, there may be a better and more specific capture agent for ovarian cancer-derived EVs, based on this limited pilot case/control study we found that using CA125 or EpCAM as the capture agents, and subsequently measuring CA125 and CYFRA21-1, respectively, may offer improved specificity and sensitivity for ovarian cancer. Time will tell once expanded sample sets become available and are evaluated.

Overall, these and other studies provide the framework for the initial characterization of circulating ovarian cancer-derived EVs and exosomal protein discovery for future development
of microfluidic-based devices utilized for screening purposes. This study shows that there are subpopulations of EVs in plasma samples of women with ovarian cancer as well as healthy individuals. Each of these subpopulations has a unique protein content which can be measured and used for analysis as biomarkers.
Chapter 6. References


27. Bourne TH, Campbell S, Reynolds KM, Whitehead MI, Hampson J, Royston P, Crayford TJ, Collins WP. Screening for early familial ovarian cancer with transvaginal ultrasonography and colour blood flow


